

PAX6 Mutation as a Genetic Factor Common to Aniridia and Glucose Intolerance

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A paired homeodomain transcription factor, PAX6, is a well-known regulator of eye development, and its heterozygous mutations in humans cause congenital eye anomalies such as aniridia. Because it was recently shown that PAX6 also plays an indispensable role in islet cell development, a PAX6 gene mutation in humans may lead to a defect of the endocrine pancreas. Whereas heterozygous mutations in islet-cell transcription factors such as IPF1/IDX-1/STF-1/PDX-1 and NEUROD1/BETA2 serve as a genetic cause of diabetes or glucose intolerance, we investigated the possibility of PAX6 gene mutations being a genetic factor common to aniridia and diabetes. In five aniridia and one Peters' anomaly patients, all of the coding exons and their flanking exon-intron junctions of the PAX6 gene were surveyed for mutations. The results of direct DNA sequencing revealed three different mutations in four aniridia patients: one previously reported type of mutation and two unreported types. In agreement with polypeptide truncation and a lack of the carboxyl-terminal transactivation domain in all of the mutated PAX6 proteins, no transcriptional activity was found in the reporter gene analyses. Oral glucose tolerance tests revealed that all of the patients with a PAX6 gene mutation had glucose intolerance characterized by impaired insulin secretion. Although we did not detect a mutation within the characterized portion of the PAX6 gene in one of the five aniridia patients, diabetes was cosegregated with aniridia in her family, and a single nucleotide polymorphism in intron 9 of the PAX6 gene was correlated with the disorders, suggesting that a mutation, possibly located in an uncharacterized portion of the PAX6 gene, can explain both diabetes and aniridia in this family. In contrast, the patient with Peters' anomaly, for which a PAX6 gene mutation is a relatively rare cause, showed normal glucose tolerance (NGT) and did not show a Pax6 gene mutation. Taken

together, our present observations suggest that heterozygous mutations in the PAX6 gene can induce eye anomaly and glucose intolerance in individuals harboring these mutations. *Diabetes* 51:224–230, 2002

The development and differentiation of organs such as the pancreas require the coordinated activation of a unique set of transcription factors. In rodents, a homozygous disruption of genes encoding the islet cell-related transcription factors causes severe abnormalities in pancreas development and early death due to diabetes. Diabetes, though in a milder form, can also be seen in human individuals with a heterozygous gene mutation that leads to a haploinsufficiency of those transcription factors. To date, heterozygous mutations of IPF1 or NEUROD1, for example, are associated with maturity-onset diabetes of the young (MODY) or human type 2 diabetes (1,2), suggesting a gene-dosage effect for those transcription factors in humans.

A paired domain-containing transcription factor, Pax6, has recently emerged as a transcription factor regulating the differentiation of the endocrine pancreas. Disruption of the Pax6 gene in mice caused marked reduction of all four types of endocrine cells in the pancreas (3). Whereas Pax6 binds to a common cis element called PISCES (pancreatic islet cell enhancer sequences) shared by the glucagon, insulin, and somatostatin gene promoters and activates their transcription, the amount of hormone production from the remaining cells is also substantially decreased in Pax6 mutant mice. Indeed, even in a mouse with a heterozygous mutation of Pax6, the mRNA and protein levels of insulin in the pancreatic islets were reduced by 40 and 25%, respectively (4). Although the physiological significance of the reductions was not evaluated in those mice, they may potentially contribute to the onset of diabetes if present in individuals genetically or environmentally predisposed to diabetes. Also, support for the gene-dosage effect of Pax6 comes from the phenotype of a transgenic mouse overexpressing Pax6 in the pancreas; in the mouse, the increased Pax6 appears to induce islet neogenesis from hyperplastic epithelial cells of pancreatic ducts (5). Thus, it is likely that the PAX6 gene mutations, if seen in humans, may affect the function of the endocrine pancreas and thereby contribute to the onset of diabetes.

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ADA, American Diabetes Association; EMSA, electrophoretic mobility shift assay; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; IVTT, in vitro transcription and translation; MODY, maturity-onset diabetes of the young; NGT, normal glucose tolerance; OGTT, oral glucose tolerance test; PCR, polymerase chain reaction; PISCES, pancreatic islet cell enhancer sequences; SNP, single nucleotide polymorphism.

