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Comprehensive validation of array comparative genomic hybridization platforms: how much is enough?

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Clinical testing using various array comparative genomic hybridization platforms is being incorporated rapidly into cytogenetic testing algorithms. Comprehensive validation of these complex assays presents unique challenges and very few studies reporting the validation of commercially available array platforms have been published. Sixty-seven patients with previously defined subtelomere abnormalities, representing deletions and/or duplications of all 41 clinically relevant sites, were tested in a blinded study using the Spectral Genomics Constitutional Chip 3.0. Overall, 72 of 74 (97%) subtelomeric abnormalities were concordant with previous cytogenetic studies. However, two false-negative results were documented, and issues with mismapped and suboptimal clone performance were identified that may result in failure to detect 6q and 20q subtelomeric abnormalities. The results of this study indicate that comprehensive validation is necessary before implementation of array comparative genomic hybridization platforms into a clinical setting. Specific suggestions for validation are discussed in the context of the recently proposed American College of Medical Genetics guidelines for microarray analysis for constitutional cytogenetic abnormalities. ***Genet Med* 2007:9(9):632–641.**

Key Words: *microarray validation, array comparative genomic hybridization, array CGH, subtelomeric abnormalities*

Microarray-based comparative genomic hybridization (array CGH) testing has been shown to be efficacious in the diagnostic setting and is being rapidly incorporated as an adjunct to traditional cytogenetic techniques in clinical diagnostic laboratories.[1,2] Although array CGH testing has diagnostic value, this technique is highly complex, involving hundreds to thousands of individual probes targeted to various regions of the genome. Appropriate validation for platforms of this complexity is difficult at best and has not been standardized across laboratories. Few, if any, validation studies have been published based on commercially available array CGH platforms. To address the lack of these studies, we have undertaken a large, blinded comparison of a commercially available array CGH platforms with fluorescence in situ hybridization (FISH) for the detection of subtelomeric abnormalities.

Cryptic deletions and duplications in the subtelomeric regions of chromosomes have been implicated in more than 2.5% of cases of idiopathic mental retardation, dysmorphism, and developmental delay.[3] Diagnostic testing for these abnormalities has become a widely used adjunct to routine cytogenetic and molecular genetic testing in this group of patients. Currently, the detection of such subtelomeric abnormalities is most frequently performed using FISH with chromosome-specific telomere probes.[4] However, subtelomere FISH testing is time-consuming and labor-intensive and provides only limited information regarding the size of any detected abnormality. Array CGH has the potential to decrease the time and labor involved in this testing while providing more complete information regarding the nature and extent of these anomalies.[5,6] The ability of array CGH to detect copy number changes in subtelomeric rearrangements has been described.[7,8] However, no comprehensive assessment of the utility of array CGH as a replacement for subtelomere FISH studies in a clinical setting has been reported.

Herein, we report the blinded comparison of a commercially available targeted bacterial artificial chromosome (BAC) array CGH platform (Spectral Genomics Constitutional Chip 3.0; Spectral Genomics, Inc., Houston, TX) to standard subtelomere analysis by FISH in a clinical setting. Sixty-seven patients with previously identified subtelomeric deletions and duplications representing the vast majority of the 41 clinically relevant sites were studied, and the sensitivity and specificity of this array CGH platform is reported. To our knowledge, this is the most comprehensive analytic validation of any commercially available array CGH platform reported to date.

MATERIALS AND METHODS

Patients and sample preparation

A database of patient samples with previously identified subtelomeric abnormalities identified by subtelomere FISH

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analysis (ToTelVysion, Abbott Molecular, Des Plaines, IL) was queried to identify abnormal patient samples representing both a deletion and a duplication at all 41 clinically relevant subtelomeric loci. Where multiple samples were available for a particular abnormality, samples that were normal by chromosome analysis or those with the most subtle chromosomal rearrangements were preferentially chosen to minimize detection bias due to large rearrangements. Controls were selected from specimens that were reported as normal by subtelomere FISH analysis. Genomic DNA from fixed cell pellets was extracted using a QIAamp DNA Mini kit (Qiagen, Valencia, CA). Genomic DNA from products of conception (POC) specimens was extracted using standard phenol-chloroform extraction procedures. DNA was quantified using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Informed consent and approval from the Mayo Clinic Institutional Review Board was obtained before initiating this study.

