Selection against mutant alleles in blood leukocytes is a consistent feature in Incontinentia Pigmenti Type 2

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Incontinentia Pigmenti 2 (IP2) is an X-linked dominant disorder with male lethality. Affected females display a characteristic skin eruption that evolves through four classic stages, frequently accompanied by dental and retinal abnormalities. Non-random (skewed) X-inactivation in peripheral blood leukocytes and in fibroblasts has been observed in females with IP2; however, sample sizes have been small and methods of analysis varied. We have examined X-inactivation in a large group of multigenerational IP2 families, in smaller families, and in isolated cases. Ninety-eight percent of affected females in multigenerational IP2 pedigrees show completely skewed patterns of X-inactivation, while only ~10% of a normal control population is skewed. Results both in small families and in new mutation cases with subsequent segregation consistent with Xq28 linkage are similar. Isolated cases show a lower percentage (85%) of skewed affected individuals; this difference may be due to inaccurate clinical ascertainment. The parent of origin of new mutations could be determined in 15 families; paternal new mutations were twice as common as maternal. Fibroblast subclones from a biopsy at the boundary of a skin lesion in a newborn IP2 patient were isolated, and clones with either one or the other X active were identified, demonstrating that cells with the active disease-bearing X chromosome are still present in stage I skin lesions.

INTRODUCTION

Familial Incontinentia Pigmenti (IP2) is a neurocutaneous genodermatosis which segregates as an X-linked dominant trait with prenatal male lethality (for review, see ref. 1). In affected females, its most prominent phenotypic features involve the skin and its derivatives, the eye and the central nervous system. This disorder is diagnosed in affected females at or soon after birth by the presence of a progressive erythematous and vesicular rash. This rash becomes sequentially verrucous, pigmented, then atrophic, and may leave adolescents and adults with areas of linear and reticular hypopigmentation. Other manifestations include cicatricial alopecia, hypodontia or anodontia, eosinophilia (in the early stages), peripheral vascular abnormalities and cicatrization of the retina, and other secondary ocular findings. The penetrance of the disease approaches 100%, but its expressivity is highly variable, even within families.

Linkage of IP2 to Xq28 markers was demonstrated in 1989 (2); subsequently, IP2 was localized more precisely to the region surrounding and most likely telomeric to *DXS52* (3). More recently, the region of interest has been narrowed to Xq28 distal to Factor VIII; the highest LOD scores are found with *DXS1108* ($\theta = 0.00$) (4).

Non-random X inactivation in patients with IP2 has been reported by several groups. Migeon *et al.* (5), using G6PD variants and an RFLP at the HPRT locus which detects a differentially methylated *Hpa*II site, observed that the chromosome bearing the normal allele at the IP locus is the active X in the majority of skin fibroblasts in affected females in three families. Variable skewing in peripheral blood leukocytes was also observed in these families, which was unexpected since this tissue had not previously been thought to be involved in this disease. Analysis of G6PD isozymes showed a more random distribution in red blood cells, suggesting that red cell progenitors are less likely to be affected negatively by the IP mutation than are white cell progenitors. Other investigators (6) used somatic cell hybrid analysis to compare skin biopsies taken from normal and hyperpigmented (stage III) areas of the skin; the same X chromosome was found to be active in every clone, regardless of whether it was derived from normal or hyperpigmented skin. These authors concluded that invasion of affected skin by normal cells most likely takes place at the transition from the inflammatory (I) to the verrucous (II) stage, and that their results support the model that normal cells have a proliferative advantage. A third study (7), again with RFLP analysis, produced contradictory results. Extreme skewing was found in a minority of cases, and in most of these cases it was not possible to determine parental origin of the inactive X chromosome; in the one fully informative family with extreme skewing, the X

carrying the IP mutation was found to be preferentially active. Finally, identification of a female patient with both IP and hemophilia (8) indicates that skewing must be present in some populations of liver cells.

