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

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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.

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

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.
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 ascer-
tained 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

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 prepara-
tion for analysis, the samples were whole genome-amplified, frag-
memented, 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 chromo-
some 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
gene-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
gene 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 per-
formed 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>
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<th>Proband #</th>
<th>Age</th>
<th>Amnio</th>
<th>Blood (Gband, FISH)</th>
<th>Skin (Gband, FISH)</th>
<th>Buccal</th>
<th>Blood aCGH</th>
<th>Current age</th>
<th>Age difference</th>
<th>% i(12p) blood</th>
<th>Concordance with previous testing (blood)</th>
<th>% i(12p) fibroblasts</th>
<th>Concordance with previous testing (skin)</th>
<th>Estimated cross-over position (hg18)</th>
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<td>8 days</td>
<td>nt</td>
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<td>nt</td>
<td>nt</td>
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<td>nt</td>
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<td>2</td>
<td>2 weeks</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
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<td>nt</td>
<td>nt</td>
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<td>nt</td>
<td>1 year 6 and months years and 6 months</td>
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<td>1 year and 3 months</td>
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<td>nt</td>
<td>4 years and 6 months years and 9 months</td>
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<td>Yes</td>
<td>nt</td>
<td>nt</td>
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<td>16%</td>
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CGH, array comparative genomic hybridization, nt, not tested. For diagnosis: Yes, positive testing. No, negative testing.
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 from 11 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.
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 and 14) 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 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 fail 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).

**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.
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 supernumerary isochromosome 12p 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.
ACKNOWLEDGMENTS

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