Array CGH

Following the manufacturer's protocol, 1 μg each of test and opposite-gender control DNA was labeled by random priming using Cy3 and Cy5 dyes and buffers (Spectral Genomics, Inc.). A reciprocal labeling reaction in opposite fluorochromes was concurrently performed. The labeled DNA was hybridized to a Spectral Genomics Constitutional Chip 3.0 Human BAC array slide at 37°C for 16 hours. Slides were washed in 0.5% sodium dodecyl sulfate, 50% formamide, 0.1% Igepal, 0.2xSSC, and 80% ethyl alcohol and dried with a nitrogen gas stream. Slides were scanned and TIFF images acquired using a GenePix 4000B scanner (Molecular Devices, Union City, CA). Raw array data were generated with GenePix Pro V6.0.1.26 software (Molecular Devices, Union City, CA). Array clones with an SD of triplicates >0.2 were investigated closely for background and other array artifacts and edited to remove outliers. Clones with only one remaining replicate were failed. Data were normalized using global linear regression, and fluorescence intensity ratios of Cy5/Cy3 were plotted for all array clones in two parallel dye-swap experiments with SpectralWare V2.2.40 (Spectral Genomics, Inc., Houston, TX). A priori thresholds of copy number change were set at 0.8 and 1.25 ($\log_2 \approx \pm 0.3$) for the detection of deletions and duplications, respectively. Clones exceeding thresholds for both labeling reactions were denoted as gain or loss.

FISH verification

In regions where gain or loss was expected, BAC clones that did not exceed the thresholds in one or both labeling reactions were studied by FISH. BAC DNA provided by Spectral Genomics was labeled by nick translation (Abbott Molecular). Performance of the FISH probes was evaluated on five normal male controls to document appropriate chromosomal location and to detect any cross-hybridization. Metaphase and/or interphase FISH was then performed on the abnormal specimen to evaluate the copy number status. In addition, poorly performing clones were checked for low copy repeat (LCR) content using the University of California Santa Cruz genome browser (http://genome.ucsc.edu),[9] and the scanned array image was reviewed to rule out poor clone spotting or other technical artifacts that could explain suboptimal detection of copy number change.

RESULTS

Sixty-seven patients with subtelomere anomalies previously confirmed by FISH studies and 10 normal controls were selected for a blinded analysis using the Spectral Genomics Constitutional Chip 3.0 Human BAC array. The results of previous cytogenetic studies used to characterize the abnormalities in these patients are listed in Table 1. The 67 patients harbored a total of 61 subtelomeric deletions and 45 subtelomeric duplications (Fig. 1). At least one sample was tested for each of the 41 clinically relevant subtelomeric sites. Of the 82 possible subtelomeric abnormalities (both deletion and duplication at 41 loci), 74 were tested. Patient samples were not available to test the following subtelomeric abnormalities: 1pter+, 2pter−, 8qter−, 16qter−, 17pter+, 19pter−, 19qter−, and 21qter+.

Overall, the arrays performed quite well. The DNA spots on the chips were consistent and had very few failed spots due to printing irregularities, chip scratches, or artifactual material on the slides. Technically, the DNA labeling, hybridization, and washing steps were straightforward and robust. Only one experiment failed to produce acceptable data due to poor DNA quality. This sample with a 16qter deletion was removed from the blinded study and replaced with a similar sample (Table 1, sample 77) in a nonblinded fashion. The quality of the scanned data were highly interpretable with high signal/noise and signal/background ratios. Few false-positive or uninterpretable data points were encountered. Software analysis with SpectralWare V2.2.40 was fast and the data generated by the software was acceptable and without major flaws.

