Microarray-Based Comparative Genomic Hybridization Using Sex-Matched Reference DNA Provides Greater Sensitivity for Detection of Sex Chromosome Imbalances than Array-Comparative Genomic Hybridization with Sex-Mismatched Reference DNA

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In array-comparative genomic hybridization (array-CGH) experiments, the measurement of DNA copy number of sex chromosomal regions depends on the sex of the patient and the reference DNAs used. We evaluated the ability of bacterial artificial chromosomes/P1-derived artificial and oligonucleotide array-CGH analyses to detect constitutional sex chromosome imbalances using sex-mismatched reference DNAs. Twenty-two samples with imbalances involving either the X or Y chromosome, including deletions, duplications, triplications, derivative or isodicentric chromosomes, and aneuploidy, were analyzed. Although concordant results were obtained for approximately one-half of the samples when using sex-mismatched and sex-matched reference DNAs, array-CGH analyses with sex-mismatched reference DNAs did not detect genomic imbalances that were detected using sex-matched reference DNAs in 6 of 22 patients. Small duplications and deletions of the X chromosome were most difficult to detect in female and male patients, respectively, when sex-mismatched reference DNAs were used. Sex-matched reference DNAs in array-CGH analyses provides optimal sensitivity and enables an automated statistical evaluation for the detection of sex chromosome imbalances when compared with an experimental design using sex-mismatched reference DNAs. Using sex-mismatched reference DNAs in array-CGH analyses may generate false-negative, false-positive, and ambiguous results for sex chromosome-specific probes, thus masking potential pathogenic genomic imbalances. There-

fore, to optimize both detection of clinically relevant sex chromosome imbalances and ensure proper experimental performance, we suggest that alternative internal controls be developed and used instead of using sex-mismatched reference DNAs. (*J Mol Diagn* 2009, 11:226–237; DOI: 10.2353/jmoldx.2009.080064)

Array comparative genomic hybridization (array-CGH) is a high resolution genome analysis technique used to detect DNA copy number alterations, ie, segmental genomic gains and losses. The implementation of array-CGH in clinical diagnosis and research is a fundamental step toward the elucidation of the etiology of congenital malformations frequently associated with genomic disorders,¹ mental retardation,² autism and behavioral abnormalities,³ cancer,⁴ and benign human genome copy number variants.^{5,6} Array-CGH compares genomic DNAs isolated from test and reference samples that are differentially labeled with red (Cy5) and green (Cy3) fluorescent dyes and competitively hybridized to known mapped segments of human genomic DNA (eg, bacterial artificial chromosomes/P1-derived artificial [BAC/PAC] or oligonucleotide probes) attached to a slide. The fluorescent signal intensity of the two fluorochromes at each spot on the microarray is proportional to the amount of test and reference DNA samples bound to the DNA sequence at a given genomic position. The presence of chromosomal imbalance can be detected and quantified

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by calculating the ratio of signal intensities of test DNA versus reference DNA. For statistical purposes, these ratios are usually converted to a $\log_2(\text{Cy5/Cy3})$ ratio for interpretation and analysis (see Supplemental Table 1 at <http://jmd.amjpathol.org>). The $\log_2(\text{Cy5/Cy3})$ scaling has the consequence of centering the ratios at approximately zero, making DNA copy number losses appear negative, and copy number gains positive. In general, a patient's test genome may contain 0, 1, 2, 3, or more copies of a genomic interval, when compared with the reference diploid genome. The ability of array-CGH to detect single copy changes (ie, ratios 1:2, 3:2, 0:1, 2:1) has been previously demonstrated.^{7,8} In contrast, distinguishing homozygous deletions, triplications, and other amplifications (ie, ratios 0:2, 4:2, 3:1, 5:2, and so forth), as well as ascertainment of DNA copy numbers in samples with a mixed population of abnormal and normal cells, such as in tumor tissue or a blood specimen with mosaicism, remains a challenge.

The use of array-CGH is expanding rapidly as a tool for the identification of genomic copy number abnormalities in patients.^{9–15} Abnormality rates of 10 to 15% are widely reported for heterogeneous patient populations typically studied by cytogenetic methods in the past.^{2,16,17} Sex chromosome aneuploidy, microdeletion, and microduplication abnormalities are relatively common, and are of special interest because of their frequent association with congenital anomalies, mental retardation syndromes, infertility, and lethal conditions. Abnormalities involving the X and Y chromosomes can be quite complex, are frequently present in mosaic form, and are associated with a variable phenotype between males and females, the latter complicated by random X-inactivation. Identification of sex chromosome rearrangements is particularly important for adequate genetic counseling.

In array-CGH experiments the measurement of DNA copy number of sex chromosomal regions depends heavily on the sex of the patient and reference DNA used. Although the difference in copy number between X and Y chromosomes in array-CGH studies using sex-mismatched reference DNAs serves as a positive experimental control,¹⁸ the sensitivity of various CGH platforms to detect constitutional sex chromosome imbalances when using sex-mismatched as compared with sex-matched references has not been systematically evaluated. This study evaluates the sensitivity of two array-CGH platforms (BAC/PAC and oligonucleotide microarrays) to detect sex chromosome abnormalities using sex-matched versus sex-mismatched reference DNA samples and demonstrates the importance of experimental design for the interpretation of array-CGH results in clinical diagnosis.

Materials and Methods

Specimens and DNA Isolation

The samples analyzed were selected from 6400 patients whose blood samples were submitted to the Baylor College of Medicine Clinical Cytogenetics Laboratory for array-CGH analysis between August 2005 and March 2007. Patient genomic DNAs were isolated from peripheral blood using a

PureGen kit (Gentra Systems, Minneapolis, MN) according to the manufacturer's protocol. Genomic DNA obtained from whole blood of either a normal male or female individual, was used in all experiments as reference DNA.