TABLE 1
PAX6 mutation in aniridia patients

Patient	Phenotype	Inheritance	Exon	Domain	Position	Mutation	Nucleotide change	Outcome
A	Aniridia	Familial	6	PD	590	7-bp Insertion (TAAACCG)	c.590ins7	TAA stop
B	Aniridia	Sporadic	7	LNK	790	4-bp Deletion (ATGA)	c.790del4	Frameshift→TAA stop
C	Aniridia	Familial	8	LNK	969	C→T	c.969C→T	R203X
D	Aniridia	Familial	8	LNK	969	C→T	c.969C→T	R203X
E	Aniridia	Familial				Not detected		
F	Peters' anomaly	Sporadic				Not detected		

ins, insertion; del, deletion.

Before its recent recognition as a pancreas factor, Pax6 had been known for years as a master regulator of eye development. Pax6 is expressed in the developing eye, nose, and central nervous system in addition to the pancreas, and mutations of the *Pax6* gene cause severe derangement in eye development in mammals as well as *Drosophila* (6–8). Although many of the islet cell-related transcription factors, such as *NeuroD1* and *Isl-1*, are also expressed and have functions in neural tissues, *Pax6* is a factor whose heterozygous mutation causes a developmental defect in the central nervous system of the affected animals. At least in some mammals, mutant alleles of *Pax6* are semidominant, because they cause various eye anomalies in individuals harboring the mutant allele in heterozygotes. In rodents, the heterozygous mutation causes a phenotype called “small eye” and, in humans, it typically causes aniridia, although there are cases of Peters' anomaly, keratitis, or isolated foveal hypoplasia (9–11).

Aniridia is a rare congenital eye anomaly characterized by the almost complete absence of the iris, often associated with cataracts, optic nerve hypoplasia, and glaucoma (12). Approximately one-third of aniridia cases are sporadic, but two-thirds are familial, with an autosomal dominant inheritance. In the familial cases, the penetrance is high, although the expressivity is variable. Importantly, *PAX6* gene mutations are the only cause identified to date (13). Peters' anomaly is an even rarer congenital eye anomaly characterized by dysgenesis of the ocular anterior segment and central corneal opacity. Although *PAX6* gene mutations were shown to be a cause of this disease in a few cases, the genetic cause is not known in most of the cases.

In this study, we tried to evaluate the possible significance of the *PAX6* gene mutation as a cause of glucose intolerance or diabetes. After screening for a *PAX6* gene mutation in patients with aniridia and Peters' anomaly, glucose tolerance was evaluated, and all of the aniridia patients studied were found to display glucose intolerance. In the family of one subject with both aniridia and diabetes, diabetes was very well cosegregated with aniridia. Although we were unable to identify a *PAX6* gene mutation in this patient, there was a correlation between a single nucleotide polymorphism (SNP) within the *PAX6* gene and the diseases in her family. Thus, our present observations indicate that a heterozygous *PAX6* gene mutation, which causes aniridia in humans, can also be a cause of glucose intolerance in affected individuals.

RESEARCH DESIGN AND METHODS

We recruited six unrelated Japanese patients diagnosed as having aniridia or Peters' anomaly by ophthalmologists. Four patients had familial aniridia, one had sporadic aniridia, and one had sporadic Peters' anomaly (Table 1). After obtaining written consent to participate in our study, we performed a general physical examination, blood sampling for DNA extraction and biochemical measurements, and an oral glucose tolerance test (OGTT). We also obtained written consent for participation from one family with familial aniridia. To diagnose diabetes, impaired fasting glucose (IFG), and impaired glucose tolerance (IGT), we used the American Diabetes Association (ADA) criteria. The study was approved by the ethical committee of Osaka University and was in accordance with the principles of the Helsinki Declaration.

Mutation screening. Genomic DNA was obtained from peripheral leukocytes using a QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA). The entire coding exons (exons 4–13), including the flanking exon-intron junctions of the *PAX6* gene, were amplified from the genomic DNA using previously reported polymerase chain reaction (PCR) primers and conditions (14). We purified the PCR products using centrifugal filter devices (Millipore, Tokyo) according to the manufacturer's instructions and sequenced them directly by the dideoxy-chain termination method using an ABI Prism 310 (PE Applied Biosystems, Osaka, Japan). To confirm the sequence of mutations, we subcloned each PCR product into the TA cloning vector pCR2.1 (Invitrogen, Carlsbad, CA) and sequenced it.