The samples were unblinded after completion of the array CGH experiments. All 10 normal controls were correctly interpreted as normal. In the 67 patient samples, abnormalities at 72 of the 74 (97%) clinically relevant loci tested demonstrated at least one abnormal clone on the array and were concordant with previous cytogenetic studies (Table 1). An additional subtelomeric abnormality not identified by FISH in previous studies was identified in sample 27, which demonstrated a 9pter deletion in addition to the expected 4qter duplication. Two patients with 20q deletion or duplication previously detected by FISH (Table 1, samples 47 and 59) did not demonstrate a 20q abnormality on the array (Fig. 2a). In addition, the most distal 20q subtelomere clone (GS-81-F12) was normal in another patient with a larger 20q duplication (Table 1, sample 9; array data not shown). To investigate the apparent failure of clone GS-81-F12, DNA from a POC specimen with trisomy 20 confirmed that GS-81-F12 does not detect copy number changes on chromosome 20q (Fig. 2, B). In addition, two patients with 12p subtelomere abnormalities (Table 1, Patients 18 and 74) showed GS-81-F12 deviation simultaneously with 12p deletion or duplication (data not shown). Array CGH data from a POC specimen with trisomy 12 confirmed that the

Table 1
Patient samples included in the blinded study

Sample	Karyotype	FISH	Array results	Concordance
1	n/a	Ypter−	arr cgh Xp22.33/Yp11.31(LLNOYCO3M15D10→GS-839-D20) × 1	Yes
2	n/a	5pter−/14qter+	arr cgh 5p15.33p14(RP11-811I15→RP11-91L13) × 1,14q32.33(RP11-894P9→GS-200-D12) × 3	Yes
3	n/a	14qter−	arr cgh 14q32.33(RP11-894P9→GS-200-D12) × 1	Yes
4	n/a	Normal	arr cgh 1-22(519 BAC/PAC) × 2,X(63 BAC/PAC) × 1,Y(16 BAC/PAC) × 1	Yes
5	n/a	8pter+/13qter−	arr cgh 8p23.3(RP11-555E9→RP11-82K8) × 3,8p23.1(RP11-252K12) × 3,13q34(RP11-75F3→GS-1-1L6) × 1	Yes
6	46,XX,add(18)(p11.32)	9qter+/18pter−	arr cgh 9q34.3(RP11-432J22,RP11-216L13) × 3,18p11.32(GS-52-M11,GS-74-G18) × 1	Yes
7	46,XX	Normal	arr cgh 1-22(519 BAC/PAC) × 2,X(63 BAC/PAC) × 2,Y(16 BAC/PAC) × 0	Yes
8	46,XY,add(7)(q34)	7qter−/12qter+	arr cgh 7q36.3(GS-3-K23) × 1,12q24.33(RP11-669N7→RP11-897M7) × 3	Yes
9	46,XX	7pter−/20qter+	arr cgh 7p22.3p22.2(RP11-482G13→RP11-96L18) × 1,20q13.33(RP11-157P1) × 3	Yes