Array-CGH Experiments

Initial array-CGH analyses with sex-matched reference DNA were performed using targeted BAC/PAC-based microarray-CGH (Baylor College of Medicine, Version 5 or Version 6.1). The latter microarray contained a total of ~1400 probes spotted in duplicate, and included 120 and 15 probes specific for the X and Y chromosomes, respectively, and four probes from the X/Y short arm pseudoautosomal region (PAR1) and one from the long arm pseudoautosomal region (PAR2). Among the X- and Y-specific probes, approximately half were positioned within clinically relevant and subtelomeric targeted regions, whereas the other half represented backbone clones at a density of one probe per 5 Mb. Labeled genomic DNA from the patient and sex-matched reference DNA were hybridized to a BAC/PAC microarray as previously described.^{10,16}

Array-CGH analyses with sex-mismatched reference DNA using the BAC/PAC microarray platform were performed for 22 patients selected for this study. In addition, 8 patients of the 22 had microarray analysis using a commercially available whole genome oligonucleotide-based array-CGH (Agilent Human Genome CGH Microarray Kit 244A; Agilent Technologies, Inc., Santa Clara, CA). According to the manufacturer, the 244A format has extensive probe coverage including coding and noncoding regions, well-known genes, promoters, miRNAs, and telomeric regions. It contains ~11,000 and 1300 oligonucleotide 60-mer probes specific for X and Y chromosomes, respectively. Probe design has been optimized for maximum sensitivity and specificity within unique sequence.⁷ The 244A platform has no coverage of the X/Y pseudoautosomal regions. Whole genome array-CGH with the 244A array was performed according to the manufacturer's protocol.

Array-CGH Data, Statistical Analysis, and Interpretation

In all array-CGH platforms, the accurate identification of copy number gains and losses requires a combination of visual and computational analysis. For the BAC/PAC microarrays, the fluorescent signal intensities were scanned using a GenePix 4000B microarray scanner (Axon Instruments, Union City, CA) and quantified using BlueFuse for Microarrays version 3.2 software (BlueGnome Ltd, Cambridge, UK). Although the same hybridization conditions apply to all probes on the array-CGH, the hybridization signal intensity is affected by a number of factors. In practice, the performance of the DNA labeling reaction is a particularly critical variable. Even if there is no copy number change, signal intensity and $\log_2(\text{Cy5/Cy3})$ ratios for each individual probe can be variable among a consecutive series of experiments.

To correct for experimental artifacts, array-CGH data are normalized, so that the ratio for a diploid genome is

set to a standard value, usually 1.0 on a linear scale (0.0 on a $\log_2$ scale), and threshold values are ascertained for loss, no change, and gain status relative to the diploid reference DNA (see Supplemental Table 1 at <http://jmd.amjpathol.org>). Threshold ratios were established experimentally for a particular array-CGH platform by validating DNA samples from individuals with known chromosomal gains and losses.¹⁹ The typical mean normal ratio for BAC/PAC-derived array-CGH has been established at 0.0 ± 0.2 on the $\log_2$ scale, indicating equal copy number of test and reference sample DNA. A change in $\log_2(\text{Cy5/Cy3})$ ratio greater than +0.2 or less than -0.2 threshold range typically suggests the possibility of a true gain or loss in DNA dosage at the given interrogating probe locus. Additional computation can refine the cutoff values and generate *P* values for individual patient results.¹⁹

The mean $\log_2$ ratio (patient/reference) of intensity for each single probe was integrated for data processing. Raw data from all probes and dye reversal pairs were combined for subsequent comparative statistical analysis.²⁰ For each sex-matched experiment, a chromosomal region was classified as containing a gain (likely duplicated) or loss (likely deleted) when the value for the $\log_2(\text{Cy5/Cy3})$ ratio of an interrogating BAC/PAC probe or oligonucleotide probe fell outside the fixed threshold (+0.2 and -0.2). The *T*-statistics (>3.0) and *P* values (<0.05) for each probe were also calculated, furnishing additional objective statistical criteria to determine whether deviation of each probe from zero is a significant change as described.^{10,16} The normal array-CGH profile of the $\log_2(\text{Cy5/Cy3})$ ratios clustered around zero for all autosomal and sex chromosome-specific probes considered to have a normal copy number at each interrogating locus (test/reference ratio = 1.0 regardless of whether the locus is autosomal or sex chromosome specific).

Array-CGH plots and quantified data from sex-mismatched experiments were analyzed and independently interpreted by four observers experienced in analysis of clinical arrays who were masked to affection status. For the sex-mismatched experimental design in a female, the array-CGH genomic profile is considered normal when $\log_2(\text{Cy5/Cy3})$ ratios of all X-specific probes showed a gain [$+1.0 > \log_2(\text{Cy5/Cy3}) > +0.2$; test/reference ratio is 2:1, see Supplemental Table 1 at <http://jmd.amjpathol.org>] and all Y-specific probes showed a loss [$\log_2(\text{Cy5/Cy3}) < -0.2$; test/reference ratio is 0:1, see Supplemental Table 1 at <http://jmd.amjpathol.org>]. In contrast, a male array-CGH profile is considered normal when $\log_2(\text{Cy5/Cy3})$ ratios for all X-specific probes show a loss [$-1.0 < \log_2(\text{Cy5/Cy3}) < -0.2$; test/reference ratio is 1:2, see Supplemental Table 1 at <http://jmd.amjpathol.org>] and all Y-specific probes show a gain [$\log_2(\text{Cy5/Cy3}) > +0.2$; test/reference ratio is 1:0, see Supplemental Table 1 at <http://jmd.amjpathol.org>]. Thus, in sex-mismatched CGH, variable test/reference ratios occur depending on the genomic region interrogated; 2:2 for autosomal probes, 2:1 or 1:2 for X-specific, and 1:0 or 0:1 for Y-specific probes.

