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ORIGINAL INVESTIGATION

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# Population-based risk estimates of Wilms tumor in sporadic aniridia

## A comprehensive mutation screening procedure of PAX6 identifies 80% of mutations in aniridia

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**Abstract** Aniridia is a severe eye disease characterized by iris hypoplasia; both sporadic cases and familial cases with an autosomal dominant inheritance exist. Mutations in the *PAX6* gene have been shown to be the genetic cause of the disease. Some of the sporadic cases are caused by large chromosomal deletions, some of which also include the Wilms tumor gene (WAGR syndrome), resulting in an increased risk of developing Wilms tumor. Based on the unique registration of both cancer and aniridia cases in Denmark, we have made the most accurate risk estimate to date for Wilms tumor in sporadic aniridia. We have found that patients with sporadic aniridia have a relative risk of 67 (confidence interval: 8.1–241) of developing Wilms tumor. Among patients investigated for mutations, Wilms tumor developed in only two patients out of 5 with the Wilms tumor gene (*WT1*) deleted. None of the patients with smaller chromosomal deletions or intragenic mutations were found to develop Wilms tumor. Our observations suggest a smaller risk for Wilms tumor than previous estimates, and that tumor development requires deletion of WT1. We report a strategy for the mutational analysis of aniridia cases resulting in the detection of mutations in 68% of sporadic cases and 89% of familial cases. We also report four novel mutations in *PAX6*, and furthermore, we have discovered a new alternatively spliced form of *PAX6*.

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## Introduction

Aniridia is a panocular disease affecting 1 in 50,000–100,000 individuals. It is characterized by an overall underdevelopment of the eye with a lack of the iris, often associated with cataracts, optic nerve hypoplasia, and glaucoma. It exists both as sporadic cases and as familial cases with an autosomal dominant mode of inheritance, with high penetrance but variable expressivity. A considerable number of the sporadic cases have large chromosomal deletions in 11p13 detectable by fluorescence in situ hybridization (FISH) analysis, and some are also visible with conventional cytogenetic banding. The large chromosomal deletions are associated with WAGR syndrome (Wilms tumor, aniridia, genitourinary abnormalities, and mental retardation; for a review, see Hanson and van Heyningen 1995). As many as 33% of cases with deletions have been reported to develop Wilms tumor (van Heyningen and Hastie 1992). However, this was based on either small numbers (Drechsler et al. 1994) or selected material (Fraumeni and Glass 1968).

Aniridia is caused by mutations in the *PAX6* gene (Hill et al. 1991; Glaser et al. 1992), which was cloned in 1991 (Ton et al. 1991). Several *PAX6* isoforms exist arising from alternative splicing (Carriere et al. 1993; Epstein et al. 1994; Jaworski et al. 1997; Grønskov et al. 1999). *PAX6* is a universal master control gene for eye development, and homologs are known from both mammals, amphibians, fish, amphioxus, sea squirts, sea urchins, squid, nematodes, ribbonworms, and planarians (for a review, see Gehring and Ikeo 1999).

Human *PAX6* spans 22 kb on the short arm of chromosome 11 (11p13) and consists of 14 exons (Glaser et al. 1992). The encoded protein contains a paired box and a paired-type homeobox separated by a linker region. The paired box contains two functional sub-domains, the N-terminal domain and the C-terminal domain, which both show DNA-binding capabilities. Furthermore, the *PAX6* protein contains a proline, serine, and threonine rich (PST) domain, which resembles the activation domain of

transcription factors CTF-1 (CAAT-box binding transcription factor; Mermod et al. 1989) and Oct2 (Tanaka and Herr 1990). Transcripts have been detected in the eye, brain, and pancreas (Ton et al. 1991).

In Denmark, a unique opportunity exists to ascertain practically all cases of aniridia, since the National Eye Clinic for the Visually Impaired (NEC) has an almost complete registry of Danish patients with aniridia dating back to the last century and including both sporadic cases without a family history and familial cases with a dominant inheritance.

The purpose of this study has been to elucidate genotype-phenotype correlations by calculating the risk of developing Wilms tumor for the sporadic cases and to develop a mutation detection strategy for analyzing patients with aniridia. Based on data in the Danish Cancer Registry, we report a lower risk of having Wilms tumor for sporadic aniridia cases than previously claimed. Furthermore, we describe a mutation detection strategy, demonstrating the necessity to use several methods in a structured manner in order to identify as many mutations as possible. We report four novel mutations and a new alternatively spliced form of *PAX6*, which is present in normal control individuals.

## Subjects and methods

### Epidemiological survey of patients with aniridia

Patients with aniridia were retrieved from the diagnostic index and the files at the NEC dating back to 1880. The retrieval of the oldest data was facilitated by the use of deposited original thesis material (Møllenbach 1947).