Plasmid construction. Full-length human *PAX6* cDNA (provided by Richard Maas) was cloned into an expression plasmid pcDNA3 to produce the *PAX6* expression vector pcDNA3-PAX6. To generate the mutated *PAX6* expression vectors, pcDNA3-c.590ins7, pcDNA3-c.790del4, and pcDNA3-c.969C→T, we used a Quick Change Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). All mutagenized constructs were checked by sequencing. The Pax-responsive firefly luciferase (Luc) reporter construct (Pax)₃TKLuc was described previously (15).

Electrophoretic mobility shift assays. Proteins were prepared by in vitro transcription and translation (IVTT) using a TNT-coupled reticulocyte lysate system (Promega, Madison, WI). Electrophoretic mobility shift assays (EMSAs) were then performed using the G3 element of the glucagon gene promoter, a putative target sequence for PAX6. The sense strand of the sequence was as follows: GluG3;5'-GTAGTTTTCACGCCTGACTGAGATTGA AGGGT-3'. The double-stranded oligonucleotide probe was end-labeled with [γ -³²P]ATP using T4 polynucleotide kinase. The binding reaction was performed as described previously using 2 μ g each of proteins prepared by IVTT (15). The electrophoresis was performed on 6% nondenaturing polyacrylamide gels in 0.5 \times TBE (44.5 mmol/l Tris, 44.5 mmol/l borate, and 1 mmol/l EDTA) for 90 min at 150V at 4°C.

Transient transfections and luciferase assays. An expression plasmid for wild-type PAX6 (pcDNA3-PAX6), mutant PAX6, or the mock vector (pcDNA3) (50 ng) was cotransfected into COS7 cells using LipofectAMINE reagent (Life Technologies, Rockville, MD) with the reporter plasmid (Pax)₃TKLuc (1 μ g) and a Renilla luciferase expression vector, pRL-TK (10 ng). At 48 h after transfection, the cells were harvested and firefly and Renilla luciferase activity were measured following the manufacturer's protocol (Toyo Ink, Tokyo). The firefly luciferase data were normalized with respect to transfection efficiencies evaluated by the Renilla luciferase activity (Toyo Ink).

RESULTS

Detection of PAX6 gene mutations in aniridia patients. Genomic DNA was isolated from the peripheral blood of five aniridia patients and one Peters' anomaly