For sex-mismatched experiments a loss (deletion) or a gain (duplication) of the chromosome region was suspected if the probe $\log_2(\text{Cy5/Cy3})$ ratios were lower or

higher than the expected values for the X and Y copy differences. In sex-mismatched experiments, the *T*-statistics with a null hypothesis of expected mean zero values and the corresponding *P* values cannot be used consistently as criteria for sex chromosome-specific probes because of the statistically significant difference for all sex chromosome-specific probes regardless of the software used. An interpretation is rendered based on graphical profiles of the hybridizations and manual analysis of signal intensity data.

Oligonucleotide microarray data were obtained using Agilent Feature Extraction software 9.5.3 and imported into Agilent CGH analytics 3.4.4 software for analysis. Detection of losses and gains were based on the $\log_2$ ratio (less than -0.3 or greater than +0.3) and z-score algorithm (cutoff 2.5) with a moving average of 50 kb (Agilent Technologies).

Fluorescence in Situ Hybridization (FISH) and Chromosome Analyses

Each abnormality tentatively identified by array-CGH analysis (BAC/PAC and oligonucleotide) was independently assessed experimentally using locus-specific FISH and/or conventional G-banding chromosome analyses. DNAs from BAC and PAC clones spotted on the array were isolated using the Perfectprep mini kit (Eppendorf, Hamburg, Germany) and directly labeled using Spectrum Red or Spectrum Green dUTPs (Abbott/Vysis, Inc., Des Plaines, IL). FISH analyses were performed according to a standard protocol on metaphase chromosomes or interphase nuclei from lymphocyte cultures of peripheral blood. At least 10 metaphase cells and 50 interphase cells were scored to detect deletions or duplications, respectively. A minimum of 100 cells were scored in all cases with suspected mosaicism. Confirmatory FISH analysis for each male patient was accompanied by control hybridization on a normal female sample. Chromosome analysis was performed using standard cytogenetic methods.

Results

Sex Chromosome Abnormalities

Array-CGH analysis using sex-matched reference DNA identified 122 patients from 117 families (51 phenotypic females and 71 phenotypic males) with constitutional imbalances involving the sex chromosomes (Tables 1 and 2). These 122 cases accounted for ~20% of all abnormal cases identified in our clinical laboratory among the screened cohort during the interval of study. Patients with a single BAC clone gain or loss of unclear clinical significance were not included in this analysis. Routine GTG-banding studies performed at different laboratories before array-CGH revealed a normal karyotype in 48 of the 122 patients. Twelve patients had an abnormal karyotype; however, the genomic content or extent of imbalance of X or Y was not determined. Chromosome analysis had not been performed in 62 patients before array-CGH analysis but was completed concurrently for 37 of these cases. Of the 97 patients with prior or con-

Table 1. Summary of Cytogenetic and Array-CGH Results for Female Representative Patients

Category of sex chromosome abnormalities	Total no. of patients	Representative patient (Pt)	Array-CGH plots	Mosaicism	Interpretation of sex-matched study/karyotype	Interpretation of sex-mismatched experiments	Concordance	Number of interrogating BAC/PAC probes
Poly-X syndrome one cell line	4	Pt 1	Figure 1, C and D	No	47,XXX	47,XXX suspected	?	120
Poly-X syndrome multiple cell lines	2	Pt 2		~40%	47,XXX[13]/46,XX[17]	46,XX	—	120
Monosomy X one cell line	2	Pt 3		No	45,X	45,X	+	120
Monosomy X multiple cell lines	4	Pt 4	Figure 1, E and F	~50%	45,X[24]/46,XX[26]	46,XX	—	120
Apparent X deletions	8	Pt 5		No	46,X,del(X)(p11.4)	46,X,del(X)(p11.4)	+	16
Apparent X duplications	4	Pt 6		No	46,X,dup(X)(q26q27)	46,X,dup(X)(q26q27)	+	9
Small X deletions	7	Pt 7	Figure 2, A and B	No	46,X,del(X)(q21q21)	46,X,del(X)(q21q21)	+	2
Small X duplications	11	Pt 8		No	46,X,dup(X)(p22p22)	46,X,dup(X)(p22p22)	?	3
		Pt 9	Figure 2, C and D	No	46,X,dup(X)(q28q28)	46,X,dup(X)(q28q28)	?	4
Complex X abnormalities	10	Pt 10	Figure 2, E and F	~55%	45,X[55]/46,X,dic(X)(q13)[45]	46,X,del(X)(q13)	—	120
Complex Y abnormalities	1	Pt 11		No	46,X,i(Y)(q10)	del(Y)(p10) or i(Y)(q10) suspected	?	8

current karyotype, 82 were reported as normal. Mosaicism was detected in 19 of the 122 patients (16%).

The majority of patients were referred for clinical indications including nonsyndromic developmental delay and mental retardation, behavioral abnormalities, dysmorphic features, and multiple congenital anomalies. A subgroup (~15%) of patients had diagnoses associated with X or Y chromosome rearrangements or an established carrier status. Approximately 36% of patients were positive for submicroscopic deletions or duplications of either the X or Y chromosome, and in some of the patients, cryptic imbalances were present as a part of a complex rearrangement. Duplication of a genomic region encompassing the *MECP2* gene (Lubs X-linked mental retardation syndrome, MRXSL; OMIM no.300260) was found as the most frequent familial aberration affecting males ($n = 19$). Approximately 25% of patients were identified to have common deletions and duplications associated with well-known sex chromosome genomic disorders such as X-linked ichthyosis (*STS*, OMIM no. 308100), Duchenne muscular dystrophy (DMD, OMIM no. 310200), Kallmann syndrome (*KAL1*, MIM no. 308700), Pelizaeus-Merzbacher disease (PMD, OMIM no. 312080), or Rett syndrome (RTT, OMIM no. 312750); however the size of the segmental aneusomy was variable between the patients. Rare deletions and duplication events of the X and Y chromosome were detected in ~20% of patients.