Due to the discrepancies in results of previous studies, and since the numbers of informative individuals were limited by the low heterozygosity of the RFLP markers used, we have undertaken further studies with a more informative system in larger numbers of carefully ascertained families. Using differentially methylated sites near the highly polymorphic CAG repeat in the human androgen receptor gene (ARA), we have analyzed the X-inactivation status of peripheral blood leukocytes of individuals from 21 multi-generational IP2 families and from 19 families with new mutations (Fig. 1). We have demonstrated significant skewing in a majority of cases; lack of skewing has prompted review of clinical findings, which has resulted in exclusion of IP as a diagnosis in one isolated case.

Both of the previous studies (5,6) of X-inactivation patterns in fibroblasts from IP patients were done from stage III (hyperpigmented) skin lesions. In order to further investigate the X-inactivation status of skin fibroblasts from stage I lesions, a skin biopsy was obtained from a newborn affected female from a multigenerational pedigree with unequivocal Xq28-linkage. A fibroblast cell line was established, and fibroblasts were subcloned and assayed for X-inactivation status. Three clones had the maternal (IP-bearing) X active, while two had the paternal (normal) X active.

RESULTS

X-inactivation patterns in familial cases

X-inactivation patterns were studied in 15 families in which affected individuals appear in at least two generations, and in which pedigree analysis strongly supports linkage to Xq28 markers. Forty-seven affected females were tested by the method of Allen *et al.* (9) in which PCR is performed on genomic DNA digested with either *Rsa*I

or *Hpa*II in the presence of labelled nucleotide. Products are resolved on a denaturing polyacrylamide gel, which is fixed, dried, and exposed to X-ray film. Skewing is determined by comparison of signal intensities between bands representing the different alleles; complete or extreme skewing means that one allele was completely undetectable by autoradiography, as was the case with most of the affected females in these pedigrees. The extent of skewing in partially skewed individuals was estimated by visual comparison.

The vast majority (98%) of affected females in these multigenerational IP2 pedigrees show complete skewing; of the 47 affected females, only one newborn girl (XL-278-03; sampled at 9 days of age) showed random X-inactivation in peripheral blood leukocytes. In each case where parental origin of the inactive chromosome could be determined, it is the chromosome inherited from the mother with the IP phenotype (Table 1).

For six families, only affected mother/daughter pairs were available. In four of these families (XL-161, XL-170, XL-183 and XL-187), both mother and daughter were extremely skewed, and the daughters were found to inactivate their mother's X chromosome. In one family (XL-224), both mother and daughter were extremely skewed, but the parental origin of the daughter's inactive X could not be determined because her father was not available and the daughter was heterozygous for the same alleles as her mother. In another family (XL-205), the mother was skewed but the daughter was homozygous (and uninformative) at the ARA locus.

X-inactivation patterns in new mutation cases

X-inactivation patterns were studied in 19 families in which IP has appeared as an apparent new mutation. Ten of these families had only a single affected individual; these are listed as isolated cases in Table 1. In the other nine families, the mutation has been passed to another generation. Haplotype data for two of these (XL-160 and XL-206) have been reported previously (10), with the conclusion that the mutations arose on the paternal X chromosome.

Table 1. Summary of X-inactivation patterns in IP2 families

Family category	IP status	X inactivation pattern[1]		Inactivated chromosome[2]	
		# skewed	# random	# maternal	#paternal
Multigeneration,	affected	46	1	23	
Xq28-linked	unaffected	1	11		
(n[4] = 15)	uncertain[3]	–	3		
Mother/daughter	affected	11	–	4	
pairs only	unaffected	–	–		
(n = 6)	uncertain	–	–		
New mutations with	affected	16	2	11	5
positive segregation	unaffected	1	12		
Data (n = 9)	uncertain	–	–		
Isolated cases	affected	8	1	2	6
(n = 10)	unaffected	4	10		1
	uncertain	1	–	1	

[1]Only informative females listed.

[2]Where origin could be determined; applies only to skewed individuals.

[3]No direct clinical confirmation of status.