Since 1920, the registration of children with severe visual impairments has been compulsory. The NEC runs the registry, and a medical file on each child has been established (Rosenberg 1987). This registry is considered almost complete with regard to aniridia. Accordingly, 1920 was chosen as the cut-off year for the prevalence calculations.

By means of their full name and date of birth, patients born 1920–1996 were linked to the Central Population Registry, established April 1, 1968, for the verification of their personal identification number and for information on vital status and migration. The personal identification number, which is unique to every Dansih citizen, incorporates sex and date of birth and permits the accurate linkage of information obtained through record linkage to the Danish Cancer Registry, which, since 1943, has collected data on all patients with cancer in Denmark (Storm et al. 1997). Four patients who had died before the start of the Central Population Registry were verified by death certificates obtained from the national mortality files and subsequently linked to the Cancer Registry by use of their name and date of birth.

### Cancer risk analysis

136 patients with aniridia born 1920–1996 were linked with the Danish National Cancer Registry with follow up until 1996. Of a total of the 147 patients with aniridia, nine were born after 1996, one was born in Greenland, and one died before 1943, and therefore these patients were excluded from this analysis. The expected number of cancers were calculated by multiplying the number of person-years at risk among patients by the sex-specific cancer incidence rates for the entire Danish population in 5-year age groups and calendar periods of observation during the period 1943–1996. The ratio of observed-to-expected cancers, serving as a measure of the relative risk, and 95% confidence intervals (CIs) were calculated, assuming a Poisson distribution of the observed cancers (Rothman and Boice 1979; Bailar and Ederer 1964).

### Mutational analysis

#### *FISH analysis and karyotyping*

Conventional cytogenetic preparations were made from phytohemagglutinin-stimulated peripheral blood. All cases were karyotyped by using routine Q-banding. In addition, FISH analysis was carried out with cosmids FAT5 (*AN2*) (11p14.1) and B2.1 (*WT1*) (11p14.1) derived from the *PAX6* gene and *WT1*, respectively, (Fantes et al. 1992). An intervening cosmid p60 (DXS324) (11p14.1) was also used to cover the interval between *PAX6* and *WT1* (Fantes et al. 1995). In one case (see below), the cosmids A1255 (11p14.1) (V. van Heyningen, personal communication) and H11148 (11p14.1) (Fantes et al. 1995) were also used. The position of the cosmids relative to each other can be seen in Fig. 1B. The cosmids were labeled with biotin by using a nick-translation kit following the manufacturer's protocol (Roche Molecular Biochemicals). The probes were preannealed with Cot1 DNA in the hybridization mix, denatured for 5 min at 75°C, and added to the denatured chromosomal slides. Hybridization was carried out overnight. Signals were detected by using 2–3 rounds of amplification with fluorescein-isothiocyanate-conjugated avidin and anti-avidin antibodies. The chromosomal slides were counterstained with propidium iodide and 4,6-diamidino-2-phenylindole. The chromosomes were viewed by using Leica FISH station Q550CW with a DMRXA microscope equipped with appropriate filters. A minimum of 20 metaphases was analyzed.

#### *DNA extraction, polymerase chain reaction, and dideoxy fingerprinting*

Genomic DNA was isolated from blood leukocytes by a standard salting-out method (Miller et al. 1988). The polymerase chain reaction (PCR) and dideoxy fingerprinting were performed as previously described (Grønskov et al. 1999). DNA was sequenced with the cycle-sequencing kit from Amersham according to the manufacturer's instructions, with radioactive end-labeled primers. All single nucleotide substitutions, except when located in splice sites, were confirmed by sequencing a second PCR product.

#### *Reverse transcription/PCR of illegitimate transcripts in lymphoblastoid cells*

Total RNA was isolated from Epstein-Barr-virus-transformed lymphocytes from patients with splice site mutations and normal controls by using the RNeasy mini kit from Qiagen following the manufacturer's instructions. The RNA (approximately 10 μg) was treated with 10 U DNaseI (amplification grade from Gibco, Life Science), following the manufacturer's instructions; 1 μg total RNA was reverse transcribed by using random hexamer primers and the SuperscriptII enzyme from Gibco (Life Science) following the manufacturer's instructions.

Nested reverse transcription-PCR (RT-PCR) was performed with respect to three regions: fragment 1 from nucleotides –51 to 962, fragment 2 from nucleotides 742 to 1298, and fragment 3 from nucleotides 1248 to 1622 (nucleotide numbering according to Ton et al. 1991) with the primers listed below: fragment 1 first PCR: forward primer: 1F: 5'-CAG AGG TCA GGC TTC GCT AA-3', reverse primer C130: 5'-CTT TCT CCA GGG CCT CAA T-3' (Hanson et al. 1993); nested PCR: forward: 4F-N: 5'-GGT CAG GCT TCG CTA ATG GG-3', reverse primer C860: 5'-AGC CTC ATC TGA ATC TTC TCC-3' (Hanson et al. 1993); fragment 2 first PCR: forward primer 794F: 5'-TTC TTC GCA ACC TGG CTA GC-3', reverse primer 1356R: 5'-GGA GCC AGA TGT

