High-resolution copy number array in the molecular cytogenetic diagnostics of pediatric malignant hematological disorders

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Abstract. The presence of genetic alterations was investigated by SNP array in combination with conventional and spectral karyotyping and fluorescence *in situ* hybridization analysis of 75 consecutive pediatric bone marrow samples. The samples were collected at diagnosis from all children diagnosed with malignant hematological disease between 2006 and 2010 at a single diagnostic center in Gothenburg, Sweden. Conventional cytogenetic and molecular genetics techniques are up to this date essential for the clinical laboratories but there is a need for higher resolution techniques in order to identify new genetic markers that eventually can improve the management of these disorders. Here, we conclude that the addition of SNP array-based karyotyping combined with conventional cytogenetics increase the diagnostic accuracy of pediatric hematological malignancies. The two techniques enhance the cytogenetic image and should not be contrasted as they meet distinct important roles. Since balanced translocations cannot be detected with the SNP arrays of today and tumor-specific translocations are very important diagnostic and prognostic indicators we suggest that SNP-based array is a valuable adjuvant tool in the cytogenetic diagnostics of pediatric leukemias but cannot replace currently used techniques, i.e. G-banding, SKY and FISH analysis.

Introduction

The importance of genetic characterization with cytogenetic and molecular genetics methods is well-established in the diagnosis, prognosis prediction and determination of therapy in the management of malignant hematological disorders. However, there are limitations in the currently used techniques that need to be overcome. The methods used have limited detection sensitivity and resolution, and are also relatively expensive. Conventional cytogenetics are time-consuming; they are dependent on cell growth *in vitro* and good quality of metaphase spreads and have low resolution. Molecular genetics techniques, i.e. fluorescence *in situ* hybridization (FISH), and polymerase chain reaction (PCR) are sensitive and highly specific, but only for selected targets, and are also relatively labor-intensive, especially since the number of possible molecular markers that need to be investigated may be overwhelming. These factors make them both costly and inefficient. It is therefore important to introduce new techniques that can overcome these obstacles. There is also a need to identify new genetic markers that eventually can improve the management of these disorders. Microarray-based genomic profiling is one technique that may help to achieve this goal.

Different array based techniques have proven to be useful complementary tools in characterizing chromosomal aberrations in many tumor types (1,2). Recently, high-density oligonucleotide based single nucleotide polymorphism (SNP) arrays have been used in the analysis of whole-genome copy number. SNP arrays, in combination with computational calculation algorithms, permit a more precise detection of common submicroscopic genomic lesions, amplifications, deletions, and allelic imbalances, such as copy number and neutral loss of heterozygosity (CNN-LOH) (3,4). So far, comparative genomic hybridization arrays, aCGH, or SNP array analysis have mainly been used as a research tool but may now be ready to be introduced in clinical laboratory routines and could possibly replace some of the currently used laboratory procedures and improve the genetic characterization of malignant hematological neoplasms.

In order to validate the use of a genome-wide high resolution DNA copy number analysis in combination with conventional cytogenetic and molecular cytogenetic analysis, a SNP array (Affymetrix, GeneChip® 250K Nsp) analysis was performed on 75 consecutive pediatric hematological malignancies diagnosed at the Sahlgrenska University Hospital, Gothenburg, Sweden between 2006 and 2010. This study shows that the SNP array could provide additional cytoge-

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netic information, more precise chromosome breakpoint information and could increase the detection rate of loss of the *CDKN2A* gene (9p21).

Materials and methods

Patients. Bone marrow aspirates were consecutively collected from 75 children referred for diagnosis and treatment at The Queen Silvia Children's Hospital during the years 2006 and 2010 (Table I). All patients were diagnosed with malignant hematopoietic disorders, including 45 patients with precursor B lymphoblastic leukemia (B-ALL), 11 with T-cell acute lymphoblastic leukemia (T-ALL), 14 with acute myeloid leukemia (AML), two with myelodysplastic syndrome (MDS), one with chronic myeloid leukemia (CML), one with acute promyelocytic leukemia (APL) and one with Burkit's lymphoma (BL). All bone marrow samples were analyzed using conventional cytogenetic, molecular cytogenetic and molecular genetics techniques. In addition, all 75 cases were analyzed using SNP array. The study was performed in accordance to the Declaration of Helsinki and approval by the Ethics Committee, registration number KI02-560.

