

Expansion of Genetic Testing in the Division of Functional Genomics, Research Center for Bioscience and Technology, Tottori University from 2000 to 2013

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ABSTRACT

Background At the Division of Functional Genomics, Research Center for Bioscience and Technology, Tottori University, we have been making an effort to establish a genetic testing facility that can provide the same screening procedures conducted worldwide.

Methods Direct sequencing of PCR products is the main method to detect point mutations, small deletions and insertions. Multiplex ligation-dependent probe amplification (MLPA) was used to detect large deletions or insertions. Expansion of the repeat was analyzed for triplet repeat diseases. Original primers were constructed for 41 diseases when the reported primers failed to amplify the gene. Prediction of functional effect of human nsSNPs (*PolyPhen*) was used for evaluation of novel mutations.

Results From January 2000 to September 2013, a total of 1,006 DNA samples were subjected to genetic testing in the Division of Functional Genomics, Research Center for Bioscience and Technology, Tottori University. The hospitals that requested genetic testing were located in 43 prefectures in Japan and in 11 foreign countries. The genetic testing covered 62 diseases, and mutations were detected in 287 out of 1,006 with an average mutation detection rate of 28.5%. There were 77 samples for prenatal diagnosis. The number of samples has rapidly increased since 2010.

Conclusion In 2013, the next-generation sequencers were introduced in our facility and are expected to provide more comprehensive genetic testing in the near future. Nowadays, genetic testing is a popular and powerful tool for diagnosis of many genetic diseases. Our genetic testing should be further expanded in the future.

Key words genetic testing; prenatal diagnosis; germline mutation

The development of new technologies such as microarray analysis and next-generation sequencing has enabled more comprehensive screening for mutations that cause inherited diseases.^{1, 2} According to GeneTests (<http://www.genetests.org/>), screening methods covering more than 2,000 genes are currently available.

In Japan, however, genetic testing has not yet become a common practice in the medical field, since public insurance covers only screenings for mutations in 36 genes. Mutation hunting on several genes is conducted on a research basis, which as expected depends on the research project and is not ensured to be provided in the future.

In the Division of Functional Genomics, Research Center for Bioscience and Technology, Tottori University, we have established a genetic testing facility that can provide the same screening procedures conducted worldwide. This system started as a service for screening mutations responsible for several types of inherited disorders of children, and now has expanded to cover more than 60 inherited disorders. Human DNA samples have been provided not only from Tottori University Hospital but also from other medical facilities all over Japan and foreign countries. Here the development of the system from January 2000 to September 2013 is reported.

SUBJECTS AND METHODS

Subjects

From January 2000 to September 2013, a total of 1,006 DNA samples were subjected to genetic testing in our facility. They were from 13 departments at Tottori University Hospital, 123 hospitals in Japan and 11 in foreign countries (Table 1). Most of the DNA was extracted from peripheral blood. Exceptions include 24 from cultured fibroblasts and 3 from tissue. For prenatal diagnosis, 40 DNA samples were from chorionic villi, 36 from cultured amnionic cells and 1 from umbilical blood. The DNA was subjected to genetic testing for 62 inherited diseases (Table 2).

The samples were collected according to “Guideline for Genetic Testing” (August 2005) approved by the Association of Genetic Medicine (10 societies and

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Abbreviations: DHPLC, denaturing high performance liquid chromatography; MLPA, multiplex ligation-dependent probe amplification; *PolyPhen*, prediction of functional effect of human nsSNPs; SSCP, single strand conformation polymorphism

Table 1. The number of medical facilities that requested for genetic testing in our laboratory*