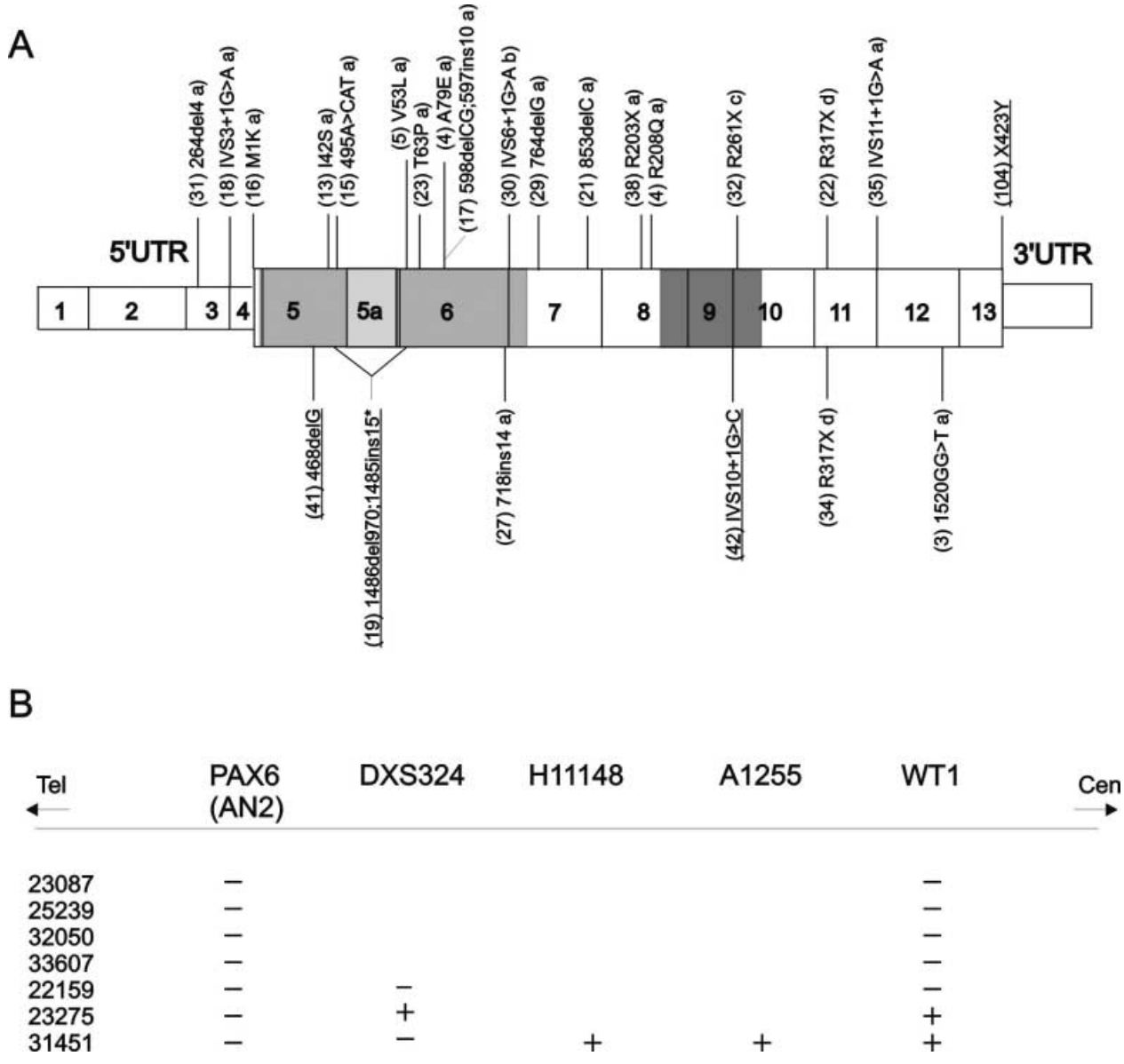


**Fig. 1** **A** Localization of intragenic mutations in *PAX6*. Mutations listed *above* and *below* the gene are found in familial and sporadic cases, respectively. *Light gray box* Position of the paired domain (the alternatively spliced exon 5a is shaded in a *lighter gray*), *dark gray box* position of the homeodomain, *asterisk* genomic deletion, *numbers* according to EMBL acc. no. Z83307. *Numbers* in *brackets* in *front* of the mutations indicate the patient ID. *a)* Grønskov et al. 1999, *b)* Hanson et al. 1993, *c)* Martha et al. 1995, *d)* Davis and Cowell 1993. **B** Schematic representation of chromosomal deletions detected by FISH analysis (– signal on one chromosome, + signal on both chromosomes. *Numbers* on the *left* refer to the patient ID

GAA GGA GG-3'; nested PCR: forward primer 797F-N: 5'-TCG CAA CCT GGC TAG CGA AA-3', reverse primer 1353R-N: 5'-CCA GAT GTG AAG GAG GAA AC-3'; fragment 3 first PCR: forward primer 1300F: 5'-GTC TAC CAA CCA ATT CCA CAA CC-3', reverse primer 1680R: 5'-TAA TCT TGG CCA GTA TTG AGA CAT ATC-3'; nested PCR: forward primer: 1303F-N: 5'-ACC AAC CAA TTC CAC AAC CCA CC-3', reverse primer: 1677R-N: 5'-CTT GGC CAG TAT TGA GAC ATA TCA GGT-3'.

