RESEARCH ARTICLE

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Utility of SNP Arrays in Detecting, Quantifying, and Determining Meiotic Origin of Tetrasomy 12p in Blood From Individuals With Pallister–Killian Syndrome

Laura K. Conlin,[1]* Maninder Kaur,[2] Kosuke Izumi,[2] Lindsey Campbell,[2] Alisha Wilkens,[2] Dinah Clark,[2] Matthew A. Deardorff,[2,3] Elaine H. Zackai,[2,3] Phillip Pallister,[4] Hakon Hakonarson,[2,3,5] Nancy B. Spinner,[1,3] and Ian D. Krantz[2,3]

[1]Department of Pathology, The Children's Hospital of Philadelphia, Philadelphia, Pennsylvania

[2]Division of Human Genetics, The Children's Hospital of Philadelphia, Philadelphia, Pennsylvania

[3]The Perelman School of Medicine at The University of Pennsylvania, Philadelphia, Pennsylvania

[4]Shodair, Helena, Montana

[5]The Center for Applied Genomics, The Children's Hospital of Philadelphia, Philadelphia, Pennsylvania

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Identification of the isochromosome 12p (i(12p)) associated with Pallister–Killian syndrome is complicated by the low frequency of this supernumerary chromosome in PHA stimulated peripheral blood lymphocytes, and frequently requires cytogenetic analysis of fibroblast cells. Recently, it has been shown that array CGH techniques are able to detect tetrasomy 12p in peripheral blood, even when not identified by traditional cytogenetic techniques. We studied 15 patients with a previous cytogenetic and clinical diagnosis of Pallister–Killian syndrome using genome-wide SNP arrays to investigate the ability of this platform to identify the i(12p) in blood and tissue. Array analysis verified tetrasomy 12p in all samples from fibroblasts, but was only able to detect it in 46% of blood samples. The genotyping information available from the SNP arrays allowed for the detection of as low as 5% mosaicism, as well as suggesting a Meiosis II origin for the isochromosome in the majority of patients. Analysis of the percentage of abnormal cells with patient age at time of study suggests that the frequency of the i(12p) decreased with age in blood, but not in fibroblasts. These highlight the power of SNP arrays in detecting and characterizing the isochromosome 12p in Pallister–Killian syndrome as well as underscoring the important utility of traditional cytogenetic techniques.

Key words: Pallister–Killian syndrome (PKS); tetrasomy 12p; Teschler–Nicola–Killian syndrome; SNP array; isochromosome 12p (i(12p)); mosaicism

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INTRODUCTION

Pallister–Killian syndrome (PKS) is characterized by facial anomalies, variable developmental delay and intellectual disability, hypotonia, seizures, pigmentary skin differences, diaphragmatic hernia, congenital heart defects, and other systemic abnormalities. PKS is typically caused by the presence of a supernumerary isochromosome composed of the short arms of chromosome 12 resulting in tetrasomy 12p, which is often present in a tissue limited mosaic state [Peltomaki et al., 1987]. Identification of the isochromosome 12p (i(12p)), associated with PKS is complicated by the low frequency of this supernumerary chromosome in phytohemagglutinin (PHA) stimulated peripheral blood lymphocytes, and frequently requires cytogenetic analysis of fibroblast cells for diagnosis. It has been hypothesized that the use of PHA promotes the growth of the normal cells over the i(12p) tetrasomy cells, resulting in a representational loss of the i(12p) tetrasomy cells in peripheral blood

*Correspondence to:
Laura K. Conlin, Ph.D., Department of Pathology and Laboratory Medicine, The Children's Hospital of Philadelphia, 3615 Civic Center Blvd., Abramson Research Building, Rm. 1007B, Philadelphia, PA 19146.
E-mail: conlinl@email.chop.edu

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using standard chromosome analysis [Reeser and Wenger, 1992]. Even the cytogenetic analysis of fibroblast cells, which often contain a higher proportion of i(12p) cells, requires cell culture which may also result in skewing towards a lower estimation of the true mosaic ratio [Priest et al., 1992]. Recently, it has been shown that array CGH techniques are able to detect tetrasomy 12p in peripheral blood, even when not identified by a standard chromosome analysis [Ballif et al., 2006; Delahaye et al., 2006; Powis et al., 2007; Theisen et al., 2009]. This is likely explained by the fact that CGH is performed on a direct DNA prep from the blood and does not require cell culture with PHA, thereby giving a closer representation of the mosaic level. Therefore, array based cytogenetic technology such as array CGH and single nucleotide polymorphism (SNP) array may represent an improved methodology for the detection of mosaic chromosomal abnormalities such as seen in PKS. However, the systematic evaluation of the utility of array technology in detecting mosaic i(12p) cells has not been performed. In this study, we studied 15 patients with a previous cytogenetic and clinical diagnosis of PKS using a genome-wide SNP array to investigate the ability of this platform to identify i(12p) in blood and tissue in order to formally evaluate the diagnostic capability of SNP arrays in PKS.