Area	Number	Prefecture or Country
Hokkaido	5	
Tohoku	10	Aomori 1, Iwate 1, Miyagi 3, Akita 1, Yamagata 2, Fukushima 2
Kanto	27	Ibaraki 1, Tochigi 3, Gunma 2, Saitama 4, Chiba 2, Tokyo 11, Kanagawa 4
Chubu	20	Niigata 2, Ishikawa 2, Fukui 1, Yamanashi 1, Nagano 1, Gifu 1, Shizuoka 4, Aichi 8
Kinki	20	Mie 3, Shiga 3, Kyoto 2, Osaka 8, Hyogo 3, Nara 1
Chugoku	19	Tottori 4, Shimane 1, Okayama 5, Hiroshima 6, Yamaguchi 3
Shikoku	3	Tokushima 1, Kagawa 2
Kyusyu	19	Fukuoka 5, Saga 1, Nagasaki 2, Kumamoto 1, Oita 4, Miyazaki 1, Kagoshima 3, Okinawa 2
Total	123	
Foreign countries	11	United Kingdom 1, Macedonia 1, United Arab Emirates 1, United States 1, India 1, Singapore 1, Turkey 1, Thailand 1, China 1, Belgium 1, Russia 1

*Tottori University Hospital excluded.

1 committee), and also according to the “Guidelines for Genetic Tests and Diagnoses in Medical Practice” (February 2011) approved by the Japanese Association of Medical Sciences. Prenatal diagnosis was approved by the Ethical Committee of Tottori University.

Methods

Direct Sequencing of the PCR product is the main method to detect point mutations, small deletions and insertions. In the past, single strand conformation polymorphism (SSCP) and denaturing high performance liquid chromatography (DHPLC) were used in mutation screenings for porphyria, holoprosencephaly and Joubert Syndrome. Mutations in the aberrant PCR product detected by SSCP or DHPLC were confirmed by direct sequencing. SSCP and DHPLC were used from January 2000 to October 2001, and from July 2004 to April 2010, respectively.

The length of the triplet repeat was determined in the diagnosis of myotonic dystrophy, fragile X syndrome and dentatorubral-pallidolusian atrophy. Multiplex ligation-dependent probe amplification (MLPA) was used for diagnosis of Fabry disease, Rett syndrome, Pelizaeus-Merzbacher disease, Duchenne muscular dystrophy, von Hippel-Lindau disease and spinal muscular atrophy, because these diseases are often caused by large deletions and insertions. Real-time PCR was specially used to detect deletions or insertions in the genes that cause Pelizaeus-Merzbacher disease, Gaucher disease, Menkes disease and metachromatic leukodystrophy.

Disease and gene information

Information on diseases and causative genes was obtained from Online Mendelian Inheritance in Man and Gene database provided by the National Center for Biotechnology Information.

PCR

Before 2009, the primers were designed according to published data with variable rates of successful amplification. From 2010, almost all primers were designed originally in our laboratory using Primer3 software utilizing the gene information from Entrez Gene and RefSeq, resulting in successful amplification in almost all cases. The coding sequence with 70 bp of the flanking area of each exon was amplified by PCR. The annealing temperature was set relatively high (around 63 °C) (Table 2). The sequences of the primers are available on request.

AmpliTaq Gold (Life Technologies, Carlsbad, CA) was routinely used except for the amplification of GC rich sequences (more than 70%). For GC rich sequences, TaKaRa LA Taq (Takara Bio, Otsu, Japan) was used. The reaction mixture contained in a 10 µL reaction volume 10 to 100 ng of template DNA, 2.5 U AmpliTaq Gold, 250 µM or 400 µM dNTP, 1 µM of each primer (forward and reverse) and was run at the following conditions: initial denaturation at 95 °C for 5 min, followed by 30 cycles with denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min, extension at 72 °C for 1 min and final extension at 72 °C for 5 min. For GC-rich or relatively long sequences, 0.5 U TaKaRa LA Taq was used instead of AmpliTaq Gold. The reaction contained 400 µM dNTP and was run as follows: initial denaturation at 94 °C for 1 min, followed by 30 cycles with denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 2 min and final extension at 72 °C for 5 min.