Cytogenetic, spectral karyotype (SKY) and fluorescence in situ hybridization (FISH) analyses. Conventional cytogenetics, SKY and FISH were performed according to standard procedures at the time of diagnosis. All bone marrow aspirates were cultured, harvested and slides were prepared and G-banded. Conventional chromosome G-banding and/or SKY analysis was performed in all cases. The conditions for pretreatment, hybridization, post-hybridization washes, detection and analysis were essentially as recommended by the manufacturer (ASI-Applied Spectral Imaging Ltd., Migdal Ha'Emek, Israel) and as previously described (5). In most cases at least 20-25 metaphases were analyzed by two independent reviewers using a combination of G-banding and SKY. Karyotypes were described according to ISCN 2005 and 2009 (6,7). FISH analysis was performed on both metaphase and interphase cells using different probe panels depending on diagnosis. The FISH panel used for B-ALL and T-ALL include *ETV6-RUNX1* [t(12;21)(p13;q22)], *BCR-ABL1* [t(9;22)(q34;q11)], *MLL* (11q23), *E2A* (19p13), *CDKN2A* (9p21) and centromere specific probes for chromosomes 9 and 20, for the identification of dic(9;20)(p11~13;q11), for AML *RUNX1-ETO* [t(8;21)(q22;q22)], *PML-RARA* [t(15;17)(q22;q21)], *CBFB* [inv(16)(q22)], *MLL* (11q23), for CML *BCR-ABL1*, for APL *PML-RARA*, and for BL the *IGH-MYC* [t(8;14)(q24;q32)] probe. All probes are commercially available and purchased from Vysis (Downers Grove, IL) except for the probe for the *E2A* gene which was purchased from Cytocell (Cambridge, UK). For each case at least 200 nuclei were scored by two independent reviewers.

DNA and RNA extraction and reverse transcription. DNA was extracted from 1×10^5-5×10^6 leukocytes with a QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized from 500 ng total RNA using Superscript II reverse transcriptase (Invitrogen) and random

Table I. Diagnoses of the 75 cases from which bone marrow aspirates were obtained.

Diagnosis	No. of cases
B-ALL	45
Hyperdiploid	19
t(12;21)	10
Other	16
T-ALL	11
AML	14
t(8;21)	2
inv(16)	1
der(11)(q23)	4
Other	7
MDS	2
CML	1
APL	1
BL	1
Total	75

hexamers in 20 μl reactions according to the instructions of the manufacturer.

Reverse transcriptase-polymerase chain reaction (RT-PCR). Quantitative RT-PCR was performed with primer and probes according to consensus procedures (8), to verify the inversion of chromosome 16 (*CBFB-MYH11*), identify the *MLL* fusion partner and to verify the FISH result *RUNX1-ETO*, *RUNX1-ETV6*, *BCR-ABL1* and *PML-RARA*.

Single nucleotide polymorphism (SNP) arrays. The GeneChip® Human Mapping Affymetrix 250K (Affymetrix, Inc., Santa Clara, CA) array was used to perform a SNP array analysis. The 250K array detects ~262,000 SNPs. All samples were compared *in silico* with healthy individuals. The array experiments were performed according to the protocol provided by the supplier (Affymetrix). Total genomic DNA (250 ng) was digested with the *Nsp*I restriction enzyme and ligated to adaptors. After ligation, the template was subjected to PCR amplification using a generic primer that recognizes the adaptor sequence. The amplified DNA was fragmented with DNase I, labelled with biotin and hybridized to the GeneChip Human Mapping 250K array. The hybridized probes were washed using the Affymetrix Fluidics Station 450 and marked with streptavidin-phytoerythrin. The arrays were scanned using a confocal laser scanner, GeneChip Scanner 3000 (Affymetrix).