MATERIALS AND METHODS

Patient Cohort

All individuals with PKS were enrolled into a research protocol that was approved by the The Children's Hospital of Philadelphia (CHOP) Institutional Review Board. All individuals were ascertained as having PKS based on prior cytogenetic analyses in peripheral blood or skin fibroblasts as well as on detailed clinical examinations by dysmorphologists experienced in the diagnosis of PKS (M.A.D., E.H.Z., P.P., and I.D.K.).

Peripheral blood and skin biopsies were obtained, and DNA was extracted from non-stimulated blood, directly from skin, or from cultured skin fibroblasts for each patient.

SNP Array Analysis

The quality of the DNA was monitored by analysis of OD260/OD280 and OD260/OD230 ratios. Acceptable samples had values between 1.8 and 2.0 and ratios >2.0, respectively. Thirty microliters of a 50 to 100 ng/μl solution of genomic DNA was genotyped on the Illumina BeadStation (Illumina Inc., San Diego, CA). In preparation for analysis, the samples were whole genome-amplified, fragmented, hybridized, fluorescently tagged, and scanned, as per standard protocols [Gunderson et al., 2005]. DNA was analyzed using the Illumina Quad610 SNP array by the Center for Applied Genomics (CAG) at the Children's Hospital of Philadelphia. The Illumina Quad610 array contains 8,849 SNP probes on chromosome 12p, with an average spacing of 4.2 kb between probes. Intensity (log 2R ratio) data and genotyping (B-allele frequency) data was used to determine the molecular karyotype of each patient. Genotyping information was used to determine the presence of mosaicism to levels as low as 5% [Conlin et al., 2010]. The B-allele frequency was also used to determine the number of haplotypes present in the two cell lines to distinguish between a meiotic event with rescue (presence of three haplotypes), or a purely mitotic origin of the mosaicism (presence of only two haplotypes). The position of the crossover was determined by the position the most proximal informative SNP (e.g., a SNP with B allele frequency representing mosaicism for AA in the normal cell line and AABB in the i(12p) cell line). Genomic coordinates are reported in Human Genome Build 36, hg18, March 2006.

RESULTS

Cohort and Previous Cytogenetic Findings

We studied 15 patients with a clinical diagnosis of PKS using a genome-wide SNP array to investigate the ability of this platform to identify the i(12p) in blood and tissue. Tetrasomy 12p was previously identified using traditional cytogenetic techniques (karyotyping, or buccal FISH) in 14 probands, with the average age at diagnosis being <1 year Table I). As expected, every proband in whom cytogenetic testing was undertaken on skin fibroblast or buccal swab samples produced a positive result for tetrasomy 12p at the time of diagnosis. Blood karyotyping was positive for only 5 of 11 probands (45%). One proband only had array CGH performed on peripheral blood, with a positive test result (Proband 2). One proband was diagnosed prenatally upon amniocentesis (Proband 8). Two probands had unusual structural findings; one patient was mosaic for an additional cell line with hexasomy 12p due to presence of two copies of i(12) (Proband 7) and one patient had a derivative 12p, containing three inverted tandem copies of 12p (Proband 1; Fig. 1).

Array Findings

Proband age at the time of tissue collection was between 8 days and 6.75 years (Table I). Eleven probands had both blood and skin tissue analyzed, two probands had only blood analyzed, and two probands had only skin analyzed. Array analysis verified tetrasomy 12p in all 13 fibroblast samples. Percent mosaicism was calculated using both probe intensity and allele frequency, with the percent mosaicism ranging from 35% to 100% (Fig. 2 and Table I). One proband had two skin biopsies taken, with one biopsy from hypopigmented skin showing 85% i(12p), and one biopsy from hyperpigmented skin showing 55% i(12p) (Proband 5). Tetrasomy 12p was detected in 6 of the 13 samples (46%) from unstimulated blood, with the percent mosaicism ranging from 5% to 75%. Of the eight probands that had a blood karyotype at initial diagnosis and an array performed on blood with the current study, two probands showed new findings of mosaic i(12p) by array at 10% and 20% (Probands 6 and 14), three probands showed concordant positive testing (Probands 1, 4, and 9), three probands showed concordant negative testing (Probands 7, 11, and 12). As with the cytogenetic testing, tetrasomy 12p was identified in fibroblast samples from all probands tested by array. When comparing array findings from blood and tissue samples from the same probands, the majority of probands showed a higher percent of i(12p) in fibroblasts as compared to blood (9/11, 82%). Only two probands had a slightly higher percent mosaicism in blood than tissue (Probands 2 and 4), three probands had a much lower, but detectible percent present in blood (Probands 6, 9, and 14), and the remaining six probands had no detectable i(12p) in blood.