For diagnosis of incontinentia pigmenti and Fukuyama-type congenital muscular dystrophy, long insertion or deletion was detected according to the methods described by Bardaro et al.³ and Kato et al.,⁴ respectively.

Direct sequencing

PCR products were run on 1.5% of agarose gel electro-

Table 2. List of inherited diseases surveyed for genetic mutations in our facility

Disease	Gene symbol	Number of genetic cases	
Myotonic dystrophy	<i>DMPK</i>	250*	115‡
Fragile X syndrome	<i>FMR1</i>	173	19‡
Niemann-Pick disease type C	<i>NPC1</i> †	73*	24‡
GM1 gangliosidosis	<i>GLB1</i>	56*	20‡
Joubert syndrome	<i>NPHP1</i> †, <i>CEP290</i> †, <i>AH11</i> †, <i>TMEM67</i> , <i>RPGRIPL</i>	42*	9‡
Gaucher disease	<i>GBA</i>	40*	9‡
Tay-Sachs disease	<i>HEXA</i>	38*	12‡
Porphyria	<i>HMBS</i> †, <i>PPOX</i> †, <i>CPOX</i> †	38	3‡
Pompe disease	<i>GAA</i>	32	8‡
Cowden syndrome	<i>PTEN</i>	30	6‡
Fabry disease	<i>GLA</i>	28	5‡
Menkes disease	<i>ATP7A</i> †	23*	3‡
Metachromatic leukodystrophy	<i>ARSA</i> †	19*	4‡
Rett syndrome/MECP2-related disorders	<i>MECP2</i>	13	4‡
X-linked hydrocephalus	<i>LICAM</i>	13*	3‡
Pelizaeus-Merzbacher disease	<i>PLP1</i>	11*	3‡
Fukuyama-type congenital muscular dystrophy	<i>FKTN</i>	10*	4‡
Nephrogenic diabetes insipidus (NDI)	<i>AVPR2</i>	10	3‡
Myotubular myopathy	<i>MTM1</i> †	9	1‡
Galactosialidosis	<i>CTSA</i>	8	4‡
Duchenne muscular dystrophy	<i>DMD</i>	7*	3‡
Von Hippel-Lindau disease	<i>VHL</i> †	5	3‡
Spinal muscular atrophy	<i>SMN1</i>	5*	0‡
Dentatorubral-pallidoluysian atrophy (DRPLA)	<i>ATN1</i>	4	2‡
Tuberous sclerosis	<i>TSC1</i> †, <i>TSC2</i> †	4	2‡
Limb-girdle muscular dystrophy type 2B (LGMD2B)	<i>DYSF</i> †	4	1‡
Pendred syndrome	<i>SLC26A4</i> †	3	3‡
Enlarged parietal foramina	<i>ALX4</i> †, <i>MSX2</i> †	3	2‡
Sialidosis	<i>NEU1</i>	3	2‡
Congenital muscular dystrophy-dystroglycanopathy	<i>FKRP</i> †	3	0‡
Methylmalonic acidemia	<i>MMAA</i> †, <i>MMAB</i> †, <i>MMADHC</i> †, <i>MUT</i> †	3	0‡
Niemann-Pick disease type C2	<i>NPC2</i> †	3	0‡
Benign hereditary chorea	<i>NKX2-1</i>	2	2‡
Li-Fraumeni syndrome	<i>TP53</i>	2	2‡
Mucopolysaccharidosis type IVB (Morquio)	<i>GLB1</i>	2	2‡
Dyschromatosis symmetrica hereditaria	<i>ADAR</i> †	2	1‡
Incontinentia pigmenti	<i>IKBKG</i>	2	1‡
Melanoma	<i>CDKN2A</i> †	2	1‡
Calcium-sensing receptor	<i>CASR</i>	2	0‡
Dopamine transporter	<i>SLC6A3</i>	2	0‡
Familial dysautonomia	<i>IKBKAP</i>	2	0‡
Holoprosencephaly	<i>SHH</i> , <i>ZIC2</i> †	2	0‡
Lesch-Nyhan syndrome	<i>HPRT1</i>	2*	0‡
Mitochondrial diseases	–	2	0‡
Papillon-Lefevre disease	<i>CTSC</i> †	2	0‡
Werner syndrome	<i>WRN</i> †	1	1‡
Ceroid lipofuscinosis, neuronal, 1	<i>PPT1</i> †	1	0‡
Feingold syndrome	<i>MYCN</i> †	1	0‡
Gardner syndrome	<i>AXINI</i> †	1	0‡
GM2-gangliosidosis, AB variant	<i>GM2A</i> †	1	0‡
Greig cephalopolydactyly syndrome	<i>GLI3</i> †	1	0‡
Growth hormone receptor	<i>GHR</i>	1	0‡
Klippel-Feil syndrome	<i>GDF6</i> †	1	0‡
Leber hereditary optic neuropathy	–	1	0‡
Limb-girdle muscular dystrophy type 1C (LGMD1C)	<i>CAV3</i>	1	0‡
Nevoid basal cell carcinoma syndrome	<i>PTCH1</i> †	1	0‡
Niemann-Pick disease type B	<i>SMPD1</i> †	1	0‡
Ornithine carbamoyltransferase deficiency disease	<i>OTC</i> †	1	0‡
Pseudohypoparathyroidism	<i>GNAS</i> †	1	0‡
Sialic acid storage disease	<i>SLC17A5</i> †	1	0‡
Townes-Brocks syndrome	<i>SALL1</i>	1	0‡
Zellweger syndrome	<i>PEX26</i> †	1	0‡
Total		1006	287‡