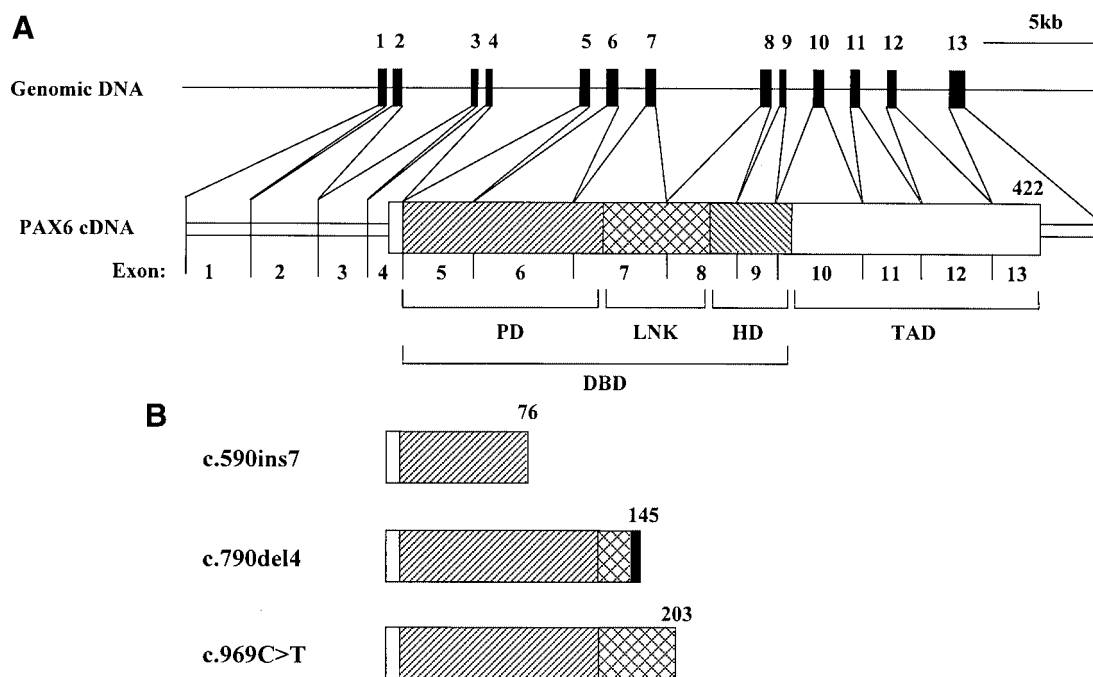


FIG. 1. Structures of human *PAX6* gene/cDNA and mutants. **A** (top): Diagram of the human *PAX6* gene. All exons (exons 1–13) are indicated. **A** (bottom): Diagram of the human *PAX6* cDNA. The coding regions are indicated by the wide bar and 5'- and 3'-untranslated regions by the thin bars. DBD, DNA-binding domain; PD, paired domain; LNK, linker region; HD, homeodomain; TAD, transactivation domain. **B**: Diagram of mutant *PAX6* cDNAs. The blackened area shows the altered open reading frame beyond the mutation site.

patient and screened for *PAX6* gene mutations. Using direct DNA sequencing, all of the coding exons and the flanking exon-intron junctions of the *PAX6* gene were examined, and three kinds of mutations were identified in four aniridia patients (Table 1). Figure 1 depicts the structures of the mutant *PAX6* cDNAs identified in aniridia patients. The human *PAX6* gene encodes a 422-amino acid protein comprised of multiple functional domains. The DNA-binding function is mediated by a paired domain and a paired-type homeodomain (residues 4–131 and 210–269, respectively) that are separated by a linker region (residues 132–209). Located in the COOH-terminal region of the protein is the transactivation domain (residues 270–422). The c.590ins7 mutation in patient A is a newly identified 7-bp insertion (TAAACCG) just after codon 76 of exon 6 (Fig. 1 and Table 1). This insertion contains a premature termination codon (TAA) and thereby generates a truncated protein that lacks part of the paired domain and all of the other domains. In patient B, the c.790del4 mutation was identified as a new type of *PAX6* gene mutation. The c.790del4 mutation is a deletion of four nucleotides (ATGA) at codon 143 of exon 7 that leads to a frame-shift of amino acid codons and creates a premature termination codon (TAA) within the linker region just two codons downstream of the deletion site (Fig. 1 and Table 1). The resulting truncated protein contains only the paired domain and some residues of the linker region and lacks the rest. The c.969C→T mutation seen in patient C and D was a C→T substitution at codon 203 that converts an Arg codon (CGA) to a termination codon (TGA). This also creates a truncated protein similar to that of c.790del4, with a slightly longer linker region (Fig. 1 and Table 1). Only the c.969C→T mutation was previously reported in several aniridia cases (16,17).

In one aniridia patient and one Peters' anomaly patient, we were not able to detect any mutation within the coding sequences or exon-intron junctions of *PAX6*. We thus surveyed the 346-bp DNA fragment of the promoter region and 5'-noncoding sequences of the *PAX6* gene for possible mutations. Moreover, to search for a chromosomal deletion or rearrangement within the region of 11p13 where *PAX6* is located, we performed Southern blot and microsatellite polymorphism analyses. The Southern blot used a *PAX6* cDNA probe and gave exactly the same pattern of positive bands as obtained with normal subjects (data not shown). The microsatellite polymorphism analysis used three polymorphic markers located between the *PAX6* and *WT1* loci (*PAX6*, *D11S929*, and *D11S914*) (18) and detected no loss of heterozygosity in any of the markers (data not shown). Thus, no apparent deletion or rearrangement of the chromosomal locus for the *PAX6* gene could be found. **Evaluation of DNA-binding activity of mutant *PAX6* proteins.** To evaluate the function of the mutant *PAX6* proteins identified in the aniridia patients, we first examined the DNA-binding capacity in gel-mobility shift analyses. A radiolabeled double-stranded oligonucleotide probe reproducing the G3 element of the glucagon gene was allowed to bind to either wild-type or mutant *PAX6* proteins that had been produced by IVTT. All of the wild-type and mutant (truncated) *PAX6* proteins could be produced properly according to the size of the radiolabeled proteins (data not shown).