An aliquot of 2 µl cDNA was amplified in a 20-µl volume in standard PCR buffer, 250 µM each dATP, dCTP, dGTP, and dTTP, 0.5 µM each primer, and 1.5 U AmpliTaq Gold from Perkin Elmer. The DNA was initially denatured for 7 min at 95°C, followed by 40 cycles of denaturing at 95°C for 30 s, annealing at 55°C for 1 min, and extension at 72°C for 3 min. The amplification was ended by a final extension at 72°C for 10 min. Of this PCR, 1 µl was amplified with the nested primers in a 20-µl volume in standard PCR buffer, 125 µM each dATP, dCTP, dGTP, and dTTP, 0.5 µM each primer, and 1 U AmpliTaq Gold. The DNA was denatured initially for 7 min at 95°C, followed by 35 cycles of amplification with denaturing at 95°C for 30 s, annealing for 1 min at 55°C, and extension at 72°C for 2 min, and a final extension at 72°C for 10 min. The products were visualized on ethidium-bromide-stained agarose gels.

*Southern blot analysis*

Southern blot analysis was carried out by using standard methods (Sambrook et al. 1989). The products of RT-PCR of the *PAX6* gene fragment 1 and of RT-PCR with primers 794F and 1680R followed by nested PCR with 797F-N and 1677R-N (see above)

were used as probes either separately or together and covered most of the cDNA sequence. The probes were labeled by using the random primed DNA labeling kit from Roche Molecular Biochemicals.

### Identification of *pax6* isoforms in human brain cDNA by RT-PCR

Fragment 3 was amplified by using 2 ng human fetal brain Marathon cDNA (cat. no. 7402–1) from Clontech (Calif., USA) as described above. Bands were excised from the gel, left in 50–100 µl $H_2O$ over night, and 13.5 µl were sequenced by using Amersham's cycle sequencing and primer 876R-N: 5'-GGT CTG CCC GTT CAA CAT CC-3'.

### BLAST search and alignments

The BLAST search was performed by using an E-value of 100 and Blosum62 for the 129 C-terminal amino acids of *PAX6*-ex12 and an E-value of 1000 and PAM250 for the most 55 C-terminal amino acids of *PAX6*-ex12. The proteins were aligned by using the ClustalW program.

## Results

### Epidemiology

#### *Prevalence of aniridia*

The aniridia database at the NEC included 170 individuals (87 females and 83 males) born 1875–1999. The corresponding number was 147 for birth year 1920–1999. Three patients born in Greenland or The Faroe Island were excluded from the prevalence calculations leaving 144 (Table 1). The number of live borns in the 80-year period from 1920–1999 was 5,695,666. Accordingly, the point prevalence rate at birth was 2.5 in 100,000 or 1:40,000 live borns.

The overall proportion familial/sporadic was 100/44. However, this proportion changed from 58/14 during 1920–1959 to 42/30 from 1960 and was inverted to 14/21 during the last 20 years. This skewness is indeed mainly attributable to the youngest age groups not yet having reached reproductive age. An estimate of the new mutations throughout the 80-year period is shown in Table 1.

**Table 1** Distribution of 141 aniridia patients with respect to birth-year decade. The point prevalence rate per 100,000 live born (*BBP*) was calculated for the whole group (*N*) and the observed new mutations (*Nnew*) separately (*BBPnew*)

| Birth year | Live births | Cases (N) | Nnew | BBP | BBPnew |
|---|---|---|---|---|---|
| 1990–99 | 669,999 | 17 | 13 | 2.54 | 1.94 |
| 1980–89 | 551,139 | 18 | 8 | 3.27 | 1.45 |
| 1970–79 | 685,604 | 18 | 6 | 2.63 | 0.88 |
| 1960–69 | 797,472 | 19 | 6 | 2.38 | 0.75 |
| 1950–59 | 765,129 | 26 | 13 | 3.40 | 1.70 |
| 1940–49 | 843,676 | 15 | 8 | 1.78 | 0.95 |
| 1930–39 | 658,572 | 17 | 7 | 2.58 | 1.06 |
| 1920–29 | 724,075 | 14 | 7 | 1.93 | 0.97 |
| 1920–99 | 5,695,666 | 144 | 68 | 2.53 | 1.19 |

#### *Risk of Wilms tumor in sporadic cases*

After linking the patients with the Danish Cancer Registry, we found eight incident cancers diagnosed between 1943 and 1996 among 136 aniridia patients (Table 2). Two of these were Wilms tumors, both found among the sporadic cases, four were lung cancers (1 sporadic and 3 from familial cases), and two cancers were in the digestive tract both from the familial cases. One case with benign gonadoblastoma had previously been reported (Ry Andersen et al. 1978).