*The number includes the cases of prenatal diagnosis

†The PCR primers were originally designed in our laboratory

‡The number indicates the cases of mutation.

phoresis and were purified by Ultrafree-DA centrifugal filter unit (Millipore, Billerica, MA). The sequence was determined by using BigDye Terminator v3.1 Cycle Sequencing Kit and capillary sequencer 3130xL Genetic Analyzer (Life Technologies).

SSCP

As described previously, PCR products were separated on 12% polyacrylamide gel at room temperature or 4 °C, and were visualized by silver staining.⁵

DHPLC

A WAVE-MD Mutation Detection System (Transgenomic, Omaha, NE) based on the DHPLC method was used to detect heteroduplex states according to the manufacturer's instructions.

Fragment analysis for triplet repeat diseases

PCR in a total volume of 10 µL contained 100 ng of DNA, 2.5 U AmpliTaq Gold DNA Polymerase, 250 µM dNTP, 10% DMSO, 1 µM each primer (forward and reverse), 0.1 µM of the fluorescent labeled probe (FAM, HEX or NED) and was run as follows: initial denaturation at 94 °C for 5 min, followed by 30 cycles with denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min, and final extension at 72 °C for 5 min. The PCR products were mixed with GeneScan 500 ROX Size Standard (Life Technologies). After incubation at 95 °C for 5 min, the samples were immediately cooled and electrophoresed by 3130xL Genetic Analyzer. Before 2005, ALFred DNA Sequencer (Amersham Pharmacia Biotech, Piscataway, NJ) was used.

MLPA

An MLPA kit (MRC-Holland, Amsterdam, the Netherlands) was used according to the manufacturer's instructions. In brief, 80 µg of DNA in 6% of glycerol was incubated at 98 °C for 15 min for initial denaturation. The final reaction mixture was electrophoresed by a 3130xL Genetic Analyzer using a 50 cm-long capillary POP-7.

Real-time PCR

When there was any inconsistency in the results of mutations in family members, real-time PCR was used to confirm the presence of deletions or duplications around the mutations.