As shown in Fig. 2, the wild-type *PAX6* revealed a markedly retarded band that competed very well against the addition of cold competitors. Although they had different mobilities, two of the mutant *PAX6* proteins, c.790del4 and c.969C→T, but not the other mutant, c.590ins7, revealed specifically formed protein-DNA com-

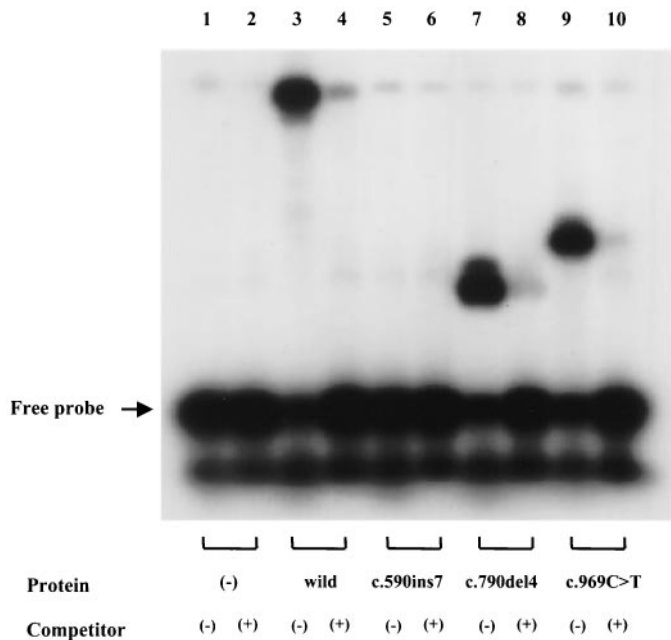


FIG. 2. DNA-binding capacity of mutant PAX6 proteins. EMSAs for the mutant PAX6 proteins are shown. An equal amount of protein yielded by IVTT was applied to each lane; wild-type PAX6 in lanes 3 and 4, mutant PAX6 (c.590ins7) in lanes 5 and 6, (c.790del4) in lanes 7 and 8, and (c.969C→T) in lanes 9 and 10. The glucagon G3 element (GluG3) is used as a probe for each lane. Unlabeled probes were applied in 50-fold excess to lanes 2, 4, 6, 8, and 10 as specific competitors. Reticulocyte lysate (2 μ l) was applied to lanes 1 and 2 as negative controls. Identical results were obtained in three independent experiments.

plexes, suggesting that DNA-binding capacity is preserved in c.790del4 and c.969C→T mutants but not in the c.590ins7 mutant. This finding agrees with the observation that c.790del4 and c.969C→T mutants preserve an intact paired domain, whereas the c.590ins7 mutant lacks both the paired domain and the homeodomain required for DNA binding. Because of the lack of a transactivation domain, those two mutants with preserved DNA-binding capacity (c.790del4 and c.969C→T) may function in a dominant-negative manner in vivo.

Evaluation of the transactivation potential of mutant PAX6 proteins. Transcriptional activity of the mutant PAX6 proteins were evaluated by reporter gene analyses (Fig. 3). Plasmids expressing either the wild-type or a mutant PAX6 were cotransfected into COS7 cells together with a luciferase reporter plasmid containing five copies of high-affinity Pax6 binding sites upstream of the minimal thymidine kinase promoter. As shown in Fig. 3, wild-type PAX6 protein activated the luciferase reporter by ~2.5-fold. In contrast, mutant PAX6 proteins did not exert any significant effects on the promoter activity of the reporter construct, in agreement with the observation that all of the mutant PAX6 proteins lacked the transactivation domain.

Evaluation of glucose tolerance in patients with aniridia. To determine whether the PAX6 gene mutations causing aniridia can also cause glucose intolerance, we examined plasma glucose and insulin levels using OGTTs. One aniridia patient (patient E) was excluded because she had overt diabetes and was on insulin injection therapy.

As shown in Table 2, all of the nonovertly diabetic aniridia patients (patients A–D), who carried the PAX6

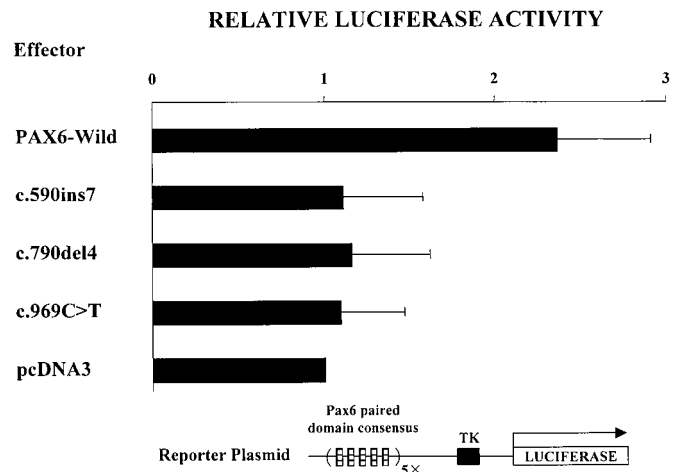


FIG. 3. Transcription properties of mutant PAX6 proteins. Wild-type or mutant PAX6 (50 ng) was transfected into COS7 cells with 1 μ g reporter plasmid [(Pax)₅TKLuc] and 10 ng pRI-TK. At 48 h after transfection, the cells were harvested and firefly luciferase activity was measured. Transfection efficiencies were normalized with respect to Renilla luciferase activity. Each experiment was repeated four times, and data are expressed as the means \pm SE.