TABLE I. Findings at Time of Diagnosis and Current Study

	Diagnosis						Current study						
Proband #	Age	Amnio	Blood (% Gband, FISH)	Skin (% Gband, FISH)	Buccal	Blood aCGH	Current age	Age difference	% i(12p) blood	Concordance with previous testing (blood)	% i(12p) fibroblasts	Concordance with previous testing (skin)	Estimated cross-over position (hg18)
1	8 days	nt	Yes (3%, nt)	nt	nt	nt	8 days	0	75%	Yes	nt		16,975,264
2	2 weeks	nt	nt	nt	nt	Yes	4 weeks	2 weeks	65%		55%		None
3	1 year and 5 months	nt	nt	Yes	nt	No	1 year and 5 months	0	0%		80%	Yes	10,387,333 and 29,770,317
4	3 months	nt	Yes	Yes	nt	nt	1 year 6 and months	1 year and 3 months	35%	Yes	30%	Yes	21,633,573
5	2 years and 6 months	nt	nt	Yes	nt	nt	2 years and 6 months	0	0%		55%, 80%	Yes	16,022,209
6	1 year and 3 months	nt	No	nt	Yes	nt	3 years	1 year and 9 months	20%	No	65%		11,240,129
7	7 months	nt	No	Yes (3%, 0.6%)	nt	nt	3 years and 6 months	2 years and 9 months	0%	Yes	35%	Yes	19,997,161
8	Prenatal	Yes	nt	nt	nt	nt	3 years and 9 months	3 years and 9 months	0%		35%		None
9	1 month	nt	Yes	Yes	nt	nt	4 years and 6 months	4 years and 5 months	5%	Yes	60%	Yes	32,080,273
10	1 year	nt	Yes (2%, 1.4%)	Yes	nt	nt	4 years and 9 months	3 years and 9 months	nt		80%	Yes	15,453,043
11	6 months	nt	No	Yes (85%, nt)	nt	nt	5 years	4 years and 6 months	0%	Yes	100%	Yes	25,908,105
12	1 year and 7 months	nt	No	Yes (60%, 50%)	nt	nt	5 years	3 years and 5 months	0%	Yes	nt		nt
13	1 month and 1 week	nt	Yes	Yes	Yes	nt	5 years and 6 months	5 years and 5 months	nt		80%	Yes	16,716,890
14	5 months	nt	No	Yes	nt	nt	6 years and 6 months	6 years and 1 month	10%	No	50%	Yes	4,424,891
15	1 years	nt	No	Yes	nt	nt	6 years and 9 months	5 years and 9 months	0%		70%	Yes	27,689,172
Average	9 months and 2 weeks						3 years and 7 months	2 years and 10 months	16%		63%		19,099,874

CGH, array comparative genomic hybridization, nt, not tested. For diagnosis: Yes, positive testing, No, negative testing.