The relative risk of developing Wilms tumor among sporadic aniridia cases showed that a patient with sporadic aniridia had a 67-times higher risk of developing Wilms tumor compared with the normal population with a very wide CI (8.1–241).

### Mutational analysis

We investigated 19 of the 44 sporadic cases and 19 of the 100 familial cases. The remainder were not available for analysis. The results are shown in Fig. 1 and Tables 3 and 4.

#### *Sporadic cases*

Seven of the sporadic cases had de novo chromosomal deletions of variable size in the 11p13 region and deleting the entire *PAX6* gene, as shown by FISH analysis (Fig. 1B).

**Table 2** Observed (*O*) and expected (*E*) incident cancers occuring between 1943 and 1996 among 136 patients with sporadic or familial aniridia in Denmark

| Site of cancer | All cases | | | Sporadic cases | | | Familial cases | | |
|---|---|---|---|---|---|---|---|---|---|
| | O | E | O/E (95% CI) | O | E | O/E (95% CI) | O | E | O/E (95% CI) |
| All malignancies | 8 | 7.0 | 1.1 (0.5–2.3) | 3 | 1.3 | 2.3 (0.5–6.7) | 5 | 5.8 | 0.9 (0.3–2.0) |
| Kidney[a] | 2 | 0.2 | 11.1 (1.4–40) | 2 | <0.1 | 67 (8.1–241) | 0 | <0.1 | – |
| Lung | 4 | 0.8 | 4.9 (1.3–13) | 1 | 0.1 | 9.3 (0.1–52) | 3 | 0.7 | 4.2 (0.9–12) |
| Digestive tract[b] | 2 | 1.1 | 1.8 (0.2–6.4) | 0 | 0.2 | – | 2 | 1.0 | 2.1 (0.2–7.6) |

[a]Two cases of Wilms tumor both diagnosed under age 3
[b]Cancer of the rectum and liver diagnosed at ages 33 and 51, respectively

**Table 3** Mutational analysis (– signal on only one chromosome, + means signal on both chromosomes, *nd* not determined)

| Patient ID (family no.) | Karyotype | FISH | ddFP/DNA sequence | RT-PCR | Southern blot |
|---|---|---|---|---|---|
| Sporadic cases | | | | | |
| 23087 | 46,XY,del(11)(p12p14) | 11p13(AN2–,WT1–) | nd | nd | nd |
| 25239 | 46,XX,del(11)(p11p13) | 11p13(AN2–,WT1–) | nd | nd | nd |
| 32050 | 46,XX,del(11)(p11.2p13) | 11p13(AN2–,WT1–) | nd | nd | nd |
| 33607 | 46,XX,del(11)(p12p14) | 11p13(AN2–,WT1–) | nd | nd | nd |
| 22159 | 46,XX | 11p13(AN2–,S324–, WT1–) | nd | nd | nd |
| 23275 | 46,XY | 11p13(AN2–, S324+,WT1+) | nd | nd | nd |
| 31451 | 46,XX | 11p13(AN2–,S324–, H11148+,A1255+, WT1+) | nd | nd | nd |
| 34 | 46,XX | Normal | R317X[a] | nd | nd |
| 3 | 46,XX | nd | 1520GG-T (b) | nd | nd |
| 27 | 46,XY | Normal | 718ins14[b] | nd | nd |
| 41 | 46,XX | Normal | 468delG | nd | nd |
| 42 | 46,XX | Normal | IVS10+1G-C | skip ex 10 | |
| 19 | 46,XX | Normal | Normal | skip ex 5, 5a, 6 | del1486–2455,ins15 |
| 2 | 46,XY | Normal | Normal | Normal | Normal |
| 20 | 46,XY | Normal | ? | nd | nd |
| 25 | 46,XX | Normal | Normal | Normal | Normal |
| 39 | 46,XX | Normal | Normal | Normal | Normal |
| 43 | 46,XX | Normal | Normal | Normal | Normal |
| 47 | 46,XX | Normal | Normal | Normal | Normal |
| Familial cases | | | | | |
| 4 (AN1S0105) | 46,XX | Normal | A79E;R208Q[b] | nd | nd |
| 5 (AN1S0108) | 46,XX | Normal | V53L[b] | nd | nd |
| 13 (AN1S0102) | 46,XX | Normal | I42S[b] | nd | nd |
| 16 (AN200111) | 46,XX | Normal | M1 K[b] | nd | nd |
| 23 (AN1S0101) | 46,XY | Normal | T63P[b] | nd | nd |
| 104 (AN200131) | 46,XY | Normal | X423Y | nd | nd |
| 22 (AN200126) | 46,XY | Normal | R317X[a] | nd | nd |
| 32 (AN200101) | 46,XX | Normal | R261X[b] | nd | nd |
| 38 (AN200102) | 46,XY | nd | R203X[c] | nd | nd |
| 15 (AN1S0104) | 46,XX | Normal | 495A-CAT[b] | nd | nd |
| 17 (AN200122) | 46,XX | Normal | 598delCG, 597ins10[b] | nd | nd |
| 21 (AN200118) | 46,XY | Normal | 853delC[b] | nd | nd |
| 29 (AN200109) | 46,XX | Normal | 764delG[b] | nd | nd |
| 31 (AN1S0103) | 46,XY | Normal | 246del4[b] | nd | nd |
| 18 (AN200105) | 46,XY | Normal | IVS3+1G-A[b] | skip ex 3, 4, 5, 5a | nd |
| 30 (AN200120) | 46,XX | Normal | IVS6+1G-A[d] | skip ex 6 | nd |
| 35 (AN1S0107) | 46,XY | Normal | IVS11+1G-A[b] | skip ex 11 | nd |
| 1 (AN200107) | 46,XY | Normal | Normal | Normal | Normal |
| 33 (AN1S0106) | 46,XX | Normal | Normal | Normal | Normal |