gene mutations, displayed glucose intolerance (patient A: IFG; patients B, C, and D: IGT), whereas the patient with Peters' anomaly (patient F), in whom no apparent PAX6 gene mutation was found, had NGT. None of the aniridia patients, including those with relatively high BMI values (patients C and D) (Table 2), were hyperinsulinemic. Instead, the insulinogenic index, which can be defined as the ratio of the increment of immunoreactive insulin (IRI) to the increment of plasma glucose (PG) 30 min after a glucose load (Δ IRI_{0–30 min}/ Δ PG_{0–30 min}) (19), was relatively low or close to the lower limit of the normal range (>0.4) in the aniridia patients. According to previous reports with Japanese subjects, an insulinogenic index <0.4 manifests early-phase insulin deficiency and is a strong predictor of the development of type 2 diabetes (20–22). Thus, it was suggested that a β -cell defect, rather than insulin resistance, is the cause of the glucose intolerance in these patients.

Cosegregation of aniridia and diabetes in pedigree of patient E. Patient E was diagnosed as being diabetic at the age of 22 years. Her diabetes continued to progress, and she began requiring insulin when she was 31 years old. As is usually the case with poorly controlled diabetes, she had suffered from microangiopathy/triopathy for the 6 years before the study. She had neither elevated levels of GAD antibody nor a history of ketosis. Written consent was obtained from family members of this patient to conduct some genetic and laboratory analyses.

As shown in Fig. 4, the proband (patient E) and her father had aniridia and diabetes. Although one subject in this family (subject 3) revealed IGT, only those two who had aniridia revealed a diabetic pattern in OGTT (Fig. 4 and Table 3). This cosegregation of aniridia with diabetes in two of the family members suggests that a certain inherited genetic disorder may cause both aniridia and diabetes in this family.

Although we could not detect mutations within the surveyed portion of the PAX6 gene, we found an SNP in intron 9 of the gene. This cannot be directly associated

TABLE 2
Clinical characteristics of aniridia patients

Patient	Age (years)	Sex	BMI (kg/m ²)	Glucose levels (mg/dl)			Insulin levels (μU/ml)			Status	Insulinogenic index
				0'	30'	120'	0'	30'	120'		
A	54	F	23.1	118	149	121	7	17	17	IFG	0.32
B	29	M	16.8	93	145	142	2	24	34	IGT	0.38
C	37	F	25.5	102	173	147	9	39	47	IGT	0.42
D	28	F	28.7	115	203	154	9	39	44	IGT	0.34
*E	37	F	19.1	—	—	—	—	—	—	D	—
F	38	M	19.8	85	149	80	5	40	42	NGT	0.54

*Patient E has diabetes and is undergoing treatment with insulin. D, diabetic.

with the onset of aniridia or diabetes because individuals with the same genotype as the proband (GT) or her father (GG) did not necessarily develop the diseases. However, the pattern of inheritance of this marker (G) does not reject the hypothesis that a mutant gene allele of *PAX6* is responsible for the onset of aniridia and diabetes in this family (Fig. 4). Thus, it is still possible that the aniridia patients in this family have a mutation in the *PAX6* gene within a portion of the gene that has not been investigated.

DISCUSSION

In the present study, screening of five unrelated aniridia patients showed that they all had glucose intolerance or diabetes. In 1993, Sekikawa et al. (23) investigated the prevalence of the then "impaired glucose intolerance," which included both IGT and IFG according to current ADA/World Health Organization diagnostic criteria, and diabetes in 868 Japanese subjects who were >45 years old (BMI [mean ± SD] 23.4 ± 3.4 kg/m² for men and 24.2 ± 3.5 kg/m² for women) (Funagata Study). They found that 15.3% (11.9% in men and 16.6% in women) had either IGT or IFG, and 10.4% (8.8% in men and 14.0% in women) had

diabetes according to the current diagnostic criteria. Considering that the average age of our aniridia subjects (37 years old) was much lower than that of the Funagata population (median age 55–64 years) (23), aniridia patients seem to have a high prevalence of glucose intolerance.