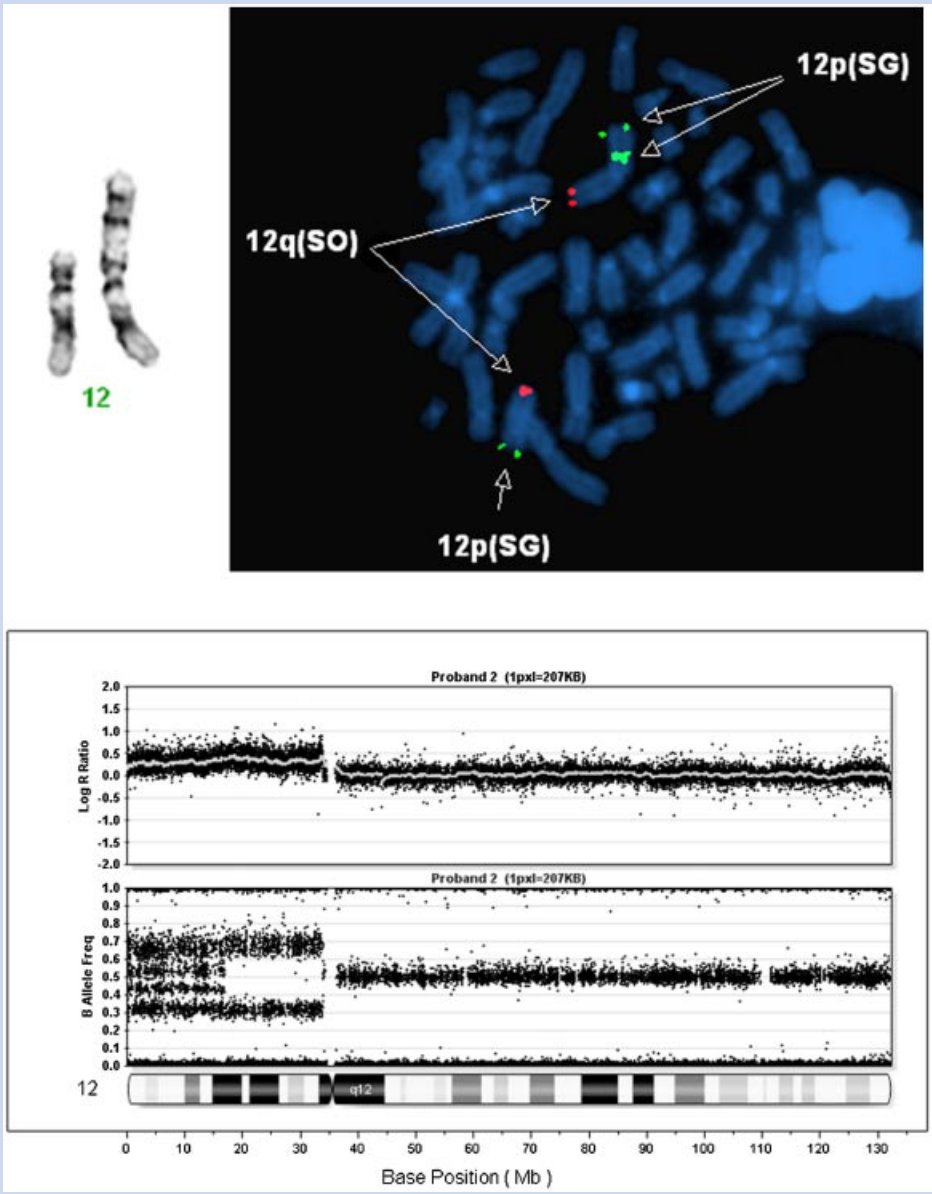

FIG. 1. Derivative 12 in Proband 1 showing inverted tandem triplication of 12p G-banded chromosome (top left) from Proband 1 showing derivative chromosome 12. Fluorescence in situ (FISH) confirms inverted and tandem triplication of 12p (top right). FISH was performed using chromosome 12 telomere probes from Abbott Molecular (Abbott Park, IL). The 12p telomere is labeled in green (12p(SG)) and the 12q telomere is labeled in red (12q(SO)). Array results (bottom) showing the presence of mosaicism for four copies of 12p, with three haplotypes at the distal end of the chromosome. Note that the tetrasomy does not extend to the centromeric region.

Trends With Age

Analysis of the percentage of the i(12p) in blood with proband age at time of study demonstrated a trend with the frequency of the i(12p) decreasing with increasing age (Fig. 3); however, of the three probands with previous cytogenetic testing performed on blood at the time of initial diagnosis (e.g., with detection of the i(12p) at the time of diagnosis), all three still demonstrated the presence of the i(12p) in blood by array, even up to 4.5 years after the initial diagnosis. No age dependent trend was observed in fibroblast samples.

Insights Into Origin of i12p

Using the haplotype calls available through the SNP array analyses, 12 of 14 probands showed evidence for three haplotypes at the distal portion of 12p. All patients demonstrated two genotypes present near the centromere, suggesting the i(12p) arose during, or after, Meiosis II (Fig. 4). Proband 2, who had tandem duplications of 12p, did not have a duplication of the centromeric region of the short arm of 12p (Fig. 1); however, this patient displayed the presence of three haplotypes suggesting that while the structure of the tetrasomy 12p is significantly different, the origin of the extra 12p