The observed sex chromosome abnormalities were classified into several categories based on the number of probes involved: poly X syndrome (gain with all X-specific probes), monosomy X (one copy with all X probes), disomy Y (two copies with all Y-specific probes), appar-

ent segmental aneusomies because of duplications/deletions (more than three consecutive BAC clones showing a gain or loss) and complex rearrangements (coexistence of loss and gain in copy number with probes representing different segments of the X or Y chromosome). Patients with segmental aneusomies were subdivided into two major categories depending on the number of consecutive interrogating probes that reveal either gains or losses. Patients with imbalances detected by four or more consecutive probes were included in the category apparent segmental aneusomies, whereas the category small duplications/deletions comprises patients in whom abnormalities were identified by less than four consecutive BAC probes. Deletions or duplications identified by four or more consecutive probes produce a pattern that can be more easily visualized on a hybridization profile, whereas imbalances detected by less than four probes are not always obvious on an array CGH graphical profile. Because interpretation of array CGH analyses using sex-mismatched reference DNA was based primarily on visual examination of hybridization profiles, we sought to determine the sensitivity of array CGH as a function of the number of consecutive data points observed from the interrogated probes.

Sex-Matched Reference DNA Detects Abnormalities Not Revealed by Sex-Mismatched References

To evaluate the ability to detect sex chromosome abnormalities by array-CGH using sex-mismatched reference DNA, we selected 22 patients with representative sex chromo-

Table 2. Summary of Cytogenetic and Array-CGH Results for Male Representative Patients

Category of sex chromosome abnormalities	Total no. of patients	Representative patient (Pt)	Array-CGH plots	Mosaicism	Interpretation of sex-matched study/karyotype	Interpretation of sex-mismatched experiments	Concordance	Number of interrogating BAC/PAC probes
Poly-X syndrome one cell line	7	Pt 12		No	47,XXY	47,XXY	+	120
Poly-X syndrome multiple cell lines	2	Pt 13		~42%	47,XXY[21]/46,XY[29]	46,XY	—	120
Monosomy X multiple cell lines	2	Pt 14		~33%	45,X[10]/46,XY[20]	46,XY	—	120
Disomy Y	7	Pt 15	Figure 3, C and D	No	47,XYY	46,XXY	?	15
Apparent X deletions	3	Pt 16		No	46,Y,del(X)(p22p22)	46,Y,del(X)(p22p22) suspected	+	12
Apparent X duplications	7	Pt 17		No	46,Y,der(7)t(X;7)(p22.31;q35)	46,Y,der(7)t(X;7)(p22.31;q35) suspected	+	16
Complex X abnormalities	2	Pt 18		No	46,Y,der(X)del(X)(p22)dup(X)(q27)	46,Y,der(X)del(X)(p22)dup(X)(q27)	+	20
Small X deletions	9	Pt 19	Figure 3, E and F	No	46,Y,del(X)(p22p22)	46,XY	—	3
Small X duplications	23	Pt 20	Figure 3, G and H	No	46,Y,dup(X)(q28q28)	46,Y,dup(X)(q28q28)	+	4
Complex Y abnormalities	5	Pt 21		No	45,X,i(Y)(p10)	45,X,del(Y)(q10)	?	8
Complex X and Y abnormalities	2	Pt 22		No	46,X,der(X)t(X;Y)	46,X,der(X)t(X;Y)	?	6

some imbalances. The results of sex-mismatched versus sex-matched experiments are shown in Tables 1 and 2 for female and male patients, respectively. An array-CGH experimental design using either sex-mismatched or sex-matched reference DNA results in two normal array-CGH profiles for a test female versus reference male DNA (Figure 1A) and a test female versus reference female DNA (Figure 1B). The array-CGH analysis of 11 samples from female patients (Table 1) were concordant in four cases (patients 3, 5, 6, and 7) performed with either sex-mismatched or sex-matched reference DNA. For the remaining seven patients, BAC/PAC array-CGH results were either ambiguous ($n = 4$) or incorrect ($n = 3$) when sex-mismatched reference DNAs were used. In the four patients with ambiguous results (patients 1, 8, 9, and 11) an abnormality (gain or loss) was suspected and additional studies were recommended for optimal interpretation. For the three patients with discordant results, the sex-matched reference design enabled a correct interpretation (patients 2, 4, and 10).