The developmental processes of the ectoderm-derived neural tissues and the endoderm-derived pancreas share some key transcription factors, such as Isl-1, NeuroD1/Beta2, and HB9 (24). When those transcription factors are totally lost (e.g., as a result of homozygous gene disruption), there is a clear phenotype in both neural tissues and the pancreas (25–28). However, in the case of a heterozygous disruption, the effect varies. For example, the heterozygous gene mutation of *NEUROD1/BETA2* in humans causes early onset diabetes (MODY), but no neural phenotype has been reported to date, indicating that the gene-dosage effect is evident only in the pancreas and not in the brain. In terms of *PAX6*, the situation was different: heterozygous gene mutations of *PAX6* in humans have been known for years to be a cause of aniridia and some other eye anomalies, whereas no data were available for their effects on the pancreas. The OGTT data obtained with nonovert diabetic aniridia patients in this study indicated that they appear to develop glucose intolerance because of a defect in pancreatic β-cells rather than because of insulin resistance, suggesting that there is a gene-dosage effect of *PAX6* on the function of the endocrine pancreas as well as on eye development.

Among the five aniridia patients, four had mutations within the coding sequences for *PAX6*; however, we could not detect any mutations in one patient with diabetes. The possibility of a chromosomal deletion or rearrangement of

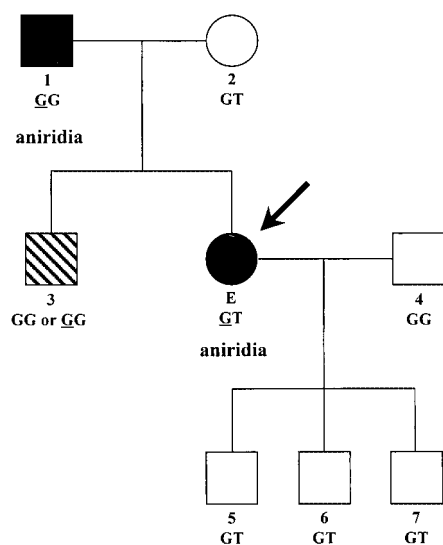


FIG. 4. Cosegregation of aniridia and diabetes in the pedigree of patient E. ■ And ●, male and female subjects, respectively, with type 2 diabetes; ▨, male subjects with IGT; ○ and □, female and male subjects, respectively, with NGT. The clinical characteristics for each subject are presented in Table 3. Numbers shown below the symbols match the subject's number in Table 3. Genotypes for the G/T SNP identified in *PAX6* intron 9 are also shown. The allele, which may carry a mutation responsible for the diseases, is underlined. An arrow indicates the proband.

TABLE 3
Clinical characteristics of family members of patient E

Subject	Age (years)	Sex	BMI (kg/m ²)	Status	Glucose levels (mg/dl)		
					0'	30'	120'
1	65	M	25.4	D	95	202	216
2	65	F	27.4	NGT	95	178	126
3	34	M	20.8	IGT	88	192	160
E	37	F	19.1	D	—	—	—
4	40	M	23.7	NGT	87	141	124
5	13	M	20.2	NGT	88	120	128
6	12	M	19.7	NGT	85	143	130
7	6	M	16.1	NGT	76	109	85

D, diabetic.

the *PAX6* gene locus, which in some cases causes a contiguous genetic syndrome, WAGR (Wilms' tumor, aniridia, genitourinary malformation, and mental retardation) syndrome, was also denied because the Southern blot analyses revealed exactly the normal band pattern, and microsatellite polymorphism analyses identified no loss of heterozygosity. Also, we found no mutations in the 346-bp fragment of the *PAX6* gene promoter by a direct sequencing analysis. However, this does not necessarily mean that aniridia is caused by some genetic factors other than the *PAX6* gene mutations in the patient. According to the Human *PAX6* Allelic Variant Database (found online at <http://www.hgu.mrc.ac.uk/Softdata/PAX6/>), *PAX6* mutations can be found in ~80% of aniridia patients. However, no other genetic cause that explains the aniridia in the remaining 20% has been identified (29). Thus, it is still possible that *PAX6* gene mutations are the only genetic cause for aniridia, although some mutations tend to be hidden in unscreened portions of the gene, such as 5'- and 3'-untranslated sequences, introns, or regulatory sequences further upstream. Because of the cosegregation of aniridia and diabetes in the family of patient E, we are tempted to consider that a mutation that causes both aniridia and diabetes in the patient and her father may be located in the uncharacterized portion of the *PAX6* gene (Fig. 4). Support for this comes from the inheritance of an SNP in the *PAX6* gene within the family, which does not dispute the hypothesis that a hidden mutation of *PAX6* gene is the cause of aniridia and diabetes in the family.