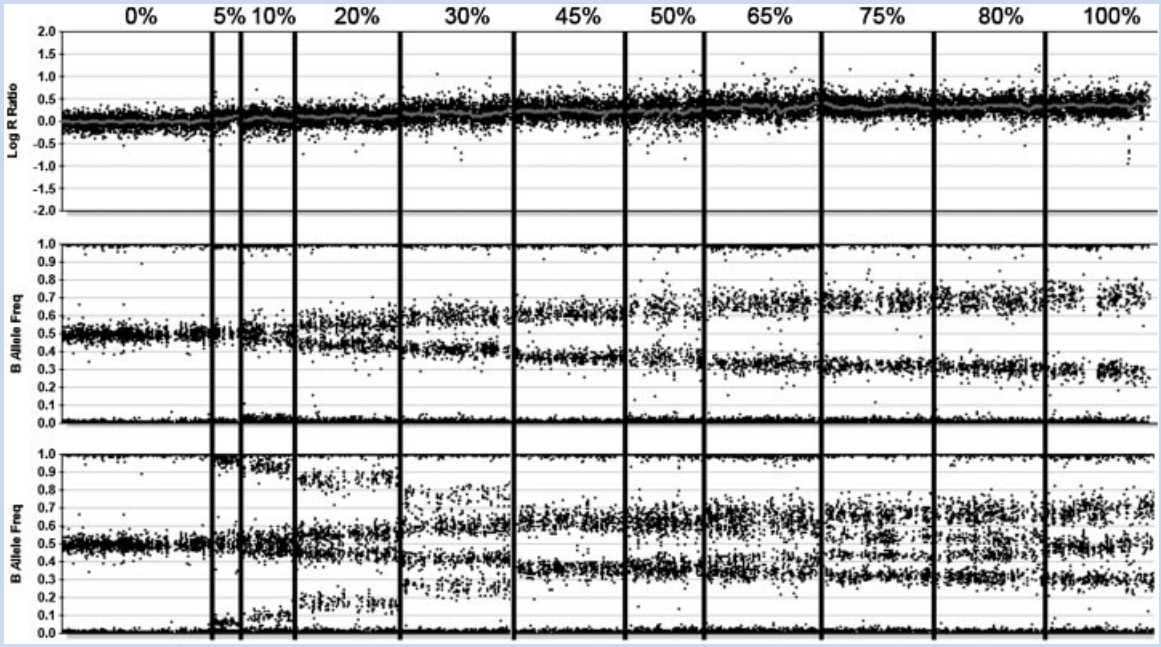

FIG. 2. Composite array results for mosaic i(12p). This figure shows chromosomal segments from11 patients illustrating mosaicism for isochromosome 12p from 0% to 100%. For all figure parts, the percentages above the data indicate the level of mosaicism, with 0% representing a region of 12p with normal copy number, and 100% representing a nonmosaic i(12p). Top: Log R Ratio. Middle: B allele frequency for i(12p) mosaicism from regions of 12p with genotypes indicating two haplotypes at a ratio of three copies to one copy. Bottom: B allele frequency for i(12p) mosaicism from regions of 12p from the same patients with genotypes indicating three haplotypes at a ratio of two copies to one copy to one copy.