[a]Davis and Cowell 1993
[b]Grønskov et al. 1999
[c]Martha et al. 1995
[d]Hanson et al. 1993

Four of the deletions were visible cytogenetically. The deletion in these four patients included the *WT1* gene, and all four displayed mental retardation in addition to aniridia. One developed Wilms tumor, and one developed nephrogenic rest (Table 4 summarizes the phenotypes of patients with chromosomal deletions). Three patients had deletions not visible by conventional cytogentic analysis; however, one had the *WT1* gene deleted and displayed the WAGR phenotype with slight retardation, aniridia, and bilateral Wilms tumor. The two remaining patients had deletion breakpoints between *PAX6* and *WT1*, and both showed normal mental development.

Dideoxy fingerprinting revealed five patients with intragenic mutations in *PAX6*. Three of these patients have previously been published (Grønskov et al. 1999), whereas two are novel mutations. The novel mutations are

**Table 4** Additional phenotypes of patients with large chromosomal deletions in 11p13 (– signal on only one chromosome, + means signal on both chromosomes)

| Patient ID | Karyotype | FISH | Development | Growth | Tumor/malformation |
|---|---|---|---|---|---|
| 23087 | 46,XY,del(11)(p12p14) | 11p13(AN2–, WT1–) | Retarded | Retarded | Testis atrophy + retention inguinal hernia |
| 25239 | 46,XX,del(11)(p11p13) | 11p13(AN2–, WT1–) | Retarded | ? | None |
| 32050 | 46,XX,del(11)(p11.2p13) | 11p13(AN2–, WT1–) | Retarded | Retarded | Unilateral Wilms tumor |
| 33607 | 46,XX,del(11)(p12p14) | 11p13(AN2–, WT1–) | Retarded | Normal | Nephrogenic rest |
| 22159 | 46,XX | 11p13(AN2–, S324–, WT1–) | Slightly retarded | Normal | Bilateral Wilms tumor |
| 23275 | 46,XY | 11p13(AN2–, S324+, WT1+) | Normal | Normal | None |
| 31451 | 46,XX | 11p13(AN2–, S324–, H11148+, A1255+, WT1+) | Normal | Normal | None |

IVS10+1G→C found in patient 42 and 468delG found in exon 5 in patient 41. The splice mutation IVS10+1G→C causes skipping of exon 10, which results in a frameshift and introduction of a stop codon in exon 12 (data not shown). The 468delG mutation also causes a frameshift and introduction of a stop codon after incorporation of 17 incorrect amino acids.

In patient 19, RT-PCR analysis of fragment 1 showed amplification of the expected fragment plus an additional fragment, which upon sequencing was shown to lack exons 5, 5a, and 6. Subsequent Southern blot analysis detected an aberrant fragment, and sequencing of genomic DNA showed a deletion of 970 bp with an insertion of 15 bp with breakpoints in exon 5 and exon 6 (g1486del970;1485ins15). Figure 1A shows the localization of the intragenic mutations.

In six out of 19 sporadic patients, we found no mutations. None of the patients with intragenic mutations or chromosomal deletions leaving WT1 intact had Wilms tumor. The other case of Wilms tumor reported to the Danish Cancer Registry was not among the 19 investigated cases.