10	46,XX	2pter+/5pter−	arr cgh 2p25.3(GS-892-G20→RP11-625N16) × 3,5p15.33p15.32(RP11-811I15→RP11-58A5) × 1	Yes
11	46,XY,add(11)(q25)	5qter+/11qter−	arr cgh 5q35.2q35.3(RP11-88L12→GS-240-G13) × 3,11q25(GS-26-N8→RP11-555G19) × 1	Yes
12	46,XX,add(1)(q44)	1qter−/4pter+	arr cgh 1q44(RP11-690C23→GS-160-H23) × 1,4p16.3p16.1(RP11-1398P2→RP11-357G3) × 3	Yes
13	46,XX,del(3)(p26.2)	3pter−	arr cgh 3p26.3(RP11-385A18→RP11-33J20) × 1	Yes
14	n/a	8pter−	arr cgh 8p23.3(GS-580-L5→RP11-45M12) × 1	Yes
15	46,XX	Normal	arr cgh 1-22(519 BAC/PAC) × 2,X(63 BAC/PAC) × 2,Y(16 BAC/PAC) × 0	Yes
16	n/a	4qter−/16qter+	arr cgh 4q35.2(GS-31-J3→RP11-463J17) × 1,16q24.2q24.3(RP11-106D4→RP11-566K11) × 3	Yes
17	n/a	2qter+/9qter−	arr cgh 2q37.3(GS-1011-O17,RP11-341N12) × 3,9q34.3(RP11-424E7→GS-135-I17) × 1	Yes
18	46,XY,del(12)(p13.3)	12pter−	arr cgh 12p13.33(GS-124-K20→RP11-407G6) × 1,20q13.33(GS-81-F12) × 1	Yes
19	n/a	6qter−/18pter+	arr cgh 6q27(RP11-91O16→RP3-495K2) × 1,18p11.32p11.31(GS-74-G18→RP11-105C15) × 3	Yes
20	46,XX,add(4)(p16.3)	4pter−/19pter+	arr cgh 4p16.3(GS-36-P21→RP11-478C1) × 1,19p13.3(GS-546-C11→RP11-554A7) × 3	Yes
21	46,XY	3qter−	arr cgh 3q29(GS-56-H22) × 1	Yes
22	n/a	18qter−	arr cgh 18q22.2q23(RP11-49H23→GS-964-M9) × 1	Yes
23	46,XY,add(9)(p22)	1qter+/9pter−	arr cgh 1q44(RP11-690C23→GS-160-H23) × 3,9q24.3(GS-43-N6→RP11-48M17) × 1	Yes
24	n/a	Normal	arr cgh 1-22(519 BAC/PAC) × 2,X(63 BAC/PAC) × 2,Y(16 BAC/PAC) × 0	Yes
25	n/a	2qter−/22qter+	arr cgh 2q37.3(RP11-202B7→RP11-341N2) × 1,22q13.31q13.33(RP11-66M5→GS-99-K24) × 3	Yes
26	46,XX,dup(20)(p1?2.2p1?3)	20pter−	arr cgh 20p13(RP11-530N10→GS-82-O2) × 1,20p13(RP4-673D20) × 3	Yes
27	46,XX,der(2)(2;9;4)(p11.2;pter;q31.1),der(9)t(2;4;9)(p11.2;pter;q31.1)	4qter+	arr cgh 4q35.2(GS-31-J3) × 3,9p24.3(GS-43-N6,RG-41-L13) × 1	Yes
28	46,XX	Normal	arr cgh 1-22(519 BAC/PAC) × 2,X(63 BAC/PAC) × 2,Y(16 BAC/PAC) × 0	Yes
29	46,XY,add(13)(q32)	5pter+/13qter−	arr cgh 5p15.33p15.2(RP11-811I15→RP11-89M18) × 3,13q34(RP11-75F3→GS-1-L16) × 1	Yes
30	n/a	21qter−	arr cgh 21q22.3(GS-2-H14,GS-63-H24) × 1	Yes
31	46,XY,22ps+	Yqter+	arr cgh Xq28/Yq11.23(cH3.1) × 3	Yes
32	n/a	17qter−	arr cgh 17q25.3(GS-50-C4→RP13-629P20) × 1	Yes
33	46,XY,add(10)(p13)	6qter+/10pter−	arr cgh 6q27(RP11-91O16→RP1-191N21) × 3,10p15.3(GS-23-B11→RP11-486H9) × 1	Yes

(*Continued*)

