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Case Report

CYTOGENETIC STUDY OF A SEVERE CASE OF PALLISTER-KILLIAN SYNDROME USING FLUORESCENCE *IN SITU* HYBRIDIZATION

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Summary Usually, the supernumerary isochromosome 12p characterizing Pallister-Killian syndrome patients was detected in cultured skin fibroblasts but not in cultured blood lymphocytes. The proband of this study was a one-day-old female, who presented with major clinical characteristics of the Pallister-Killian syndrome, and had severe malformations in the form of anal atresia, cleft palate, and severe laryngomalacia. Chromosome preparations from cultured blood lymphocytes and skin fibroblasts, as well as buccal smears, from this patient were analyzed by fluorescence *in situ* hybridization (FISH) using a chromosome 12-specific alpha satellite probe. The proportions of cells showing positive signals for i(12p) in these samples were found to be 20, 62.5, and 70%, respectively. Repeated FISH studies of buccal smears from this patient showed considerable decreases in the proportions of i(12p) containing cells to 40% at one year of age and to 32% at the age of one year and five months. The decline in the percentage of i(12p)-containing cells in buccal smears with aging supports the concept of *in vivo* loss of the marker during repeated cell division.

Key Words Pallister-Killian syndrome, i(12p) mosaicism rate, severe case, buccal smear, fluorescence *in situ* hybridization

INTRODUCTION

"Pallister mosaic syndrome," "Techler-Nicola/Killian syndrome," "Killian

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syndrome" and, most recently "Pallister-Killian syndrome" are the various nomenclatures used in the literature to refer to the original syndrome described by Pallister *et al.*, in 1977. They reported the first two cases of a new MCA/MR syndrome, which was characterized by a coarse facial appearance, hypertelorism, cataracts, pigmentary dysplasia, polymastia, and severe mental retardation. This recognizable phenotype resulted from mosaicism of a small metacentric 12p isochromosome which is usually detected in skin fibroblasts, but not in blood lymphocytes. Furthermore, a high frequency of this isochromosome was also demonstrated in direct bone marrow preparations from newborn children (Peltomaki *et al.*, 1987; Ward *et al.*, 1988; Sharon *et al.*, 1990).

Identification of the isochromosome has been based on its specific banding pattern (Pallister *et al.*, 1977), LDH-B gene dosage effect (Gilgenkrantz *et al.*, 1985) and the results of molecular studies (Hunter *et al.*, 1986). Recently, it was found that fluorescence *in situ* hybridization (FISH) using chromosome 12-specific DNA probes could be employed successfully for this purpose. After the introduction of interphase cytogenetics, FISH could also be applied to tissues for which cytogenetic analysis was not feasible (Manuelidis, 1985; Cremer *et al.*, 1986; Pinkel *et al.*, 1986; Devilee *et al.*, 1988; Kuo *et al.*, 1991). Very recently, epithelial cells from buccal mucosa were used as a substrate for FISH for the rapid, reliable and non-invasive diagnosis of the Pallister-Killian syndrome (Ohashi *et al.*, 1993).

In this paper, we describe the case of a surviving patient with the Pallister-Killian syndrome, who showed an extraordinarily high proportion (20%) of i(12p)-containing cells in her peripheral blood lymphocytes. The effect of aging on the mosaicism rate *in vivo* was also evaluated by repeated FISH analysis of buccal mucosal smears.

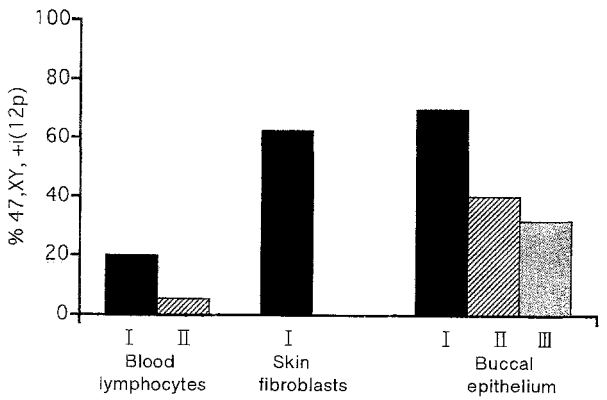


Fig. 1. This figure shows the percentage of i(12p) positive cells present in blood lymphocytes, skin fibroblasts, and buccal epithelium of the proband at birth (I), at the age of one year (II), and at the age of one year and five months (III) as detected by FISH.