*Familial cases*

All 19 familial cases had a normal karyotype, and FISH analysis with the AN2 and *WT1* probes showed no deletions. Dideoxy fingerprinting and/or DNA sequencing revealed intragenic mutations in *PAX6* in 17 patients. Six patients had missense mutations. Five of these patients have been previously published (Grønskov et al. 1999). Patient 104 had a novel mutation changing the stop codon to a tyrosine, X423Y (1631A→T), causing an extended *PAX6* protein of 14 amino acids. This mutation was also found in the patient's affected mother. Patient 38 had a nonsense mutation, R203X (Martha et al. 1995). Two additional patients had nonsense mutations, three patients had intragenic deletions, two patients had intragenic deletions and insertions, and three patients were found with mutations in splice sites; the mutations in these patients have been published by Grønskov et al. (1999). Figure 1A shows the localization of the intragenic mutations. We did not find any mutations, either with RT-PCR or Southern blot analysis, in the remaining two patients.

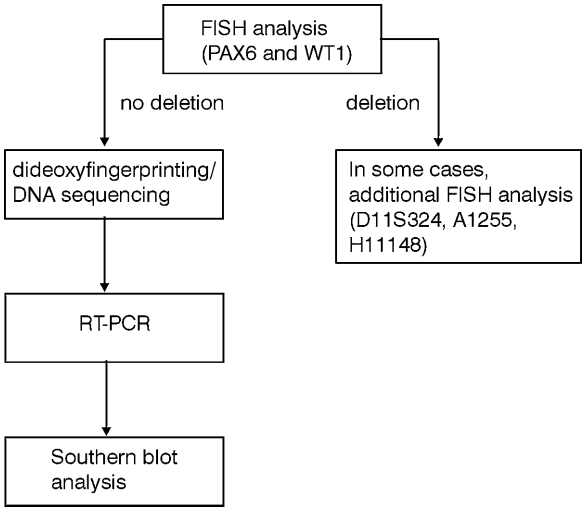


**Fig. 2** Flow diagram showing the mutational strategy

Based on these results, we proposed a mutation detection strategy as outlined in Fig. 2. With this strategy, we detected mutations in 68% of the sporadic cases and 89% of the familial cases, i.e., on average 80% among all cases.

*PAX6* isoform

RT-PCR analysis, with RNA from lymphoblastoid cell lines, of fragment 3 showed an aberrant band in one of the patients (data not shown). DNA sequencing of the RT-PCR fragment showed that this fragment skipped exon 12. However, extensive sequencing of both intron 11 and intron 12 did not reveal any mutations. Investigation of 12 normal control individuals showed three who expressed the isoform lacking exon 12 (Fig. 3A). RT-PCR analysis of a pool of brain cDNA from 24 commercially available human male/female fetuses showed the amplification of a similar fragment, which, upon sequencing, was confirmed to be a *PAX6* isoform lacking exon 12 (data not shown). This alternatively spliced isoform is identical to the canonical *PAX6* isoform as far as amino acid 344Q, after which the encoded amino acids change because of the