Table 1
Continued

Sample	Karyotype	FISH	Array results	Concordance
34	46,XX,add(4)(q33)	4qter−/10qter+	arr cgh 4q35.2(GS-31-J3→RP11-463J17) × 1,10q26.1q26.3(RP11-90K19→GS-261-B16) × 3	Yes
35	n/a	Normal	arr cgh 1-22(519 BAC/PAC) × 2,X(63 BAC/PAC) × 1,Y(16 BAC/PAC) × 1	Yes
36	46,XX,der(13)inv(13)(p12q22)dup(13)(q33.2q34)	13qter+	arr cgh 13q12.3q14.3(RP11-186J16→RP11-94N9) × 1,13q22.1q34(RP11-318G21→GS-1-L16) × 3	Yes
37	n/a	Normal	arr cgh 1-22(519 BAC/PAC) × 2,X(63 BAC/PAC) × 1,Y(16 BAC/PAC) × 1	Yes
38	46,XX,der(11)t(3;11)(p25;p15.5)	3pter+/11pter−	arr cgh 3q26.3(GS-1186-B18) × 3,11p15.5(GS-44-H16,GS-908-H22) × 1	Yes
39	46,XY	22qter−	arr cgh 22q13.33(RP3-402G11,GS-99-K24) × 1	Yes
40	n/a	Normal	arr cgh 1-22(519 BAC/PAC) × 2,X(63 BAC/PAC) × 1,Y(16 BAC/PAC) × 1	Yes
41	46,XX,-1,-18,+der(1)t(1;?)(p33;?),+der(18)t(1;18)p33;q22.2)	18qter−	arr cgh 18q21.2(RP11-160B24) × 3,18q22.2q23(RP11-49H23→GS-964-M9) × 1	Yes
42	46,XX,der(5)t(5;13)(q35;q14)	5qter−/13qter+	arr cgh 5q35.3(GS-240-G13) × 1,13q14.3q34(RP11-80H2→GS-1-L16) × 3	Yes
43	46,XX,add(6)(p25).ish der(6)t(6;17)(p25;q23)	der(6)+(6;17)(wcp6+, wcp17+, 17qter+, P53–)	arr cgh 6p25.3(GS-196-I5) × 1,17q24q25.3(RP11-79K13,GS-50-C4) × 3	Yes
44	46,XX,add(4)(q35)	4qter−/wcp15+	arr cgh 4q35.2(GS-31-J3→RP11-463J17) × 1,15q26.3(RP11-397C10→GS-154-P1) × 3	Yes
45	46,XX	Normal	arr cgh 1-22(519 BAC/PAC) × 2,X(63 BAC/PAC) × 2,Y(16 BAC/PAC) × 0	Yes
46	45,XY,-15,der(17)t(15;17)(q13;p13)	17pter−	arr cgh 15q12q13.3(RP11-80H14→RP11-231D12) × 1,17p13.3(CTD-2326F1,GS-68-F18) × 1	Yes
47	46,XY	20qter−	arr cgh 1-22(519 BAC/PAC) × 2,X(63 BAC/PAC) × 1,Y(16 BAC/PAC) × 1	No
48	46,XX,der(3)t(3;17)(p25.1;q25.1)	3pter−/17qter+	arr cgh 3p26.3(RP11-385A18→RP11-33J20) × 1,17q25.3(GS-50-C4→RP13-629P20) × 3	Yes
49	46,XX,der(8)t(8;16)(p23.3;p13.1)	8pter−/16pter+	arr cgh 8p23.3(GS-580-L5→RP11-82K8) × 1,16p13.3p13.1(GS-121-I4→RP11-81F1) × 3	Yes
50	46,XY,add(20)(p13)	20pter+	arr cgh 15q12q11.2(RP11-80H14→RP11-26F2) × 3,20p13p12(RP11-530N10→RP1-278O22) × 3	Yes
51	46,XX,der(6)t(6;7)(p25;q22)	6pter−/7qter+	arr cgh 6p25.3(GS-196-I5) × 1,7q22q36.3(RP11-12L9→RP4-764O12) × 3	Yes
52	n/a	Normal	arr cgh 1-22(519 BAC/PAC) × 2,X(63 BAC/PAC) × 1,Y(16 BAC/PAC) × 1	Yes
53	46,XY,add(10)(q26.3)	9pter+/10qter−	arr cgh 9p24.3(GS-43-N6→RP11-48M17) × 3,10q26.3(RP11-90B19→GS-261-B16) × 1	Yes
54	n/a	7pter+/10qter−	arr cgh 7p22.3(GS-164-D18→RP11-96L18) × 3,10q26.1q26.3(RP11-90K19→GS-261-B16) × 1	Yes
55	n/a	4pter−/11pter+	arr cgh 4p16.3p16.2(GS-36-P21→RP11-357G3) × 1,11p15.5(GS-44-H16→RP11-889I17) × 3	Yes
56	46,X,idic(Y)(q11.2)	Ypter+/Yqter−	arr cgh Xp22.33/Yp11.31(LLNOYCO3M15D10→RP5-1123N13) × 3,Xq28/Yq11.23(cH3.1,GS-225-F6) × 1,Yq11.221q11.23(RP11-71M14→RP11-79J10) × 1	Yes
57	46,XY,add(8)(p23)	8pter−/8qter+	arr cgh 8p23.3p23.1(GS-580-L5→RP11-79E11) × 1,8q24.3(GS-261-I1,GS-489-D14) × 3	Yes
58	46,X,rec(X)dup(Xp)inv(X)(p11.2q21.2)	Xpter+/Xqter−	arr cgh Xp22.33/Yp11.31Xp11.23(LLNOYCO3M15D10→RP11-38O23) × 3,Xq21.3q28/Yq11.23(RP11-88F12→GS-225-F6) × 1	Yes
59	46,XY,add(13)(q22)	3qter+/13qter−/15qter−/20qter+	arr cgh 3q29(GS-56-H22) × 3,13q32.3q34(RP11-122A8→GS-1-L16) × 1,15q26.3(GS-154-P1,GS-124-O5) × 1	No
60	n/a	Normal	arr cgh 1-22(519 BAC/PAC) × 2,X(63 BAC/PAC) × 1,Y(16 BAC/PAC) × 1	Yes
61	46,XY,der(12)t(8;12)(q24.1;q24.3)	8qter+/12qter−	arr cgh 8q24.23q24.3(RP11-172M18→GS-489-D14) × 3,12q24.33(RP11-897M7,GS-221-K18) × 1	Yes
62	46,XX,der(4)t(4;7)(q33;q32)	4qter−/7qter+	arr cgh 4q35.2(GS-31-J3→RP11-463J17) × 1,7q36.3(GS-3-K23) × 3	Yes