Data analysis. The primary data analysis was performed using GDAS software (Affymetrix), while further studies were performed using CNAG software, version 3.0 (Genome Laboratory, Tokyo University, http://www.genome.umin.jp) (9,10) and in NEXUS discovery (5.1) (Biodiscovery, El Segundo, CA).

Table II. Value of the SNP array.

Diagnosis	No. of cases	Was SNP of value?	
		Yes	No
B-ALL	45	27	18
Hyperdipoid	19	11	8
t(12;21)	10	5	5
Other	16	11	5
T-ALL	11	11	0
AML	14	2	12
t(8;21)	2	0	2
inv(16)	1	0	1
der(11)(q23)	4	1	3
Other	7	1	6
MDS	2	0	2
CML	1	1	0
BL	1	1	0
APL	1	0	1
Total	75	42	33

Results

Clinical findings, cytogenetic and molecular genetic findings.
The patient material consisted of 45 patients with B-ALL, 11
T-ALL, 14 AML, 2 MDS and 1 CML, APL and BL. The
pediatric hematological material corresponds to the expected
number of malignancies as well as the distribution of cyto-
genetic and genetic aberrations for this period at The Queen
Silvia Children's Hospital. The karyotypic findings, based
on G-banding and SKY, as well as the FISH and RT-PCR
results were reported to the referring physician according to
standard procedures.

The SNP array analysis identified gains, losses and
CNN-LOH in all the acute leukemia types, except for in the
APL. The SNP information showed different patterns in the
different leukemia entities. Gains dominated the B-ALL
patients and losses the T-ALL and AML patients. Nearly all
of the CNN-LOH was detected in the T-ALL patients. In 42
(56%) of the samples the SNP analysis was of value for the
cytogenetic report, i.e. the array result added new information
to the cytogenetic report and/or a cytogenetic finding was
confirmed and/or a breakpoint was determined (Table II). All
of the T-ALL patients took advantage of the array result while
the AML patients did not gain almost any information by the
technique. In the B-ALL material 27 of the 45 cases (60%)
showed an advantage of using the array. Altogether, the largest
advantage of using SNP array was detected in the lymphocytic
malignancies.

B-ALL. The 45 B-ALL patients included 19 patients showing
karyotypes with modal numbers in the hyperdiploid to
near-triploid region, 10 showing an *ETV6-RUNX1*/t(12;21)
(p13;q22) translocation and 16 with other cytogenetic aberra-

tions (Table I). As expected, the cytogenetically hyperdiploid
cases showed whole chromosome gains using the SNP array.
The concordance between the G-banding/SKY in these was
high but in two cases the quality of the metaphases were poor
and the array revised the karyotype. In another case a normal
G-banding and FISH was reported but the array showed a
hyperdiploid number of chromosomes. In about half of the
hyperdiploid and the *ETV6-RUNX1* positive leukemias there
was added value of the SNP array while the 'other' cases
showed added value in about 70% of the cases (Table II). The
exact breakpoints could be determined in the karyotypes with
unbalanced chromosome aberrations. In the cases with incom-
plete karyotypes (four cases) FISH analysis together with the
SNP result gave additional cytogenetic information otherwise
not identified. Twelve cases showed loss of *CDKN2A* (9p21)
by FISH analysis as well as with SNP analysis. No additional
losses of *CDKN2A* were detected by SNP of the B-ALL
patients. One of the hyperdiploid cases showed loss of one
copy of the *CDKN2A* gene as well as a dic(9;20) translocation
(Fig. 1). The translocation was seen by SKY and verified by a
SNP array.