Data analysis

Sequence data was analyzed by Sequence Scanner (Life Technologies) and Genetyx Software (Genetyx, Shibuya, Japan). Variant Reporter Software (Life Technologies) was used for detection of mutations from 2011. The mu-

Table 3. The number of cases subjected to genetic testing from January 2000 to September 2013

Year	Number of cases			Number of diseases
	Total	Tottori University Hospital	Other hospitals	
2000	6	6	0	2
2001	6	6	0	4
2002	2	2	0	1
2003	7	7	0	3
2004	18	18	0	7
2005	21	21	0	8
2006	44	25	19	13
2007	43	9	34	5
2008	87	18	69	17
2009	74	18	56	20
2010	128	55	73	24
2011	219	78	141	29
2012	198	70	128	26
2013*	153	54	99	27
Total	1006	387	619	

* January to September 2013.

tations were confirmed using the human genome mutation database (BIOBASE, Halchtersche, Germany) and SNP database. Prediction of functional effect of human nsSNPs (*PolyPhen*) was used for evaluation of novel mutations.⁶

Fragment analysis data was analyzed by Peak Scanner Software v1.0 (Life Technologies) for the repeat size. MLPA data was analyzed by Coffalyzer Software (MRC-Holland).

The personale and running processes of the facility

After October 2010, the working force to run the facility consists of 1 senior medical genetist, 1 assistant profes-

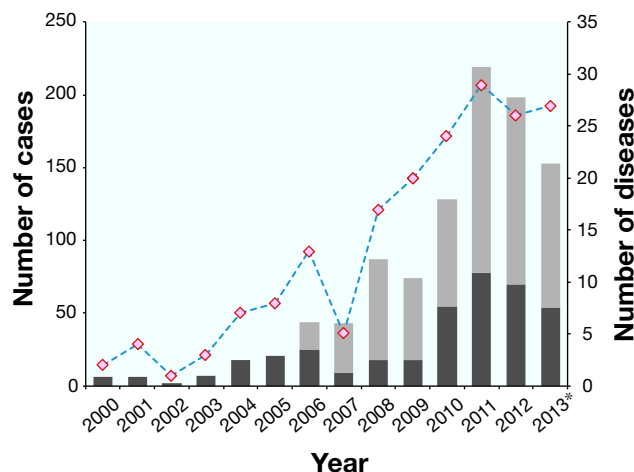


Fig. 1. The number of DNA samples subjected to genetic testing from January 2000 to September 2013. Vertical bars show the number of cases. Light gray shows the number from other hospitals, dark gray shows the number from Tottori University Hospital. Dots show the number of diseases.

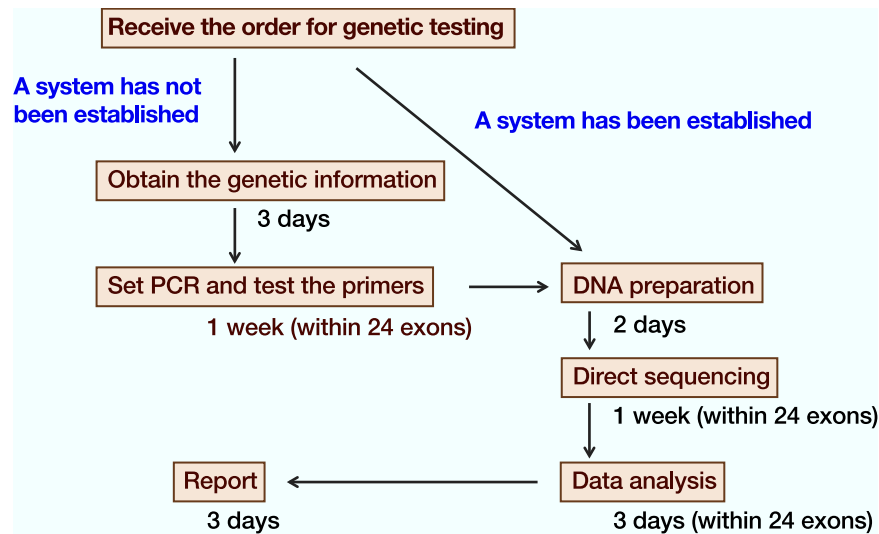


Fig. 2. A flow chart of the genetic diagnosis using direct sequencing of PCR products. When the system has not been established, it is necessary to obtain the genetic information and design new PCR primers. In this case, it takes at least 3 weeks to detect mutations. When the system has already been established, it takes 2 weeks.