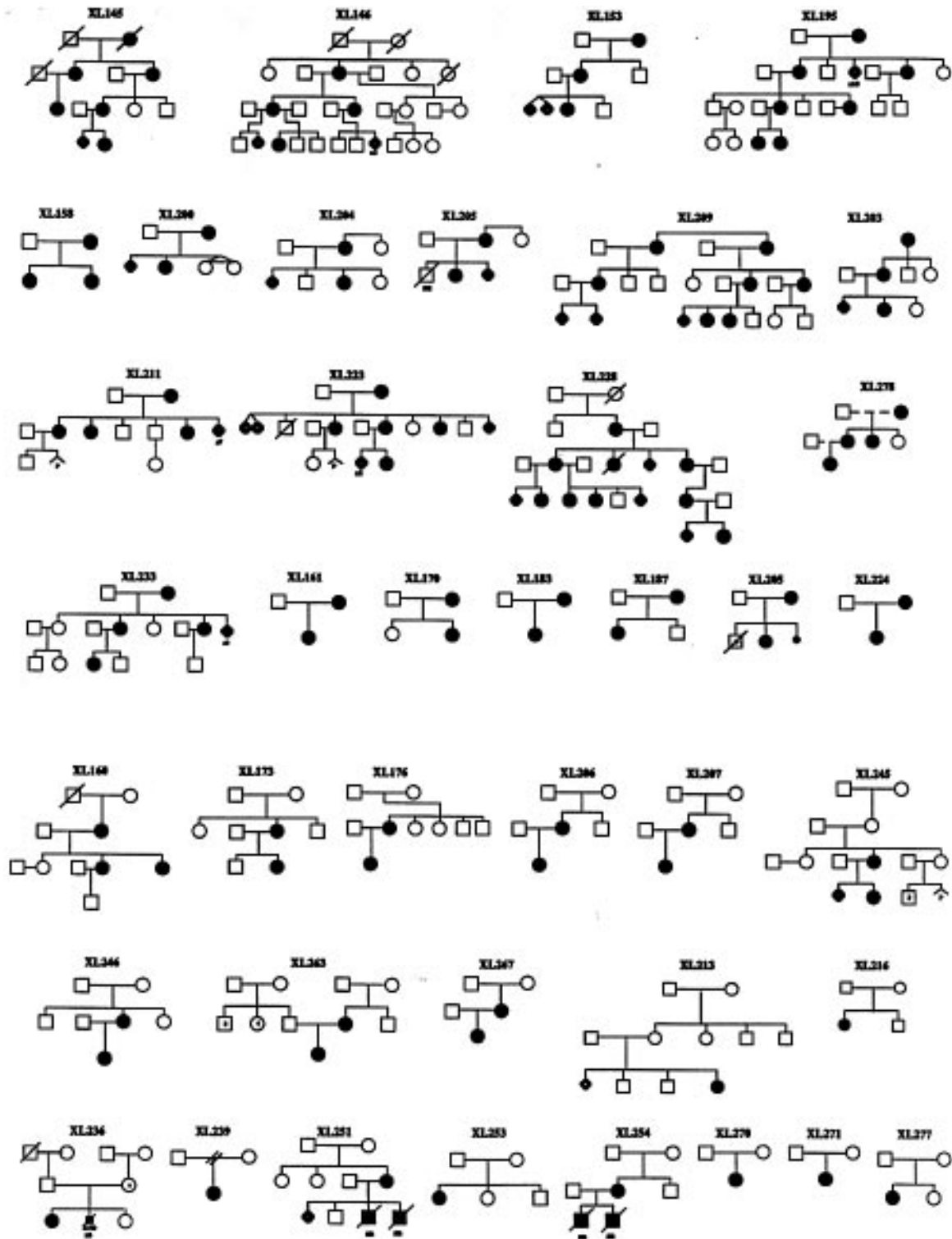[4]n = number of families in each category.

Figure 1. Pedigrees of the IP2 families included in this study. Affected individuals are denoted by blackened symbols, and deceased individuals are denoted by diagonal slashes.