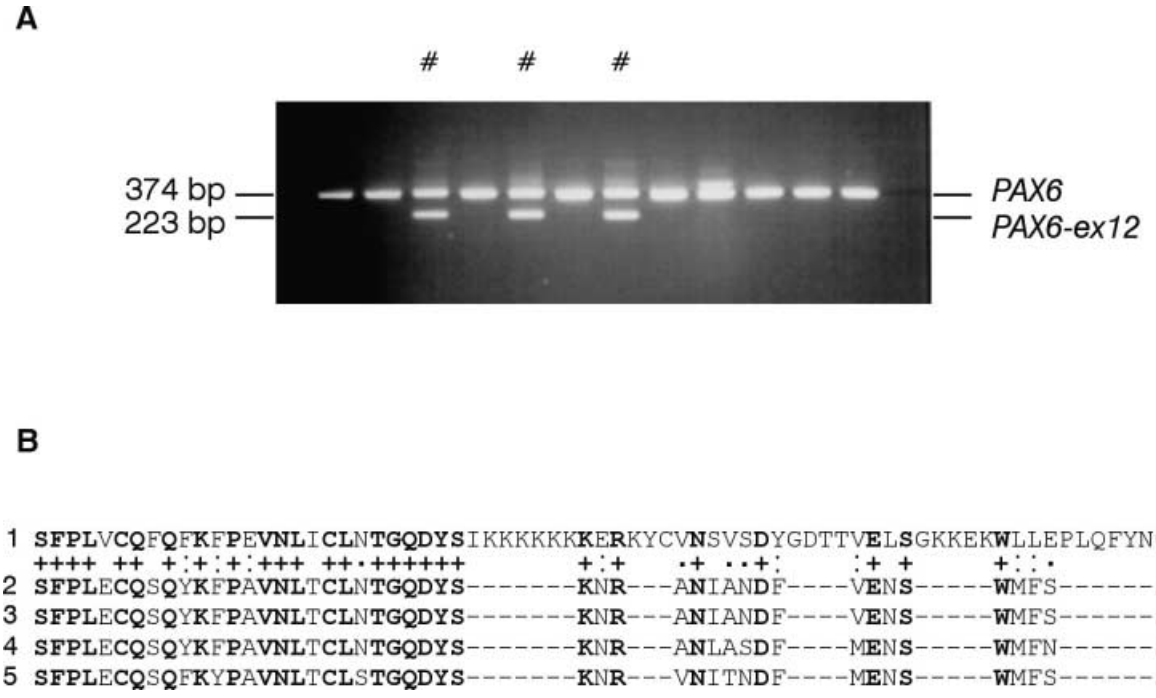


**Fig. 3** **A** RT-PCR analysis of fragment 3 of 12 control individuals. *Lanes with #* Presence of the *PAX6*-ex12 isoform. **B** Alignment of *PAX6*-ex12 sequence following exon11 of: *1* human *PAX6*-ex12, *2 Cynops pyrrhogaster PAX6*-LS, *3 Cynops pyrrhogaster PAX6*-SS, *4 Ambystoma mexicanum PAX6*, *5 Cynops pyrrhogaster PAX6*. Identical amino acids are shown in *bold*. + Identical, *:* strongly similar, . weakly similar, * stop codon

skipping of exon 12 and a change in the reading frame. Based on the published sequence (EMBL accession number M93650), this isoform is predicted to encode 129 amino acids compared with 78 amino acids for the canonical *PAX6* isoform.

## Discussion

Based on the unique registration of both aniridia cases and cancer cases in Denmark, it is possible to calculate a risk estimate of developing Wilms tumor for patients with aniridia. Seven of the 19 investigated sporadic cases were found to have large chromosomal deletions, as demonstrated either cytogenetically or by FISH, or both. Five of these were deleted for the *WT1* gene and displayed an abnormal phenotype including aniridia. Two (40%) of the five patients with deleted *WT1* developed Wilms tumor. It has previously been reported that 70% of patients with deleted *WT1* (van Heyningen and Hastie 1992) will develop Wilms tumor. Our data from the Danish Cancer Registry show that only two Danish aniridia patients developed Wilms tumors. However, the CI is wide, making accurate risk prediction difficult but suggesting a lower risk than hitherto reported. However, one patient born in 1997, not included in the statistical analysis, developed Wilms tumor when 1 year old, increasing the total number of patients with Wilms tumor to 3 of 44 sporadic cases. None of the remaining sporadic cases without *WT1* deleted developed Wilms tumor. Our observations support the notion that deletion of the *WT1* gene is a prerequisite for Wilms tumor development.

Eight of the aniridia patients were found, in the statistical analysis, to develop cancer, as was expected. However, four had lung cancer, which was above the expected. We have no explanation for this observation. However, the numbers are small.

Nineteen sporadic and 19 familial Danish aniridia cases were analyzed for mutations in the *PAX6* gene. We detected mutations in 68% of the sporadic cases and 89% of the familial cases. Some of these results have been previously published (Grønskov et al. 1999). In all, 80% of our cases could be shown to have one of several types of mutations. The high detection efficiency of this as yet incomplete mutational analysis process emphasizes that *PAX6* probably is the sole gene causing aniridia.

During our study, we have discovered yet another isoform of *PAX6* (*PAX6-ex12*), in addition to *PAX6-5a,6'* (Grønskov et al. 1999) and *PAX6-5a* (Walther and Gruss 1991). This isoform has not been reported previously and was present both as an illegitimate transcript isolated from lymphoblastoid cell lines and in human fetal brain cells, indicating that it was not an artifact of the RT-PCR method, but a true biological phenomenon. A BLAST search revealed that *Xenopus laevis*, *Cynops pyrrhogaster*, and *Ambystoma mexicanum* contain *PAX6* proteins with similar C-terminus ends (Fig. 3B), indicating the a biological importance of *PAX6-ex12*. However, it seems that the stop codon used in lower organisms has changed in humans and encodes a tyrosine instead (this has been confirmed by sequencing of three individuals), and therefore the human *PAX6-ex12* is extended by 55 amino acids

with no known homology to other proteins (as investigated by a BLAST search). We do not know the function of this alternatively spliced form, but, if RT-PCR analysis is used in mutational analysis, it is important to be aware of the existence of this isoform in normal controls.

We have demonstrated, in this study, that it is necessary to perform several mutation detection analyses in aniridia in order to obtain maximum mutation detection yield. As the disease can be caused by different types of mutations, FISH analysis, dideoxy fingerprinting, RT-PCR, and Southern blot analysis were performed. Some of the 32% sporadic cases and the 11% of the familial cases in whom we did not detect any mutation might carry mutations in the promoter region or in the 3' end of *PAX6* in the downstream region, as recently described by Lauderdale et al. (2000) but not investigated in this study.

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