sor and 6 part-time staff. The diagnostic procedures are separated into steps such as DNA preparation, PCR, purification, etc., each of which takes approximately 3 working hours, and the samples were processed sequentially by the part-time staff. The senior medical genetist is responsible for genetic counseling to the patients and family members, as well as conformation of mutations and preparation of the final report.

RESULTS

Distribution of the hospitals that requested for genetic testing

The hospitals that requested for genetic testing were located 43 in prefectures of Japan and in 11 foreign countries (Table 1).

The number of DNA samples subjected to genetic testing

A total of 1,006 genetic tests covering 62 diseases were performed from January 2000 to September 2013 (Table 2). The numbers of samples exceeded 50 in myotonic dystrophy, fragile X syndrome, Niemann-Pick disease type C and GM1-gangliosidosis. Original primers were constructed for 41 diseases. The number of genetic tests gradually increased year by year and did so rapidly after we introduced a new service system in 2010 (Table 3, Fig. 1). All samples were analyzed individually, and mutations were detected in 287 out of 1,006 with an average mutation detection rate of 28.5%. Prenatal diagnosis covered 13 diseases and 77 DNA samples were analyzed.

Flow chart of genetic testing

After 2010, it started to take at least 2 weeks for each genetic test to be completed. An additional one and a half weeks is required if it is necessary to settle new screening procedures (Fig. 2). *PolyPhen* software was used to predict whether any mutation detected was disease-causing or not.

DISCUSSION

We have received a number of requests for genetic testing from all over Japan. Institutes that provide genetic testing are rare, and our institute provides a unique function for the Japanese scientific and medical fields. Actually, genetic testing has contributed to several scientific reports.⁷⁻¹⁸ The number of cases of myotonic dystrophy was particularly high partly because the genetic testing was approved by Ministry of Health, Labour and Welfare of Japan as “highly advanced medical treatment”. It may also be because our facility is the only one approved for conducting prenatal diagnosis of this disease in Japan.¹⁹ The numbers of cases of fragile X syndrome, Niemann-Pick disease type C, GM1-gangliosidosis and other lysosomal storage diseases are also high because these diseases were the subjects of active research at Tottori University.²⁰⁻²²

Direct sequencing by a capillary sequencer is the main method of genetic testing and the size of the repeat was analyzed for triplet repeat diseases. MLPA was used for deletions or insertions of exons. For Joubert syndrome, 19 or more disease-causing genes were reported. Direct sequencing of every exon of all genes is impractical. In such cases, SSCP or DHPLC was used

for initial screening. These methods are prone to give false-negative results and it will be better to use next-generation sequencers,^{23–25} which were introduced to Tottori University in 2013.

The efficiency of genetic testing has improved and new lines of genetic testing have been enabled since 2010 by the improvement of running processes. Genetic testing is often still conducted based on research, and the expense is usually paid for by research funds. Because the expense is limited and genetic testing is necessary for clinical diagnosis of many inherited diseases, the costs should be included in medical expenses. At Tottori University Hospital, we have settled on basic original rules that enable us to refer the cost to the patient or client.

Genetic testing performed for research lacks the high quality required in clinics. In the United States and Europe, the quality of genetic testing is secured by Clinical Laboratory Improvement Amendments.²⁶ Unfortunately there is no guideline for quality control in Japan. At our facility, testing is performed according to the Standard Operating Procedures and the information is shared by each member. Genetic testing is now a popular and powerful tool for diagnosis of many inherited diseases. We should promote it further in the future.

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