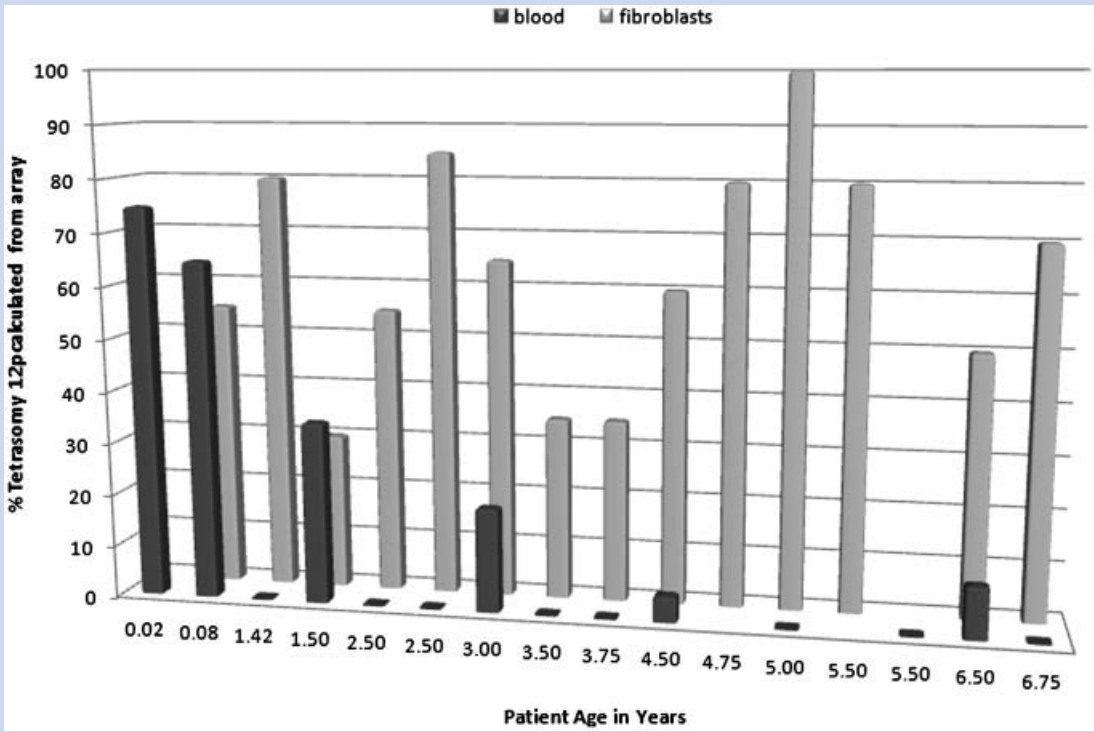

FIG. 3. Trends of i(12p) with age of patient. Array results for the 15 patients indicating percentage of tetrasomy 12p found in peripheral blood (dark gray) and cultured fibroblasts (light gray) at age of tissue collection. Graphical representation of the array findings, showing higher percent of tetrasomy 12p found in blood in younger patients.

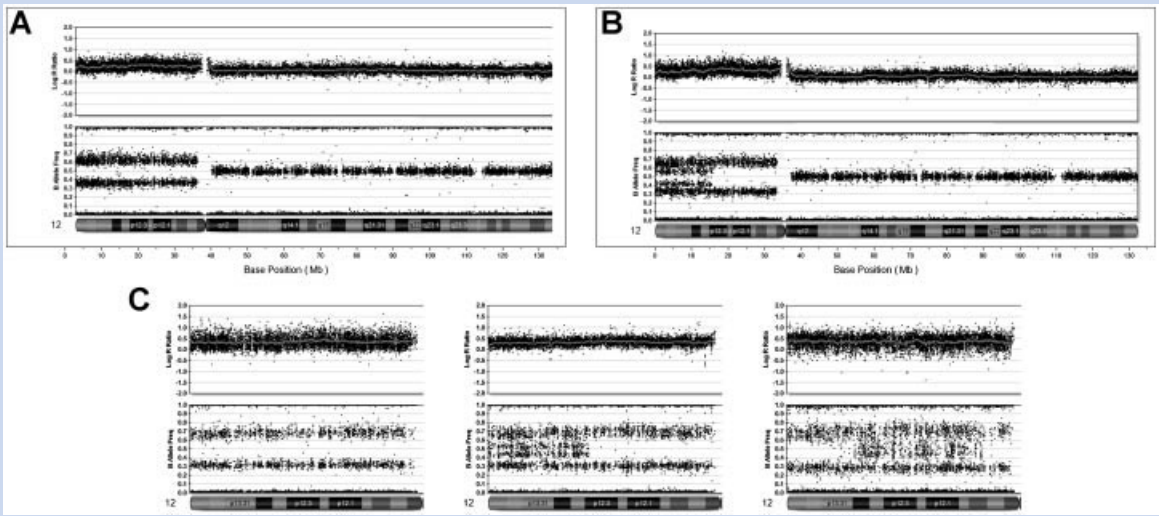

FIG. 4. Evidence for meiotic recombination. Array results for four probands. A: Array result for Proband 5 on DNA from cultured hyperpigmented skin. Percent of i(12p) is calculated at 55%. B: Array result for Proband 5 on DNA from cultured hypopigmented skin with 85% i(12p). The presence of three genotypes is more visible in the array on hypopigmented skin, as expected at these mosaicism percentages. Parental genotyping supported this finding, with both maternal genotypes present in the distal p arm in both samples. C: Array result for three probands with similar percent mosaicism of i(12p) (80%) showing the p arm with none (left), one (center), and two (right) recombination sites.

material occurred at the same time as the other patients. Locations of recombination events in 12 probands showed that a third of the probands had a recombination between 15 and 17 Mb (Table I).