In patient 1 with 47,XXX (Table 1; Figure 1, C and D), array-CGH showed an apparently normal female versus male profile, but the presence of an additional X chromosome was suspected based on the observation of $\log_2(\text{Cy5}/\text{Cy3})$ ratios for some probes that were greater than the mean 0.58 for a 2:1 ratio. Apparently, the detection of an extra chromosome X in patients with 47,XXX is obscure when sex-mismatched reference DNA is used. It becomes even more problematic in patients with mosaicism for trisomy X. The array-CGH analysis using sex-mismatched reference DNA did not identify the cytogenetic abnormality in a female with mosaicism for trisomy X (patient 2, see Supplemental Table 2 at <http://jmd.amjpathol.org>; array-CGH profile not shown). In patient 3 with monosomy X (Table 1, array-CGH profile not shown), results of experiments using both sex-mismatched and sex-matched refer-

ence DNA were concordant. In patient 4 with mosaicism for monosomy X (Table 1; Figure 1, E and F), array-CGH with sex-mismatched reference DNA did not detect a 45,X cell line. For patients 5 and 6 (Table 1, array-CGH profile not shown), results were concordant. Sex-matched versus mismatched results for patient 7 (Table 1; Figure 2, A and B) were concordant, but one clone at Xp11 showed a false-positive result. In the sex-matched experiment, the patient 7 profile (Figure 2, A and B) revealed a decreased $\log_2(\text{Cy5}/\text{Cy3})$ ratio for three probes, one probe at Xp11 and two consecutive probes at the Xq21 region, suggesting a deletion. A deletion of Xq21 was subsequently confirmed by FISH analysis. However, the probe at Xp11 showed a low signal intensity for both Cy3 and Cy5 suggesting a poor hybridization at this locus. This illustrates the need to also use objective computational analyses to render a conclusion. In the sex-matched experimental design, the same null hypothesis is applied to the autosome and sex-specific probes, so both visual and computational analyses can be performed for all probes. Probes that show poor hybridization can be identified and filtered or highlighted by the software. In the sex-mismatched experimental design, the computational analysis is complicated by the need to generate separate null hypotheses for the autosomes versus the X and Y chromosomes, thus increasing the chance of false-positive results that influence the positive predictive value of the test. In patient 8 (Table 1, array-CGH profile not shown) a duplication of Xp22 was suspected. The hybridization profiles showed slightly higher $\log_2(\text{Cy5}/\text{Cy3})$ ratios for three probes at Xp22 compared with other X-specific probes. Similarly, in patient 9 (Table 1, Figure 2C, and see Supplemental Table 3 at <http://jmd.amjpathol.org>) a duplication of Xq28 was questionable. For both patients (patients 8 and 9), gains on X chromosome were readily observed using sex-matched reference DNA (data shown for patient 9; Figure 2D).

Figure 1. Array-CGH genomic profiles of female patients with X chromosome aneuploidies hybridized versus sex-mismatched and sex-matched reference DNA. Genomic profiles of X and Y chromosomes show a hybridization of normal female (pink highlights) versus normal male (blue highlights) (A), and of normal female versus normal female (B). Each individual probe is represented by a green dot, with the horizontal green line revealing the SD of the data point. The combined (from dye-swap hybridizations) $\log_2(\text{Cy5/Cy3})$ ratios are ordered according to BAC/PAC position in the genome from Xp (top) to Yq (bottom). Borders between chromosomes are depicted by dashed horizontal lines. The dashed vertical lines on the logarithmic scale (x axis) indicate the positions of -1.0 and $+0.58$, which are the theoretical expected values for single copy loss or gain, respectively. For comparison, X and Y chromosome profiles using sex-mismatched (left) and sex-matched (right) reference DNA are shown in each column. In C and D, a patient with a 47,XXX karyotype is hybridized with a normal male and with a normal female reference DNA, respectively. The red vertical bar represents the mean value of the normalized $\log_2$ ratio across X chromosome specific probes. In C, it is difficult to recognize the fact that the patient has a third X chromosome compared with D where the presence of the extra X chromosome is obvious. In E and F, a female with 45,X/46,XX mosaicism is hybridized with a normal male and with a normal female reference DNA, respectively. With the sex-mismatched reference in E, it is difficult to recognize that the copy number for X is abnormal, but this is obvious in F.

An analysis of a sample from a patient with complex abnormalities of the X or Y chromosome provides further challenges when sex-mismatched reference DNA is used in the experimental design. In patient 10 (Table 1) the presence of two cell lines with both an isodicentric X chromosome and monosomy X complicates the array-CGH interpretation (Figure 2E). Based on reduced $\log_2(\text{Cy5/Cy3})$ ratios with multiple probes within the Xq region, the array-CGH results had been interpreted as a deletion of the long arm of the X chromosome. An array-CGH experiment using sex-matched reference DNA (Figure 2F) revealed more complex results suggestive of mosaicism and a structural rearrangement of the X chromosome, prompting the need for additional investigation.

The normal BAC/PAC array-CGH profiles of male samples using either a sex-mismatched or sex-matched reference are shown in Figure 3, A and B. The array CGH analysis of 11 samples from male patients were concordant in five cases (patients 12, 16, 17, 18, and 20) regardless of whether the experimental design used sex-mismatched or sex-matched reference DNA (Table 2). However, the BAC/PAC array-CGH analysis using sex-mismatched reference DNA did not identify cytogenetic abnormalities in males with mosaicism for disomy X (patient 13; Table 2, array-CGH profile not shown), and a 45,X cell line (patient 14; Table 2, array-CGH profile not shown). The BAC/PAC microarray

analysis of patient 15 (Figure 3C) with 47,XYY, revealed $\log_2(\text{Cy5/Cy3})$ ratios of 0.1 ± 0.04 for probes from the pseudoautosomal X/Y region indicating a gain, whereas other X-specific probes showed an expected loss because of the sex difference of the reference DNA. All chromosome Y-derived probes showed $\log_2(\text{Cy5/Cy3})$ ratios of 1.9 ± 1.2 as expected in a male versus female hybridization. Additional FISH studies uncovered XYY syndrome in patient 15. A disomy Y in patient 15 in array-CGH analysis using sex-matched reference DNA (Figure 3D) was obvious. A small deletion of Xp (patient 19, Figure 3E) was not identified with sex-mismatched reference DNA used in the experimental design. In contrast, this sex chromosome imbalance was detected by array-CGH using sex-matched references in the experimental design (Figure 3F). For patients 21 and 22 (Table 2, array-CGH profile not shown), results were ambiguous and required additional FISH and chromosome analyses studies to elucidate the genomic abnormality.