A patient with Peters' anomaly (patient F) who was also included in this study did not show glucose intolerance (Table 2). This clearly contrasts with the phenotype of the aniridia patients, all of whom display glucose intolerance, including diabetes. Although Hanson et al. (9) showed that Peters' anomaly can be caused by a missense mutation of the *PAX6* gene, the *PAX6* gene mutation does not seem to be a common cause of the disease. Calvas et al. (30) investigated the entire *PAX6* coding region in four patients with Peters' anomaly but were not able to find any mutations. Furthermore, Churchill et al. (31) investigated 15 individuals with Peters' anomaly and also found no mutations within the coding sequences of *PAX6*. Because we also failed to detect any mutations or gene rearrangement of the *PAX6* gene in our Peters' anomaly patient (patient F), the disease in the patient is not likely to be correlated with a *PAX6* gene mutation.

This study added two new types of mutations to the list of *PAX6* gene mutations and detected one previously identified mutation. All of the three mutant types give rise to truncated polypeptides lacking the carboxyl-terminal transactivation domain. However, the character of those mutants might differ because one of them (c.590ins7) has totally lost its DNA-binding capacity, whereas the other two preserve it. Although it needs to be defined whether the two mutants (c.790del4 and c.969C→T), which have an intact paired domain but lack a homeodomain, can indeed bind to DNA *in vivo* or not, there may be a dominant-negative effect in those mutants. However, we would like to note that, in agreement with previous studies that denied the correlation between the type of *PAX6* mutation and the severity of aniridia (32,33), there was no

difference in the degree of iris defect among the subjects (T.Y., Y.K., S.Y., unpublished observations).

What remains to be elucidated is how a *PAX6* gene mutation can cause glucose intolerance represented by a decrease in early insulin secretion if a *PAX6* mutation is indeed a common cause of aniridia and glucose intolerance. According to an observation by Sander et al. (4), mice with a heterozygous mutation of *Pax6* had 40% lower insulin mRNA and 25% less insulin content in their β -cells. This is consistent with the fact that Pax6 is a binding factor for the PISCES element and is thereby involved in the production of endocrine hormones such as insulin in the pancreas. In general, humans have a much longer life span than rodents, and this may be important for amplifying the effects of relatively weak gene mutations, such as a heterozygous gene mutation, to a level that can lead to disease onset. Although a 25% decrease in insulin content is not likely to cause immediate glucose intolerance in mice, it may become pathophysiologically significant during aging in humans.

All of the aniridia patients with evident *PAX6* gene mutations (patients A–D) revealed glucose intolerance but did not develop diabetes until at least 28–54 years of age, suggesting that the *PAX6* gene mutations alone are not potent enough to induce diabetes in affected humans. In contrast, overt diabetes was observed in patient E, although the involvement of a *PAX6* gene mutation in this case has yet to be determined. This difference may depend on whether there were some genetic background for diabetes/glucose intolerance independent of *PAX6* gene mutations or aniridia in those patients. In the family of patient E, apart from the two patients with aniridia (patient E and her father [subject 1]), her brother (subject 3) was glucose intolerant, despite having no anomaly in eye development (Fig. 4). In contrast, although we were not allowed to obtain laboratory data from the family members of patients A–D, there was no family history of diabetes in their families, suggesting that a common genetic background for diabetes was not prevalent in their families. Thus, a *PAX6* gene mutation do not seem to cause diabetes by itself, but when combined with other diabetes-associated genes and environmental factors such as aging or obesity, it may contribute to the onset of diabetes.

In conclusion, we identified *PAX6* gene mutations as a possible cause common to aniridia and glucose intolerance in humans. Whereas various transcription factors are involved in the development of pancreas and neural tissue, *PAX6* may be the first gene whose heterozygous mutation provokes the defect of neural tissue and islets. To confirm our present observations and further elucidate the role of *PAX6* mutations in causing glucose intolerance, more aniridia patients and their families need to be recruited and studied.

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