Eight of these 10 families behave in the same manner; that is, the Xq28 haplotype present on the chromosome which is preferentially inactivated in the first affected female in the pedigree is the haplotype that she passes to her affected daughter(s). Examples of both maternal and paternal new mutations are shown in Figure 2. In XL-263, the first affected female selectively inactivates her maternal X chromosome. She transmits her maternal haplotype at both *F8c* and *DXS1108* to her
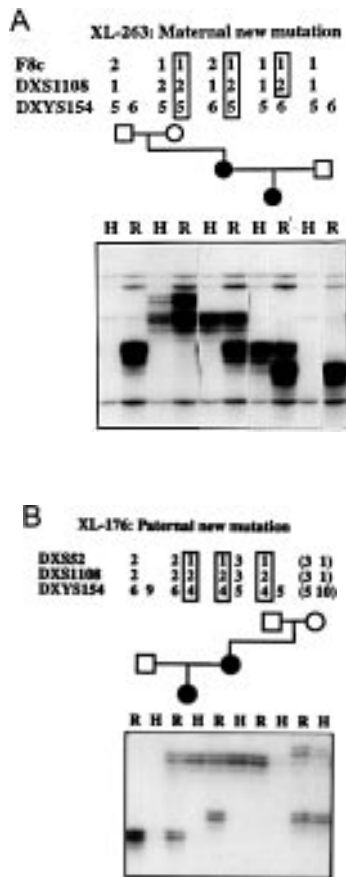
Figure 2. X-inactivation and haplotype analysis of two new mutation families. Alleles for *F8c* and *DXS1108* were arbitrarily assigned for simplicity. Filled symbols represent affected individuals. PCR across the polymorphic CAG repeat at the androgen receptor was performed on genomic DNAs digested either with *Rsa*I (R) or with the methylation-sensitive enzyme *Hpa*II (H).

affected daughter, who also inactivates this X chromosome (Fig. 2A). In XL-176, the first affected female selectively inactivates her paternal X and transmits her paternal haplotype at *DXS52*, *DXS1108* and *DXYS154* to her affected daughter, who also inactivates this chromosome (Fig. 2B). In XL-251, the affected female has no affected daughter, but she has had two stillborn sons and a single normal son who has the predicted non-IP haplotype in Xq28 (data not shown). In one family (XL-267), the affected females showed random X-inactivation.

X-inactivation patterns in the general population

A control group of 40 females, all of whom had been referred to Baylor's DNA Diagnostic Laboratory for non X-linked disorders, was assayed for X-inactivation pattern; of these, seven were homozygous at the androgen receptor. None of the remaining 33 samples showed the extreme (>20:1) level of skewing observed in IP patients. Three (9.1%) showed skewing in the 4:1–10:1 range. Normal females in the IP families, both familial and sporadic, show a slight but statistically insignificant increase in prevalence of skewed X inactivation; of 39 informative normal females in study families, 6 (15%) showed skewed (4:1 to 10:1) X-inactivation.

Confirmation of linkage

This set of families is among the largest collections of IP2 pedigrees which have been assembled. The linkage studies performed on these families support the conclusions of earlier studies. In particular, no recombination with *DXS1108*, the most tightly-linked marker, was observed in any of our multigenerational pedigrees. A recombination in one family, XL-228, places IP2 distal to *DXS52* and has the potential to narrow the interval further (Fig. 3). IP2 has been transmitted through four generations in one branch of this family. The genotype of individual XL-228-12 at *DXS52* provides evidence for recombination; she is homozygous 3/3 for this marker, while her grandmother (XL-228-08) inherited a 4 allele on her IP haplotype. The family is informative for *DXS1108*, and the 173 allele is transmitted to each affected individual. The meiosis in which the recombination event occurred could not be identified positively with the available markers, as critical members of this family (XL-228-09 and XL-228-11) are unavailable for study. Further refinement of the position of this recombination will depend on the identification of additional informative markers.

Fibroblast subclones from a newborn IP2 patient

A skin biopsy was obtained from the border of a stage I skin lesion in a newborn female, XL-145-12. The family shows unequivocal linkage of IP2 to Xq28, and all affected females in this pedigree who were available for testing show non-random X-inactivation in peripheral blood leukocytes. A fibroblastoid cell line was established from the biopsied skin and was plated out at low density for isolation of independent clones. Eighteen clones were picked, and cultures were expanded. DNA was prepared from eight clonal cell lines, and these DNAs were analyzed for X-inactivation. Five clones show complete inactivation of one allele; clones 4C, 2A and 6B have an active maternal (IP-bearing) X, while clones 5B and 7B have an active paternal (normal) X (Fig. 4).