Sex-Matched Reference DNA Should Be Used in Oligonucleotide-Based Array-CGH to Improve Detection of Sex Chromosome Abnormalities

Oligonucleotide microarrays have been shown to be more advantageous than BAC/PAC arrays for the identification of

Figure 2. Array-CGH genomic profiles of female patients with X chromosome structural abnormalities or segmental aneuploidies. In **A** and **B**, a female with a 46,X,del(X)(q21q21) karyotype is hybridized with a normal male and with a normal female reference DNA, respectively. In **A**, there appears to be a loss for one clone at Xp11 and for two clones at Xq21 (red circle). The reason for the apparent difference at Xp11 is unclear, but there is no evidence for loss in this region on the sex-matched hybridization. The deletion at Xq21 was evident in the sex-matched hybridization (red arrow) and was confirmed by FISH. In **C** and **D**, a female with a duplication in the *MECP2* region is hybridized with a normal male and with a normal female reference DNA, respectively. The duplication of Xq28 is detected by four clones (red circle) with the sex-mismatched reference, but the separation from the normal portion of the X chromosome is suboptimal by comparison with the sex-matched reference as shown in **D**, where the data reveal duplication of Xq28 detected by four clones (red arrow). In **E** and **F**, a female with a 45,X/46,X,dic(X)(q13) karyotype is hybridized with a normal male and with a normal female reference, respectively. **E**: The hybridization with sex-mismatched reference DNA shows a loss of Xq-specific clones suggestive of a terminal deletion encompassing the long arm of X chromosome (red vertical bar). The log₂ mean value of Xp-specific probes (black vertical bar) is within the normal limits as expected in a female versus male hybridization. **F**: Hybridization with a sex-matched control indicates a complex result suggestive of mosaicism and structural rearrangement of X chromosome. Xp-specific and Xq-specific clones showed a loss of different magnitude (black and red vertical bar), additionally two clones (red circle) at the breakpoint showed intermediate log₂(Cy5/Cy3) values that prompted additional investigations. Oligonucleotide-based array-CGH profile for this patient is shown in Figure 4, A and B.

segmental aneuploidies because multiple interrogating oligonucleotides increase the number of data points that can be analyzed. Also, oligonucleotide-based microarrays usually provide better signal-to-noise ratio, and more robust dynamic range with the observed log₂ ratios for a loss or gain of DNA copy number approaching the theoretical expected value of -1.0 or $+0.58$, respectively. These more ideal values can be obtained with oligonucleotide arrays perhaps primarily because repetitive sequences can be systematically avoided. To test the hypothesis that experimental design using sex-matched reference DNA is required for optimal detection of sex chromosome imbalances, we present the oligonucleotide array-CGH analysis for three female patients (patients 8, 9, and 10), and three male patients (patients 15, 19, and 20) with ambiguous and discordant results by BAC/PAC array CGH.

Using the oligonucleotide-based array, results were concordant when either sex-mismatched or sex-matched reference DNA was used in two female patients (patients 8 and 9) and one male patient (patient 20) (Figure 3, G and H; and see Supplemental Figure 1 at <http://jmd.amjpathol.org>). Based on the oligonucleotide array-CGH study using sex-mismatched reference DNA, patient 10 (Figure 4A) is interpreted as being mosaic for multiple cell lines with deletions of Xq as suggested by the variable signal intensities of probes within Xq12-q13.2 and Xq13.2-qter. However, the presence of a 45,X cell line

was not detected by the sex-mismatched experiment whereas it was detected in the experiment using sex-matched reference DNA (Figure 4, B and D). In patient 15 (Figure 4C) oligonucleotide array-CGH analysis failed to detect an additional Y chromosome when sex-mismatched reference DNA was used. It was not even suspected because of the lack of coverage within the pseudoautosomal X/Y region on the oligonucleotide CGH microarray. The presence of an additional chromosome Y is obvious when sex-matched reference DNA was used (Figure 4E).

The Ability to Identify Abnormalities by Array-CGH Studies Using Sex-Matched Reference DNA May Be Influenced by the Number of Interrogating Data Points

In patients 8 and 20, duplications were detected with 80 and 100 oligonucleotide probes, respectively, versus three and four probes available for BAC/PAC microarray analysis, when sex-mismatched (see Supplemental Figure 1, A and C, at <http://jmd.amjpathol.org>) or sex-matched references (see Supplemental Figure 1, B and D, at <http://jmd.amjpathol.org>) were used. Thus, detection of sex chromosome imbalances using either experimen-

Figure 3. Array-CGH genomic profiles of male patients with chromosome X and Y imbalances hybridized versus sex-mismatched and sex-matched reference DNA. Genomic profiles of X and Y chromosomes show a hybridization of normal male (blue highlights) versus normal female (pink highlights) (A), and normal male versus normal male comparison (B). In C and D, DNA from a 47,XYY male is hybridized with a normal female and with a normal male reference, respectively. C shows clear evidence of X and Y sequences in the patient as expected for a male versus female. There is a slight gain for clones from pseudoautosomal X/Y region (red circle), but there is no reliable indication of increased copy number for Y in the test sample. Hybridization with sex-matched DNA (D) shows a gain for all Y chromosome clones and for clones from the pseudoautosomal X/Y region (red arrows) suggestive of an XYY chromosome pattern. In E and F, a male with a 46,Y,del(X)(p22.31p22.31) karyotype is hybridized with a normal female and with a normal male reference, respectively. E: The profile with the sex-mismatched reference DNA appears normal. The red circle indicates clones that were shown to be deleted by sex-matched experiment and FISH studies. F: The profile with sex-matched reference demonstrates a deletion of Xp22 region detected by three clones (red arrow). In G and H, a male with a duplication in the *MECP2* region is hybridized with a normal female and with a normal male reference DNA, respectively. G: With the sex-mismatched reference careful inspection shows no change ($\log_2 = 0$) in copy number for the Xq28 clones (red circle), which is contrary to a normal result for a male versus female. H: With sex-matched reference the duplicated clones are more easily detected (red arrow).