CASE REPORT

The proband of this study (Fig. 2, A and B) was a female born in 1992 at 38 weeks of gestation to a 26-year-old G2P1 mother and 32-year-old father, both of whom were healthy and non-consanguineous Japanese. Pregnancy was complicated by moderate toxemia and polyhydramnios from the 33rd week of gestation, which were controlled by appropriate routine medication. Shortly after birth, the baby was referred for genetic evaluation, because she had dysmorphic features. At birth, she weighed 2,820 g, was 50 cm long, her OFC was 33.5 cm, and chest circumference was 30 cm (average values).

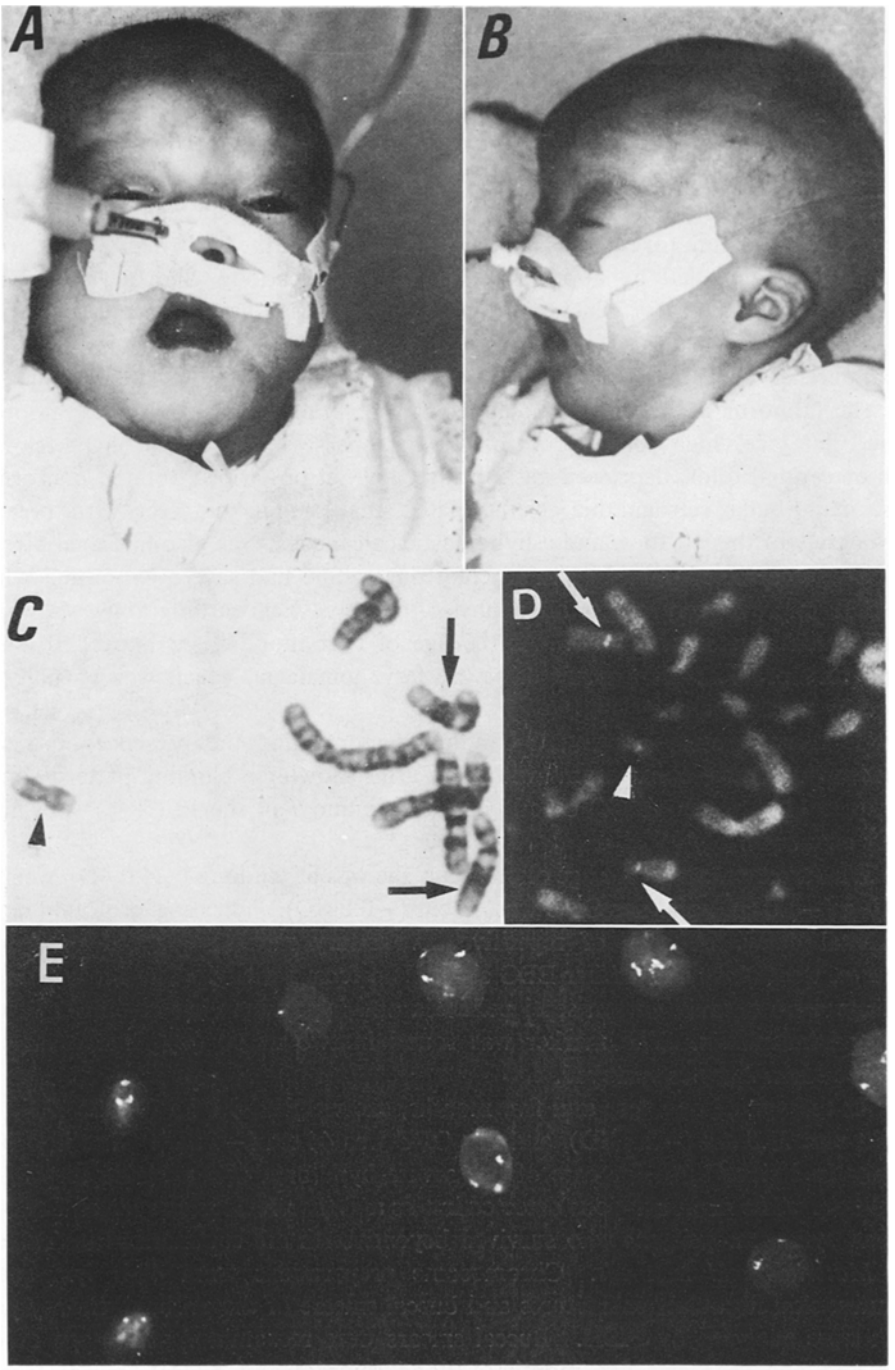
Her abnormalities at birth included sparse scalp hair, bitemporal alopecia, coarse flat face, high forehead with bossing, sparse left eyebrow, hypertelorism with epicanthal folds, depressed nasal bridge, low-set posteriorly-rotated malformed ears, mandibular retrognathia, cleft palate, small hands and feet with preaxial polydactyly of the left foot, and a hypoplastic calcaneus. She also had anal atresia, which was corrected later by simple colostomy. She had severe hypotonia which did not improve very much after physio-therapy. Echocardiography showed a small patent ductus arteriosus. At the age of four months, she started to suffer respiratory problems as a result of severe laryngomalacia, which was partially relieved by tracheostomy.