For the two probands that showed no evidence for three genotypes, the possibilities of Meiosis II origin without crossover or mitotic origin cannot be distinguished. No probands demonstrated an isodisomy on the long arm of chromosome 12, which would be consistent with a trisomy rescue [Kearney et al., 2011]. Taken together, these findings suggest that the origin of the i(12p) was occurred in, or after, Meiosis II, after crossing-over took place in Meiosis I.

DISCUSSION

Recently, with the widespread use of array based genome-wide copy number analysis including CGH and SNP arrays, the utility of array based genome-wide analysis in the evaluation of PKS has been documented as case reports. Our study confirms the utility of genome-wide copy number analysis in demonstrating the capacity of these platforms to identify tetrasomic cells from peripheral blood even in 6/13 (46%) of probands including two probands (6 and14) whose standard cytogenetic chromosome analyses were normal at time of initial diagnosis. In these two individuals, the SNP array analysis was performed at a later age than standard karyotype analysis, but was still able to detect the presence of tetrasomic cells from the peripheral blood. This suggests that array-based cytogenetic methodologies may be more sensitive than standard G-banding karyotyping methods in identifying tetrasomic cells in peripheral blood, allowing for cytogenetic diagnosis to be established more often using peripheral blood. Such higher sensitivity of array-based cytogenetic methodologies may obviate the need for undertaking a skin biopsy for fibroblast analysis, although, due to the occasional absence and lower level mosaicism of i(12p) cells in older PKS patients, skin biopsy or buccal swab sample may be required as a second tier testing if CGH and SNP array fails to demonstrate i(12p) cells [Hodge et al., 2012]. The utilization of array-based cytogenetic methodologies would also likely help improve diagnostic capture as the clinical features of PKS are not always recognized, and the need to perform chromosomal analysis on a tissue other than blood is not always appreciated. Therefore, we propose that SNP array analysis be used as first tier testing when PKS is considered as a differential diagnosis.

Our study demonstrates that younger patients tend to have a higher mosaic ratio of i(12p) cells in their peripheral blood, while in older patients the likelihood of detecting the i(12p) cells in peripheral blood declines. These findings concur with previous suggestions of a differential growth advantage between the karyotypically normal cells and the i(12p) cell population [Tang and Wenger, 2005]. In this model the selective growth advantage of the karyotypically normal cell population replaces the stem cells in the bone marrow over time making it more difficult to detect the ever decreasing number of i(12p) cells in the peripheral circulation. The advantage of CGH and SNP arrays to detect low levels of mosaicism in the peripheral blood is likely due to the use of a direct DNA prep from the blood cells, thereby avoiding cell culture, as is needed for standard karyotyping, which would further select against the i(12p) cells in T cells [Reeser and Wenger, 1992]. In addition, DNA is extracted from multiple blood tissues, in which the i(12p) may be found at a different mosaic levels. Therefore, the earlier the array is performed, the better chance of identifying i(12p)

cells in individuals with PKS. We were not able to identify a specific age cutoff after which the i(12p) cells are not observed in peripheral blood, as the timing of the disappearance of i(12p) cells from peripheral blood was quite variable amongst the probands tested. The earliest that the i(12p) cells were unable to be detected in the peripheral blood using the SNP array platform was at the age of 1 year and 5 months in Proband 3 while in Proband 14 we were still able to detect the i(12p) cells at 6 years of age. The reason for this wide variability remains unknown. Turleau et al. [1996] described a fetal case whose tetrasomic cells were not identified in fetal lymphocytes in utero. In such cases with the absence of tetrasomic cell in peripheral blood, it remains to be determined whether hematopoietic cells have lost tetrasomic cells at a very early stage of embryogenesis or hematopoietic progenitors did not include any tetrasomic cells from the initiation of hematopoiesis.