tal design is improved with an increasing number of interrogating data points. The benefit of multipoint data analysis using oligonucleotide arrays with sex-matched references in the experimental design is demonstrated in patient 10 (Figure 4A). The mean $\log_2(\text{Cy5/Cy3})$ ratios of Xpter-q12-specific oligonucleotide probes (Figure 2F) were notably different from Xq12-qter-specific probes in a BAC/PAC array, suggesting a complex rearrangement for this patient and possible mosaicism. However, two probes at the Xq12-q21.1 breakpoint region showed intermediate $\log_2(\text{Cy5/Cy3})$ ratios (Figure 2F, red circle), which were challenging to interpret as an altered pattern. Using an oligonucleotide CGH microarray, three regions of X chromosome loss were detected readily (Figure 4B, red arrows). These results demonstrate the importance of

using both sex-matched reference DNA and a multitude of data points afforded by oligonucleotide- versus BAC/PAC-based array-CGH to maximize detection rates for genomic abnormalities and for the proper interpretation and statistical analysis of array-CGH results.

Thus, oligonucleotide-based array-CGH can provide better visual analysis of hybridization plots that may resolve some ambiguities when sex-mismatched reference DNA is used. Nevertheless, objective computational analysis, which is more critical with increased array resolution, still remains an issue with the sex-mismatched design. The above results demonstrate the importance of using sex-matched reference DNA as an experimental design to maximize the detection of sex chromosome imbalances when performing array-

Figure 4. The X and Y chromosome profiles of patients with discordant results studied by oligonucleotide based array-CGH with sex-mismatched and sex-matched reference DNA. **A:** Hybridization profile obtained for female patient 10 using sex-mismatched reference DNA shows variable magnitude for three segments on X chromosome (**red arrows**): **1)** a gain of the Xpter->Xq12 segment is consistent with the expected difference between female versus male DNA, therefore the result for this region is interpreted as normal; **2)** a slight gain within the Xq12->Xq21.1 region requires additional analyses; and **3)** there are no changes detected for probes within the Xq21.1->Xqter region, which indicates a loss in copy number in this female patient suggesting a deletion. Additionally, a homozygous loss at Xq21.31 region can be suspected from this hybridization profile.²¹ The hybridization profile of the same design using the BAC/PAC microarray is shown in Figure 2E. **B:** Profile of X chromosome obtained after hybridization of female patient 10 DNA versus normal female control demonstrates loss for all X-specific oligonucleotide probes. Note three segments of the X chromosome: **1)** Xpter->Xq12; **2)** Xq12->Xq21.1; **3)** Xq21.1->Xqter (**red arrows**) showed distinguishable deviation from the normal copy number. Multiple probes located within the Xq12-q21.1 segment form a distinguishable hybridization pattern different from Xpter->q12 and Xq21.1-qter segments, whereas BAC/PAC array-CGH showed inconclusive intermediate $\log_2(\text{Cy5}/\text{Cy3})$ ratio for two clones Xq12-q21.1 segment (Figure 2, E and F). **C:** DNA from a 47,XYY male is hybridized with a normal female. **C** shows a clear difference in copy number for all Y-specific probes, the same as expected in normal male versus female hybridization (**D**). There is no reliable indication of increased copy number for Y in the patient 15 sample. Thus, in experiments using sex-mismatched DNA ratios 2:0 and 1:0 are undistinguishable (**C** and **D**), but the 47,XYY pattern is obvious in experiments using sex-matched DNA (**E**).

CGH analysis using either BAC/PAC or oligonucleotide platforms.

Discussion

We evaluated the ability to detect sex chromosome imbalances using different experimental paradigms that used either sex-matched or sex-mismatched reference

DNAs in array-CGH. At many academic, research, and private laboratories array-CGH is applied by comparing the patient's DNA sample hybridized against an opposite sex (ie, sex-mismatched) reference DNA to provide a positive internal control for each experiment. The results presented here show that utilization of sex-mismatched reference DNA may generate false-negative, false-positive, and ambiguous results for sex chromosome-specific

probes in up to 45% of sex chromosome abnormalities. In our study, imbalances involving sex chromosomes were not detected in 6 of 22 cases, and ambiguous results were obtained in 7 additional cases, when sex-mismatched reference DNA was used in the array-CGH. A sex-mismatched design for BAC/PAC microarrays did not optimally detect abnormalities in patients with mosaicism for trisomy and monosomy X, whereas the same abnormalities were identified unambiguously using sex-matched reference DNA. The presence of BAC/PAC probes within the pseudoautosomal regions of the X and Y chromosomes assist the detection of sex chromosome numerical abnormalities. However, not all array-CGH platforms currently available for clinical diagnosis contain probes covering the pseudoautosomal region. Therefore, sex chromosome aneuploidies such as 47,XXX or 47,XYY can be missed.^{11,22} Furthermore, the correct interpretation of results in female patients with X chromosome duplications and males with deletions is challenging when using sex-mismatched reference DNA, and are greatly dependent on the number of probes interrogating the region of genomic imbalance in the patient.