DISCUSSION

The results of this study establish clearly that non-random (skewed) X-inactivation is present in the peripheral leukocytes of a vast majority of individuals with Xq28-linked IP. Within the set of families with clear Xq28 linkage, only a single affected newborn girl was found to have random (non-skewed) X-inactivation. Her blood sample was obtained at 9 days of age. The three other affected females in this family, including the baby's mother, are skewed. Selection against leukocyte progenitors with the IP2 mutation may be an ongoing process and will result in skewing later in some individuals than in others; that skewing can be present in early infancy is demonstrated by the complete skewing in XL-145-12, whose blood was drawn at 4 days of age. Alternatively, this individual may be an exceptional IP2 case with random X-inactivation. Analysis of additional blood samples at a later date may resolve this question. In any case, it is clear that the overwhelming majority (98%) of patients in these pedigrees show complete skewing, and that the X chromosome that is inactive is the maternal (IP2-bearing) X.

Families with new mutations which subsequently segregate in a manner consistent with Xq28 linkage provide compelling evidence that the skewing observed is due to the IP mutations. In each of these nine families, the first affected female was completely skewed; furthermore, the Xq28 haplotype that she
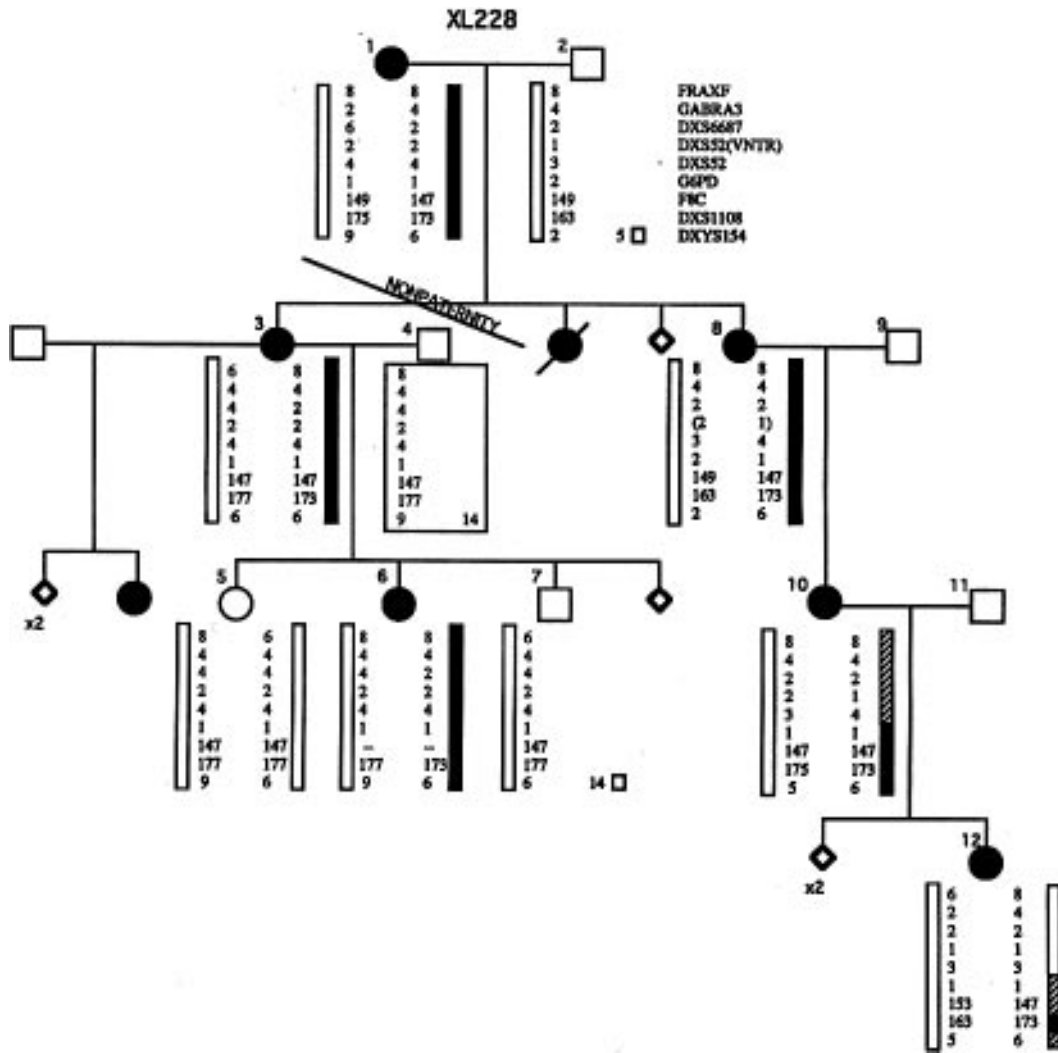
Figure 3. Pedigree analysis of family XL-228. Recombination occurred in either the meiosis between –08 and –10 or that between –10 and –12 (see text). White bars represent the non-IP2 haplotype; black bars represent the IP haplotype; hatched bars represent regions of uncertainty due to lack of informative markers.