T-ALL. Cytogenetically, four T-ALL showed complex
karyotypes, four a normal karyotype and two showed loss
of chromosome material and one monosomy 21. The T-ALL
patients in this material showed loss of chromosome mate-
rial. The four patients with apparently normal karyotypes
showed 1-7 different losses of chromosome material each.
Four areas of gained material were seen, including an extra
copy of chromosomes 8, +8q24, +9q21-q34 and +13q21. In the
cases with complex karyotypes the unbalanced translocations
were verified and the breakpoints were determined. Loss of
CDKN2A using FISH was seen in four patients; these losses
were verified with SNP that also detected four more. Two other
patients showed CNN-LOH in the 9p24-p21 area. In total, 10
of 11 T-ALL had deletion of one or both copies of *CDKN2A*
and/or CNN-LOH in the area, using the array technique.

AML. The 14 AML patients were divided into two patients
with a *RUNX1-ETO*/t(8;21)(q22;q22) translocation, one with
an inversion of chromosome 16/ *CBFB-MYH11*, four with
aberrations in the *MLL* gene (11q23) and seven patients
showing other cytogenetic changes (Table I). A split signal
for the MLL probe, using FISH analysis, was detected in all
four cases and RT-PCR showed that these consisted of three
MLL-AF9 fusions and one *MLL-AF10* fusion. Most cytoge-
netic aberrations were identified by conventional cytogenetics
and FISH and the SNP array did not contribute with additional
cytogenetic information (Table II). In two cases (14%), both
with complex karyotypes, the array added cytogenetic infor-
mation.

The two MDSs showed normal karyotypes and showed no
aberrations with the SNP array (Table II). The CML showed a
BCR-ABL1 fusion using both FISH and RT-PCR analyses and
the array showed extra material of chromosome 8 as well as
loss of material from chromosome 12. The BL showed a t(8;22)
(q24;q11) translocation and split signal for *C-MYC* (8q24) and
the array showed several chromosome changes including both
gains and losses. There were no signs of the t(8;22)(q24;q11)
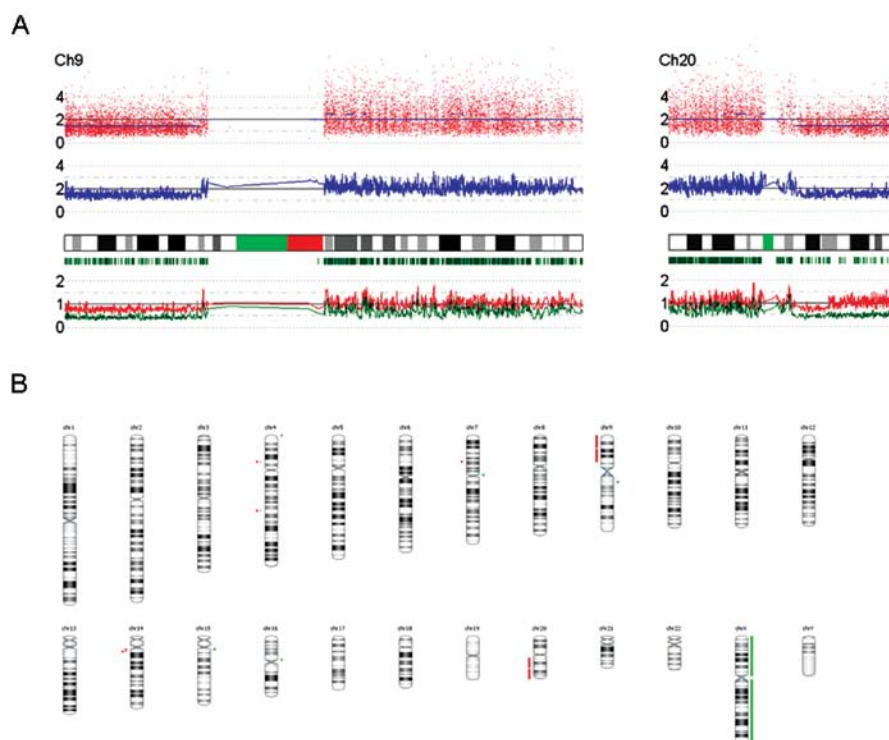translocation indicating that the translocation was indeed

Figure 1. Dic(9;20)(p11~13;q11) seen by using (A) the CNAG and (B) the NEXUS software.

balanced. The APL had the characteristic t(15;17)(22;q21) translocation and the *PML-RARA* fusion and the array did not show any additional changes.