The mechanism leading to the i(12p) cell lines has not been evaluated systematically; however several theories have been proposed including: (1) Meiosis I or II non-disjunction events generating a disomic gamete that results, upon conception, in a trisomic zygote; isochromosome 12p formation occurs postzygotically from one of the chromosome 12s (with loss of the 12q); (2) isochromosome formation associated with non-disjunction at Meiosis I resulting in a gamete with both a normal chromosome 12 as well as an isochromosome 12p [Van Dyke et al., 1987]; (3) normal gametes and zygote, with post-zygotic mitotic non-disjunction and isochromosome formation; (4) pre-meiotic mitotic centromeric misdivision with nondisjunction at Meiosis I or centromeric misdivision at either Meiosis I or II [Rivera et al., 1986; Struthers et al., 1999]. The majority of previously published studies, which used low-resolution microsatellite markers to determine the number of alleles present on the i(12p), suggest maternal Meiosis II nondisjunction as a mechanism of mosaic tetrasomy 12p [Los et al., 1995; Turleau et al., 1996; Cormier-Daire et al., 1997; Schubert et al., 1997]. There has been one report of a patient with concurrent trisomy 12 and i(12p)/uniparental disomy12, suggesting that isochromosome formation was a result of a trisomy rescue [de Ravel et al., 2004]. The haplotype information provided by the high-resolution SNP array enables us to speculate as to the timing of the origin of the i(12p) genetic material. Since we were able to demonstrate the presence of crossing-over within 12p, with parental haplotype patterns showing only two haplotypes at the centromere and three haplotypes at the telomere in the majority of our probands, the timing of isodisomic chromosome formation can be placed after Meiosis I. In all of our patients, we saw no evidence of uniparental disomy, which is unexpected if isochromosome formation occurred as a rescue of trisomy. The lack of uniparental disomy suggests that the origin of the isochromosome is likely to occur before fertilization, thus placing it between the beginning of Meiosis II and fertilization. The mechanism of formation of a monocentric isochromosome is not known, and several mechanisms like centromeric misdivision [Dutly et al., 1998], centromere cleavage [Jin et al., 2000], and intrachromosomal recombination [Koumbaris et al., 2011] have been proposed.

SNP array demonstrated the presence of a cluster of recombination around 16 Mb. Interestingly, only small numbers of genes reside around this recombination hotspot. Previous studies demonstrated that the recombination rates are typically low near the transcription starts sites of genes and recombination rates are known to increase with gene density [Kong et al., 2002; Coop et al., 2008]. Our observation of the recombination cluster around 16 Mb is in agreement with these previous studies. It remains unclear whether this recombination cluster is related to the isochromosome formation in PKS probands or not.

Several features of SNP arrays make it an ideal tool for the evaluation of PKS. First, as we stated above, it does not require cell culture eliminating the effect of differential growth rates between normal and i(12p) cells coexisting in a mosaic state in individuals with PKS. Second, SNP arrays have been shown to detect a lower percent of mosaicism as compared to array CGH, because of the added power of the available haplotype information [Conlin et al., 2010]. Third, the haplotype information obtained from SNP arrays can provide mechanistic insight into tetrasomic cell formation which can be useful for appropriate genetic counseling. Although PKS is known as a sporadic condition, the exact mechanistic basis of i(12p) can provide an additional reassurance arguing against the possible recurrence for the future pregnancies. Fourth, SNP arrays are capable of detecting small interstitial duplications of 12p. The overlapping clinical features between 12p duplication and PKS have been well documented and should be considered in the differential diagnosis of PKS [Zumkeller et al., 2004; Inage et al., 2010].

Although this study highlights the utility of SNP arrays in the evaluation of PKS, atypical cytogenetic abnormalities seen in two PKS probands (1 and 7) emphasize the importance of combinatorial use of traditional cytogenetic techniques along with SNP array analysis. Proband 1 had a derivative 12p, containing tandem copies of 12p without the presence of a marker chromosome. Proband 7 had hexasomy 12p due to the presence of two i(12p) marker chromosomes. PKS resulting from two supernumerary isochromosome 12ps has been previously described [Vogel et al., 2009]. The characterization of these atypical cytogenetic abnormalities required the utilization of G-band karyotyping and fluorescent in situ hybridization (FISH) methodologies.

A limitation of this study includes the possibility of sample bias as the interpretation of the SNP array results was not conducted in a blinded manner; therefore, resulting in improved identification of lower percent mosaicism. However, given that the strength of the SNP array in detecting low-percentage mosaicism is well-known, the contribution of this bias is suspected to be minimal if at all. The other possible limitation is the applicability of our research findings to the regular clinical settings. Among the 6 probands whose i(12p) was detected in the peripheral blood, the percentage of i(12p)detected was very low (5% and 10%) in two probands. Such a low level mosaicism may not be interpreted as abnormal or readily detectable in routine clinical diagnosis. Therefore, we still recommend buccal swab or a skin biopsy when the clinical suspicion of PKS remains high after a negative peripheral blood array result, especially in older patients.

In conclusion, we demonstrate the utility of SNP arrays in conjunction with traditional cytogenetic techniques for the evaluation of PKS. Given that the percentage of mosaic tetrasomic cells in peripheral blood decreases as the individual with PKS ages, SNP arrays should be performed as early as possible to avoid the need for skin biopsy when possible.

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