(*Continued*)

Table 1
Continued

Sample	Karyotype	FISH	Array results	Concordance
63	46,XX,der(2)t(2;11)(q37.3;q22.2)	2qter−/11qter+	arr cgh 2q37.3(RP11-463B12→RP11-341N2) × 1,11q23q25(RP11-45N4→RP11-555G19) × 3	Yes
64	n/a	Ypter−	arr cgh Xp22.33/Yp11.31(LLNOYCO3M15D10→GS-839-D20) × 1	Yes
65	n/a	5pter−/Xqter+	arr cgh Xq28/Yq11.23(cH3.1,GS-225-F6) × 3,5p15.33p15.2(RP11-811I15→RP11-72C10) × 1	Yes
66	46,XX	Xpter−	arr cgh Xp22.33/Yp11.31(LLNOYCO3M15D10→GS-839-D20) × 1	Yes
67	46,XY,rec(10)dup(10)(p)inv(10)(p13q26.1)	10pter+/10qter−	arr cgh 10p15.3p14(GS-23-B11→RP11-796C22) × 3,10q26.3(RP11-90B19→GS-261-B16) × 1	Yes
68	n/a	Yqter−	arr cgh Xq28/Yq11.23(cH3.1,GS-225-F6) × 1	Yes
69	46,XX	Xqter−	arr cgh Xq28/Yq11.23(cH3.1,GS-225-F6) × 1	Yes
70	46,XX,der(10)t(10;18)(q26.1;q21.3)	10qter−/18qter+	arr cgh 10q26.1q26.3(RP11-90K19→GS-261-B16) × 1,18q23(RP11-91C19) × 3	Yes
71	n/a	5qter−/6pter+	arr cgh 5q35.3(GS-240-G13) × 1,6p25.3(GS-196-I5→RP11-299J5) × 3	Yes
72	n/a	5pter−/19qter+	arr cgh 5p15.33p15.2(RP11-811I15→RP11-72C10) × 1,19q13.43(RP11-45K21,RP11-1129C9) × 3	Yes
73	46,XY	Normal	arr cgh 1-22(519 BAC/PAC) × 2,X(63 BAC/PAC) × 1,Y(16 BAC/PAC) × 1	Yes
74	46,XY	12pter+/12qter−	arr cgh 12p13.33(RP11-598F7→RP11-407G6) × 3,12q24.33(RP11-897M7,GS-221-K18),20q13.33(GS-81-F12) × 3	Yes
75	n/a	Normal	arr cgh 1-22(519 BAC/PAC) × 2,X(63 BAC/PAC) × 2,Y(16 BAC/PAC) × 0	Yes
76	n/a	1pter−	arr cgh 1p36.33(GS-62-L8→RP11-547D24) × 1	Yes
77	n/a	16pter−	arr cgh 16p13.3(GS-121-I4→RP11-616M22) × 1	Yes