Cytogenetic analysis using G-banding demonstrated the presence of a submetacentric marker chromosome with both arms showing a banding pattern similar to 12p in 11 of the 50 (22%) blood lymphocytes and 7 of the 12 (58%) skin fibroblasts examined.

When reexamined at the age of one year, she was 65 cm long (−4.0 SD), weighed 7,810 g (−1.0 SD) and her OFC was 43 cm (−0.5 SD), indicating profound developmental delay. Attacks of repetitive involuntary movements, but no typical seizures, were observed and her EEG showed no focal activity. The brain CT-scan findings were a small cavum septi pellucida and hypoplastic left cerebral and cerebellar hemispheres. The marker was found in two of 50 (4%) metaphases of the blood lymphocytes surveyed.

At the age of one year and five months, she weighed 8.1 kg (−1.25 SD), her height was 71.5 cm (−2.5 SD), and her OFC was 44 cm (−1 SD). She had been hospitalized since birth for care of her severe hypotonia and unstable respiratory condition. The malformations detected in this and the previously reported Pallister-Killian syndrome patients who survived beyond birth are summarized in Table 1.

Materials and Methods. Chromosome preparations from 48-h lymphocyte cultures, skin fibroblast subcultures and buccal smears of the proband shortly after birth were analyzed by FISH. Buccal smears were reexamined at the age of one year and one year and five months. Blood samples and buccal smears from a female of the same age with a normal chromosome complement were used as con-


A
B
C
D
E

trols. A biotin-labeled alpha satellite DNA probe for the chromosome 12 centromere (D12Z3; Oncor, Inc.) was used for FISH.

The blood and fibroblast cultures were set up and chromosome slides prepared using standard techniques. Buccal smears were prepared by scraping the mucosa of the cheek with a tongue depressor and spreading the cells on APS coated super frost micro-slides (Matsunami), which were fixed in 100% methanol for 20 min, air dried and stored at −20°C until required for use. Prior to use, the slides were dried at 65°C for 2 or 3 h, after which FISH was carried out as described previously (Pinkel *et al.*, 1986; Satoh *et al.*, 1993). In brief, the slides were pretreated with ribonuclease (100 μg/ml in 2×SSC) for 1 h at 37°C. For the buccal smears, additional pepsin pretreatment (300 μg/ml in 0.01 M HCl) for 10 min at 37°C was carried out (as recommended by Julia and James (1991) for tumor cell suspensions). Then the slides were denatured in 70% v/v formamide/2×SSC for 2 min at 75°C, dehydrated immediately in cold 70% ethanol followed by 100% ethanol and air dried. For each slide 10 ng probe was denatured in 50% formamide/2×SSC/10% dextran sulphate for 5 min at 70°C then cooled on ice. The hybridization mixture was added to the denatured slides and allowed to react in a moist chamber under a parafilm cover (American National Can) overnight at 37°C. They were given two 15-min post-hybridization washes with 50% v/v formamide/2×SSC at 45°C followed by several washes in 4×SSC buffer.