Microarrays are hybridization-based platforms containing thousands of probes, encompassing a broad range of hybridization thermodynamics, with variable reproducibility between probes. To determine potential chromosome imbalances, several statistical parameters should be evaluated because of the scale of the array-CGH data and the multiple individual tests performed. For the analyses of test DNA samples hybridized versus sex-matched reference DNA, our laboratory uses visual inspection of ratio profiles and review of statistical factors (*T*-statistics and *P* value) in addition to the $\log_2$ intensity ratio calculated for each probe. This allows an automated, uniform, objective evaluation of thousands of genomic loci for each patient.

Array-CGH analyses using sex-mismatched reference DNA were based mostly on visual examination of hybridization profiles and manual review of $\log_2$ (Cy5/Cy3) values across the X and Y chromosomes. The use of *T*-statistics or *P* values for this design is complicated by the need to generate separate null hypotheses for the autosomes versus the X chromosome, and indeed is theoretically invalid for the Y chromosome where the expected ratios (1:0 or 0:1, see Supplemental Table 1 at <http://jmd.amjpathol.org>) would be infinite. This leaves only a subjective visual approach, for analysis with the sex-mismatched design. The disadvantages include the lack of objective criteria that can be used in the principles used for determining sex chromosome-specific copy number alterations, nonuniform criteria for analysis of the same genome (ie, sex chromosomes versus autosomes), increased interpretation time for each analysis, an increased risk of human error, and expenses associated with investigation of samples with ambiguous or false-positive results. Although it may be possible to devise alternate computational procedures for the sex-mismatched design, the complexity and heterogeneity introduced to the interpretation is undesirable.

One justification for the use of sex-mismatched references is the positive control these probes provide for the

hybridization procedure. The development of positive internal controls for assessment of hybridization in microarray experiments is crucial for evaluation of the experimental procedure. Differential hybridization intensities of dye fluorescence may result from concentration-dependent effects between test DNA and probes spotted on microarrays. One alternative potential positive control for assessment of hybridization might consist of a spike-in of a single locus DNA hybridized to a series of dilutions of the complementary probe that would produce variable hybridization intensity ratios, and serve as an internal positive control for hybridization as well as DNA copy number scale. Alternatively, highly variable CNV loci such as the gene for amylase²³ or known CNVs in the sex-matched reference DNA may serve as positive controls. Additionally, in the sex-matched experimental design the same null hypothesis is applied to both the autosomes and sex-specific probes, so probes that show suboptimal hybridization can be filtered or highlighted by the software. We suggest that assessment of fluorescent signal intensity and saturation for every probe should be performed in each array-CGH experiment to ensure proper hybridization. In the sex-mismatched experimental design when X- or Y-specific probes show the expected copy number differences, an optimal hybridization for autosome-specific probes can be anticipated, but not assured. As human genome analysis moves forward with greater resolution using more probes for interrogation, objective statistical analyses will be even more imperative, and an alternative internal positive control, other than opposite sex reference DNA, should be used for clinical diagnostic purposes.

Many individuals with mental retardation, developmental delay, and congenital anomalies have chromosomal rearrangements that result in dosage imbalances for various genomic regions including the X and Y chromosomes.^{24–29} Based on our studies, unbalanced rearrangements involving sex chromosomes account for approximately one-fifth (~20%) of the overall detected genomic imbalances, and the duplication of the Xq28 region is the most common abnormality among all of the subtelomeric rearrangements.³⁰ Such a high incidence highlights the importance of a comprehensive evaluation of different array-CGH platforms and the experimental design used in diagnostic applications. Whereas some deletions and duplications of X and Y chromosomes are associated with characteristic phenotypes such as X-linked ichthyosis; Kallmann syndrome, Duchenne muscular dystrophy, Pelizaeus-Merzbacher disease, and *SRY*-related sex reversal, clinical manifestation in males and females can be significantly variable and nonspecific, challenging the establishment of a definitive diagnosis. Furthermore, small X chromosome deletions and duplications, and even those involving individual exons of a gene, can be associated with nonsyndromic mental retardation.^{28,29}

Often it is not possible to suspect an X-linked abnormality in these families until a number of affected male patients have been born. Furthermore, many duplications and deletions occur *de novo* in association with sporadic traits.³¹ In our cohort of 122 individuals with a sex chro-

mosome abnormality, the majority of patients (~85%) were not referred with either a diagnosis suggesting sex chromosome imbalances or an established X-linked carrier status. Whereas numerous cytogenetic laboratories are ready to offer array-CGH analysis for clinical diagnosis, appropriate validation studies and experimental designs maximizing sensitivity should be established. Until other alternative internal controls are validated for use, one must weigh carefully the presented advantages of enhanced detection of sex chromosome imbalances and objective statistical analyses versus the internal control afforded by the sex-mismatched design.

Our investigation of patient samples with sex chromosome abnormalities of various types (whole chromosome aneuploidy, segmental aneuploidy, complex rearrangements) using BAC/PAC and oligonucleotide microarray platforms revealed that array-CGH analysis with sex-matched reference DNA provides for optimal discovery of sex chromosome dosage abnormalities and thus, more accurate results. Using a sex-matched reference DNA experimental design enables the hybridization intensities to be evaluated by automated statistical approaches, and apply a uniform null hypothesis for the entire genome, thus reducing false-negative results. Abnormalities are visualized readily by inspection of genome profiles and the correct interpretation can be rendered based on multiple statistical parameters. Our data demonstrate that the sex of reference DNA used and number of interrogating data points are important parameters for experimental design. Maximum ascertainment of sex chromosome genomic imbalances and breakpoint boundaries are afforded by using sex-matched reference DNAs, enabling more accurate diagnosis, genotype-phenotype correlations, and genetic counseling.

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