Discussion

The presence of genetic alterations was investigated by SNP array in combination with conventional and spectral karyotyping and flourescence *in situ* hybridization analysis of 75 pediatric bone marrow samples. The samples were collected at diagnosis from all children diagnosed with malignant hematological disease between 2006 and 2010 at a single diagnostic center in Gothenburg, Sweden. Conventional cytogenetic and molecular genetics techniques are up to this date essential for the clinical laboratories but there is a need for higher resolution techniques in order to identify new genetic markers that eventually can improve the management of these disorders.

The SNP array analysis identified gains, losses and CNN-LOH in all the acute leukemia types, except for in the APL. The SNP information showed different patterns in the different leukemia entities. Gains dominated the B-ALL patients and losses the T-ALL and AML patients. In 42 (56%) of the samples the SNP analysis was of value for the cytogenetic report, ie the array result added new information to the cytogenetic report and/or a cytogenetic finding was confirmed and/or a breakpoint was determined. The impact of the array data was particularly significant in the lymphocytic malignancies (68%) while the myeloid malignancies did not gain as much information (17%) by the technique (Table II).

The number of good quality metaphases to analyze is often low in ALL patients yet the genetic characterization is dependent on conventional G-banding and molecular cytogenetic techniques. It could be discussed if it is necessary to perform conventional G-banding for the T-ALL patients and the hyperdiploid B-ALL patients. FISH analysis, using the ALL FISH panel, together with the array data, covered all the cytogenetic aberrations in this T-ALL material as well as contributed with additional cytogenetic information. Flow cytometry analysis always precedes the G-banding analysis at this laboratory, thus giving the diploidity of the malignant cells indicating the hyperdiploid cases.

Moreover, the use of the FISH probe for analyzing suspected loss of the tumor suppressor gene *CDKN2A* (9p21) could also be debated. In this study 10/11 patients showed loss of *CDKN2A* and only four of these were detected using FISH. The SNP array identified eight cases and two were detected using multiplex ligation-dependent probe amplification (MLPA). One problem with the FISH probe is that it covers around 222 kb and a very small deletion may occur within the probe and the signal pattern will appear normal. Five of the eleven T-ALL patients showed CNN-LOH in the 9p24-p13 area. It has been reported previously that loss of *CDKN2A* is often seen in 9p CNN-LOH in pediatric T-ALL (11). Also, it has been shown that the pathophysiology of CNN-LOH may result in a similar gene expression pattern as in deletions (12). This proves to be a great disadvantage of the FISH technique for this particular probe and also it may suggest that there is a higher incidence of loss of *CDKN2A* in pediatric T-ALL than what is reported. One reason why the probe for *CDKN2A* is still used is for the detection for the dic(9;20) translocation patients, as these show loss of one copy of *CDKN2A* (13). One of the cases in this material showed a dic(9;20) translocation (Fig. 1). The FISH pattern for these can be hard to interpret but both analysis programs for the array (CNAG and NEXUS) clearly showed the dic(9;20) translocation, which indicated the importance of this new technique.

There is often a low number of copy number changes (CNCs) in the pediatric AML patients (14). In this study, all but one AML patients showed aberrations using one or the other technique. Most cytogenetic aberrations were identified by conventional cytogenetics as well as with SNP but six CNCs were added after the array. In the cases with a complex karyotype the unbalanced abnormalities were verified and the chromosomal breakpoints were determined. None of the balanced translocations were identified by the array. The results from data collected on the AML patients indicate that there is a value in performing both conventional routine techniques and SNP array but the gain of using SNP for the karyotype is not as obvious as for the ALL patients (Table II).