The hybridization signals were detected by applying a layer of FITC-conjugated avidin and amplified with biotinylated anti-avidin, followed by another layer of FITC-avidin. Finally, the slides were mounted in antifading solution with propidium iodide as a counter stain and observed under a fluorescence microscope (Olympus).

Results. The FISH study of the proband's blood lymphocytes cultured on the first day of neonatal life showed that 14 of 70 cells (20%) were positive for the extra isochromosome. At the same time, cultured skin fibroblasts showed a higher frequency (62.5%) of the marker. However, the highest rate was detected in the epithelial cells of the buccal smears; 140 of 200 cells (70%) showed three signals. After the first year of life, the marker was detected at lower frequencies of 5.5 and 40% in blood lymphocytes and buccal epithelial cells respectively. A further decline in the number of i(12p)-containing cells was also evident in the third buccal smear sample obtained at the age of one year and five months, when it reached 32%. The results are summarized in Table 2 and Fig. 1.

Fig. 2. A and B are photographs of the proband at three month of age. C and D are partial metaphases from cultured blood lymphocytes after G-banding (C) and FISH (D) using the probe (D12Z3; Oncor, Inc.) for chromosome 12 centromere showing the normal chromosome No. 12 (pointed to by arrows) and iso 12p (pointed to by arrow heads). E shows interphases of buccal smear after pepsin pretreatment and FISH using the same probe.

Table 1. Clinical and cytogenetic findings of this and the previously reported Pallister-Killian syndrome patients who survived beyond birth.

	Present case	Reynolds et al										Hunter et al		Warburton et al		Kawashima	Speleman et al			Ohashi et al	
Case number / Features		1	2	3	4	5	6	7	8	9	10	1	2	2	4	0	1	2	3	1	2
Age (yrs)	1 5/12	45	40	18	7 3/12	5	3	2	1 5/12	1 1/12	8/12	2	2/12	4 6/12	7	3 8/12	11	6	2	1 9/12	1 3/12
Sex	F	M	M	F	M	F	F	F	M	M	F	M	M	F	F	M	F	M	F	M	F
Growth failure	+	+	-	+	+	-	-	+	-	-	-	+		+	-	+	+	+	-	+	-
Mental retardation	+	+	+	+	+	+	+	+	+	+	+	+		+	?		+	+	+	+	+
Seizures	?	+	+	+	+	+	-	-	+	-	+	+	+	+	-	-	+	+	-		
Hypotonia congenita	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
Pigmentary dysplasia	+	+	+	+	+	+	+	-	-	+	-	+	-		+	+	+	+	-	+	
Bitemporal alopecia	+	+	+	-	+	+	+	+	+	+	+	+				+	+	-	-	+	
Sparse scalp hair	+	+	+	-	+	+	-	+	+	+	+	+				+		-	-	+	+
Coarse face	+	+	+	+	+	+	+	+	+	+	+	+	+			+	+	+	+	+	
High forehead	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
Hypertelorism	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+
Epicanthal folds	+	+	+	+	+	+	+	+	+	+	+	+	-		+	+	+	+	+		
Broad nasal bridge	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Macrostomia	+	+	+	+	-	+	+	-	+	+	+		+	+		+	+	+	+		
Mandibular anomaly	+	-	+	+	-	+	-	-	-	-	-	+	-	+		+	+	+	+		
Abnormal ears	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+		-			
Accessory nipples	-	-	-	+	+	+	+	-	-	-	+	+	-					-			
Hernia	-	-	-	-	+	-	-	+	+	+	+	+	+	+		+		-			+
Congenital heart defect	+	-	-	-	-	-	-	-	+	+	-	-	-		-			-			
Optical abnormality	-	+	-	-	-	+	-	+	-	-	-	-	-			+		-			
GIT abnormalities	Anal atresia											-	-					-			
Others	Laryngomalacia															· Undescended testis · Skeletal anomalies · Pilonidal sinus					
Chromosome study: i(12p) mosaicism percentage in																					
· Blood lymphocytes	22	0	0	0	0	0	0	0	0	12	0	0	3.3	10	11.5	0	0	0	0	1	0
· Skin fibroblasts	58	50	75	80	100	80	90	100	47	98	100	100	100	ND	85.5	100	95	60	97	0	57
· Buccal smears (FISH)	70→40→32	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	68	53

*ND = Not done. *blank = no comment.