Figure 4. X-inactivation analysis of fibroblast subclones from affected individual XL-145-12. Subclones 5B and 7B have the normal (paternal) X active, while clones 4C, 2A and 6B have the IP2-bearing (maternal) X active.

received from the same parent from whom she received her inactive X chromosome (which must therefore be the haplotype present on that inactive X) was passed only to affected daughters, and the opposite haplotype was passed only to unaffected daughters and sons. In the maternal new mutation cases, the mothers of the first affected individuals always showed random

X-inactivation. The coincident appearance of skewed X-inactivation with IP and its subsequent transmission along with the disease phenotype strongly suggest that the IP2 mutations are the causative factor of the skewing.

Based on these observations, and on the assumption that they hold true in the sporadic cases, parental origin of mutation has been established for 15 new mutations, including eight of the nine mentioned above. Ten cases were identified as paternal in origin, and five as maternal (Table 2). Average age of the parent of origin at birth of the affected daughter was 29.3 (range 21–40) for paternal and 29.2 (range 24–32) for maternal mutations. The two-fold greater prevalence of paternal new mutations is the only difference between the groups. Significant differences in parent of origin of new mutations have been reported for several genetic disorders, including Apert syndrome (11), hemophilia A (12) and hemophilia B (13). In particular, mutations at CpG dinucleotides occur far more frequently in the male germ line [11:1 for Factor IX in hemophilia B (13); 57:0 for FGFR2 in Apert syndrome (11)]. Inversions disrupting the Factor VIII gene, causing hemophilia A, have been found to originate almost exclusively in

male germ cells (302:1) (12). That new mutations in IP2 show only a two-fold increase in male germ cells suggests that deletions or point mutations other than those at CpG dinucleotides may be the more common mutations. It has been suggested (14) that disorders with apparent gestational lethality in males may actually reflect a high male:female ratio of germline mutations. This does not appear to be the case with IP2, since most of the predictions of the model do not hold true for this disease.

Table 2. Parental origin of new mutations

Pedigree #	Origin of mutation[1]	Parent's age at birth of affected child (years)
160	P	39
173	M	32
176	P	40
206	P	21
207	M	24
213	M	30
216	M	30
239	P	23
245	P	27
246	P	34
251	P	28
253	P	23
263	M	30
270	P	27
271	P	31

[1]M = maternal; P = paternal.

Six normal females in the IP2 pedigrees show non-random X-inactivation patterns (4:1–10:1). Three of these women belong to the older generations in their respective pedigrees; their average age is 65. It has been observed by others that older women as a group are skewed more frequently than younger women (15). Two instances of non-random inactivation were found in a mother and daughter from one of the new mutation pedigrees (XL-277). The affected daughter and her normal sister both preferentially inactivate their paternal X, although the affected daughter is skewed to a greater degree than her unaffected sister. The unaffected mother is completely skewed. Skewing can be inherited as a Mendelian trait in humans (16,17); Naumova *et al.* found that a constitutively active X chromosome was transmitted from a paternal grandmother to her seven granddaughters (17). In XL-277, one could postulate that the tendency toward skewing was passed from mother to daughters. The fact that the daughters selectively inactivate their father's X suggests that this trait may be acting in a positive manner, as in the family studied by Naumova *et al.* (17).