The number of MDS, CML, APL and BL patients were too low to comment but in order to find secondary chromosome aberrations and to further characterize these entities karyotypically SNP seems to be a good platform in combination with the conventional techniques. One of the CML patients showed extra material of chromosome 8 (+8p12-q24.3) as well as loss of material from chromosome 12. Trisomy 8 emerges frequently at the time of acute transformation and is therefore important information.

In this study, the microarray-based genomic profiling technique (Affymetrix, GeneChip® 250 K Nsp) was used. The advantage of SNP compared to array CGH is that SNP can distinguish between the two alleles and can thus detect CNN-LOH, which has been shown to be of value not only in a clinical genetic settings, but also when studying acquired chromosome changes. A disadvantage with the SNP array platform is that the SNPs are not evenly distributed in the genome as opposed to the BAC (Bacterial Artificial Chromosome) arrays.

Today, there are Affymetrix platforms with higher resolution available than used in this article. The 250 K array improves the resolution of the cytogenetic information a lot (15,16). The additional information gained by increased probe density was marginal, if any. In Mohamedali *et al* (15) patients with myelodysplastic syndromes were analyzed using 50K SNP, 250K SNP and 500K SNP arrays. The concordance in the detection of uniparental disomies (UPD) were 100% and the 500K array did not detect more regions of loss of heterozygosity than the 250K. The higher cost for the very high density arrays is maybe not in parity with the gained information.

There are a few issues to take in consideration when setting up array analysis in the routine diagnostic laboratory. It is important to remember that the cell population analyzed is a mixture of clonal and none clonal cells and that the cell populations are not purified. Some studies have shown that the sensitivity of purified cells is higher and others have shown that the gain is insignificant (17,18). Also, when using DNA arrays it is important to bear in mind that if the clone constitutes less than 20% of cancer cells the aberrations can be undetectable. Sidelines and subclones are common in malignant neoplasms and there is no array information about these as the proportion of these cells is often too small. Another disadvantage to consider is that DNA arrays detect allelic dosage and therefore does not detect balanced translocations. Thus, conventional cytogenetics, FISH analysis and PCR techniques still must be performed according to standard protocols.

It is important clinically to distinguish between germline CNN-LOH, also called UPD and somatically acquired UPD (19). UPD occurs when both copies of a chromosome pair, or part of a chromosome, originate from one parent (20). In this study, normal DNA samples were not available. Thus, it is impossible to know whether the UPD/CNN-LOH appears as a consequence of the malignancy or if it is a result of inheritance. Normal DNA reference material is needed in order to be able to distinguish between somatically acquired and inherited CNN variations or segments of homozygosity. In the future, it is desirable to compare the tumor DNA with a normal DNA sample (paired studies) at the time of diagnosis. It has been suggested that a skin biopsy would be the best choice of control DNA (21) and the most realistic may be DNA from saliva or buccal smears.

In the future, it is possible that second-generation DNA sequencing (or next generation sequencing) methods will enter the diagnostic arena and perhaps even replace the conventional cytogenetic methods as well as DNA array methods. Second-generation sequencing can possibly solve the analytic problem of tumor samples with a low tumor cell quantity, heterogeneity within the sample and variable ploidy. With this technique it will also be conceivable to analyze circulating cells which could be of future interest (22).

In conclusion, SNP array-based karyotyping combined with conventional cytogenetics increases the diagnostic accuracy of pediatric hematological malignancies. The two techniques enhance the cytogenetic image and should not be contrasted as they meet distinct important roles. Since balanced translocations cannot be detected with the SNP-arrays of today and tumor specific translocations are very important diagnostic and prognostic indicators we suggest that SNP-based array is a valuable adjuvant tool in the cytogenetic diagnostics of pediatric leukemias but cannot replace currently used techniques, i.e. G-banding and FISH.

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