Table 2. Prevalence of i(12p) containing cells in the different tissues examined.

Sample	Cells with i(12p)	Total number scored	Percentage (%)
Blood lymphocytes			
I	14	70	20
II	11	200	5.5
Skin fibroblasts			
I	125	200	62.5
II	ND	ND	ND
Buccal smears			
I	140	200	70
II	80	200	40
III	64	200	32

ND, not done.

DISCUSSION

In this study, the frequency of aneuploid cells carrying the extra chromosome iso 12p in blood lymphocytes detected in this patient was relatively high (20%) compared with the frequencies in other surviving cases previously reported in the literature, which ranged from 0–12%. The very early chromosomal analysis carried on the first day of neonatal life was of great help in this respect. Pauli *et al.* (1987) reported a frequency of 20% in blood lymphocytes in a stillborn female infant with lethal malformations. The proportion of iso 12p-containing cells was found to be high (62.5%) in the skin fibroblast subculture of our patient. The highest frequency (70%) occurred in the epithelial cells of the buccal smears, which agrees with the results obtained recently by Ohashi *et al.* (1993). They explained this finding by assuming that this reflected minimal selection against i(12p)-positive cells in the buccal epithelial tissue during the process of cell division either *in vivo* due to its slow turnover rate or *in vitro* as direct analysis without culture was carried out.

We had a chance to reassess the mosaicism rate of iso 12p a year later in both blood lymphocytes and buccal smear epithelial cells, but not in skin fibroblasts, due to the instability of the proband's general condition as a result of respiratory problems. The i(12p) frequency was found to have decreased significantly in both samples to about 5.5% in blood lymphocytes and 40% in epithelial cells. This rate declined even further in the third buccal smear sample taken when the patient was one year and five months old, when only 32% of the scored cells retained the marker. This reduction demonstrated the effect of aging and successive cell division on the mosaicism rate. This decline was observed after direct analysis of

the epithelial cells without subjecting them to culture and thus strongly supports the concept of *in vivo* marker loss. It was evident that epithelial cells still possessed a very high number of cells containing i(12p). This may be very useful for diagnosing the Pallister-Killian syndrome in older children, specially if the marker is not detected in blood lymphocytes or skin fibroblasts.

When buccal smears were used directly without any special pretreatment, the epithelial cells showed high auto-fluorescent background activity, due to the remaining cytoplasm, which diminished the intensity of the FISH signals considerably. Therefore, we pretreated the buccal smears with pepsin to digest the cell membranes, cytoplasm and part of the nuclear protein. This facilitated probe penetration to a great extent and increased the accessibility of the target DNA and hence improved the hybridization efficiency. Furthermore, it increased the number of usable cells so that some hundred cells could be counted easily in a short time.

In conclusion, the case of a surviving patient with the Pallister-Killian syndrome with an extraordinarily high frequency of isochromosome 12p in her blood lymphocytes is presented. Repeated analysis of pepsin-pretreated buccal smears using FISH suggested that *in vivo* loss of the marker isochromosome occurred as the child grew older.