Isolation of fibroblast subclones retaining an active maternal (IP-bearing) X chromosome confirms that cells bearing the IP2 mutation on the active X are present in stage I lesions. Results of a previous study (5) demonstrated that such cells are absent from stage III (hyperpigmented) lesions. These findings suggest that progression of the inflammatory lesions to hyperpigmented skin

is concurrent with the disappearance of cells expressing the mutated form of the IP2 gene. These cell lines will provide an important resource for understanding the nature of IP2 at the cellular level.

MATERIALS AND METHODS

Collection of families and preparation of samples

Families with IP were ascertained through the Incontinentia Pigmenti Research Program approved by the Institutional Review Board for Human Subject Research at Baylor College of Medicine and by local, regional and national referral, most through direct contact with dermatologists, ophthalmologists, geneticists, obstetricians and genetic counselors in North America. All affected females exhibit or have a history of blistering and at least one other stage of skin lesions, and compatible dental and/or eye abnormalities. Affected versus unaffected status in each pedigree was assigned by one or more of three clinicians (R.A.L., M.L.L., A.E.S.) who are intimately familiar with the variable expressivity of IP2. No clinician was familiar with the haplotype analysis or X-inactivation results at the time of assignment of status.

DNA was extracted from patient blood samples by a proteinase K/SDS treatment followed by phenol and chloroform extractions. DNA was extracted from cultured lymphoblasts, amniocytes and CVS cells as described (15), except that the DNA was precipitated with one volume of isopropanol rather than two volumes of absolute ethanol. DNAs prepared directly from fresh, anticoagulated blood were used in the X-inactivation assay when possible. Data from cultured lymphocytes are included when a few individuals from study families would otherwise be excluded; the six such individuals are all in the affected/skewed category.

Cell culture and subcloning

A skin biopsy was obtained from the boundary of a skin lesion in a newborn girl diagnosed with IP2. Fibroblasts were cultured in DMEM with 10% fetal calf serum (Gibco BRL). Cells were plated at low density in 10 cm culture dishes. Clones were picked into the wells of a 24-well dish; surviving clones were expanded and frozen, and DNA was isolated as described above.

X-inactivation assay

X-inactivation status at the androgen receptor locus was determined essentially as described (9). In our study, 250 ng genomic DNA was digested with 10 U of either *Rsa*I or *Hpa*II in a 10 μl reaction; 2.5 μl of each digest was then used in a 10 μl PCR reaction.

Polymorphism at G6PD

Sequence from the RCP/GCP–G6PD region (18) was scanned for simple sequence repeats. A (CA)n repeat tract was identified near the G6PD gene, and primers were designed to amplify across this repeat. Primer sequences were 5′-CTA CTT GGT TAT CTA GTA GCC TTC TC-3′ and 5′-GTT AAT TGC TCA GTG TGA TCA GAT CGG-3′. The forward primer was end-labeled using T4 polynucleotide kinase, and PCR was carried out in standard buffer with AmpliTaq (Perkin-Elmer Cetus), using 0.4 μM each primer and 100 ng genomic DNA per 15 μl reaction. Initial denaturation was for 5 min at 95°C, followed by 28 cycles of (94°C, 1 min; 65°C, 30 s; 72°C, 1 min); final extension was for

5 min at 72°C. Products were mixed 1:1 with standard DNA sequencing stop buffer, denatured at 80°C for 2 min, then electrophoresed on 5% polyacrylamide/8 M urea/1× TBE gels. Allele designations were arbitrarily assigned.

ACKNOWLEDGEMENTS

ABBREVIATIONS

IP2, Incontinentia Pigmenti Type 2 (familial); ARA, androgen receptor; G6PD, glucose-6-phosphate dehydrogenase; R/GCP, red/green cone pigment; FGFR2, fibroblast growth factor receptor 2; HPRT, hypoxanthine phosphoribosyl transferase.

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