REFERENCES

Cremer T, Landegent J, Bruckner A, Scholl HP, Schardin M, Hager HD, Devilee P, Vander Ploeg M (1986): Detection of chromosome aberrations in the human interphase nucleus by visualization of specific target DNA with radioactive and non-radioactive *in situ* hybridization technique: Diagnosis of trisomy 18 with probe L1.84. Hum Genet **74**: 346–352

Devilee P, Thierry RF, Kievits T, Kolluri R, Hopman AHN, Willard HF, Pearson PL, Cornelisse CJ (1988): Detection of chromosome aneuploidy in interphase nuclei from human tumors using chromosome-specific repetitive DNA probes. Cancer Res **48**: 5825–5830

Gilgenkrantz S, Droulle P, Schweitzer M, Foliquest B, Chadefaux B, Lombrad M, Chery M, Prieur M (1985): Mosaic tetrasomy 12p. Clin Genet **28**: 495–502

Hunter AGW, Clifford B, Cox DM (1985): The characteristic physiognomy and tissue specific karyotype distribution in the Pallister-Killian syndrome. Clin Genet **28**: 47–53

Hunter AGW, Macdonald IM, Wyatt P, Wang HS (1986): *In situ* hybridization and the Pallister-Killian syndrome. Am J Hum Genet **39**: A117

Julia MP, James O'DM (1991): *In situ* hybridization principles and practice. World distribution, Oxford University Press, Oxford, New York, Tokyo

Kawashima H (1987): Brief clinical report: Skeletal anomalies in a patient with the Pallister-Teschler-Nicola/Killian syndrome. Am J Med Genet **27**: 285–289

Kuo WL, Tenjin H, Segraves R, Pinkel D, Golbus MS, Gray J (1991): Detection of aneuploidy involving chromosomes 13, 18 and 21, by fluorescence *in situ* hybridization (FISH) to interphase and metaphase amniocytes. Am J Med Genet **49**: 112–119

Manuelidis L (1985): Individual interphase chromosome domains revealed by *in situ* hybridization. Hum Genet **71**: 288–293

Ohashi H, Ishikiriyama S, Fukushima Y (1993): New diagnostic method for Pallister-Killian syndrome: Detection of i(12p) in interphase nuclei of buccal mucosa by fluorescence *in situ* hybridization. Am J Med Genet **45**: 123–128

Pallister PD, Meisner LF, Elejalde BR, Franke U, Hermann J, Spranger J, Tiddy W, Inhorn SL, Optiz JM (1977): The Pallister mosaic syndrome. Birth Defect **XIII(3B)**: 103–110

Pauli RM, Zeier RA, Sekhon GS (1987): Mosaic isochromosome 12p. Am J Med Genet **27**: 291–294

Peltomaki P, Knuutila S, Rituanen A, Kaitila I, De La Chapelle A (1987): Pallister-Killian syndrome: Cytogenetic and molecular studies. Clin Genet **31**: 399–405

Pinkel D, Straume T, Gray JW (1986): Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. Proc Natl Acad Sci USA **83**: 2934–2938

Reynols JF, Daniel A, Kelly TE, Gollin SM, Stephan MJ, Carey J, Adkins WN, Webb MJ, Char F, Jimenez JF, Opitz JM (1987): Isochromosome 12p mosaicism (Pallister mosaic aneuploidy or Pallister-Killian syndrome): Report of 11 cases. Am J Med Genet **27**: 257–274

Satoh H, Nagai F, Homma H, Mori S, Matsui M (1993): Regional assignment of rat androsterone UDP-glucuronosyl transferase gene (UGT2B2) to chromosome 14p21.2-p22. Cytogenet Cell Genet **62**: 49–51

Sharon LW, Leslie YB, Mark WS (1990): Mosaicism in Pallister i(12p) syndrome. Am J Med Genet **35**: 523–525

Speleman F, Leroy JG, Van Roy N, DdPaepe A, Suijkerbuijk R, Brunner H, Looijenga L, Verschraegen-Spae M, Orye E (1991): Pallister-Killian syndrome: Characterization of the isochromosome 12p by fluorescent *in situ* hybridization. Am J Med Genet **41**: 381–387

Warburton D, Anyane-Yeboa K, Francke U (1987): Mosaic tetrasomy 12p: Four new cases, and confirmation of the chromosomal origin of the supernumerary chromosome in one of the original Pallister-Mosaic syndrome cases. Am J Med Genet **27**: 275–283

Ward BE, Hayden MW, Robinson A (1988): Isochromosome 12p mosaicism (Pallister-Killian syndrome): New diagnosis by direct bone marrow analysis. Am J Med Genet **31**: 835–839