FISH, fluorescence in situ hybridization.

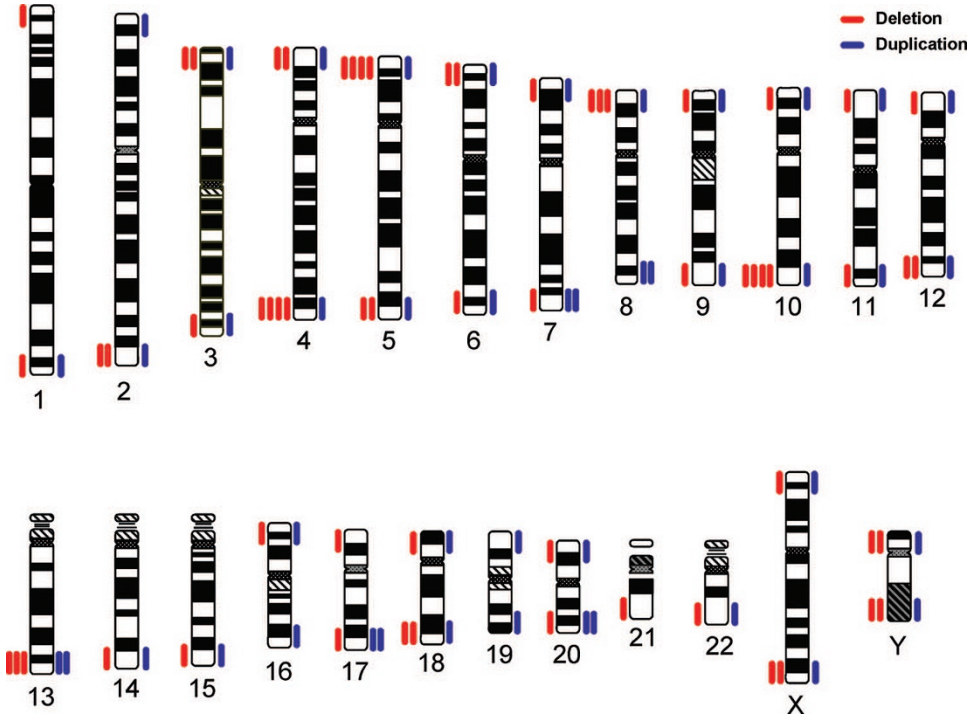

Fig. 1. Schematic representation of the type and location of the subtelomeric abnormalities in the samples tested. Red bars indicate deletion. Blue bars indicate duplication.

DNA spotted on the array for GS-81-F12 is actually derived from a clone on chromosome 12p (Fig. 2c).

Although no other subtelomeric abnormalities in the patient samples included in this study were completely undetected by the array, several additional clones on the array did not perform as expected and could have led to ambiguous or misleading clinical interpretations (Tables 2 and 3). All clones listed in Tables 2 and 3 were investigated with additional

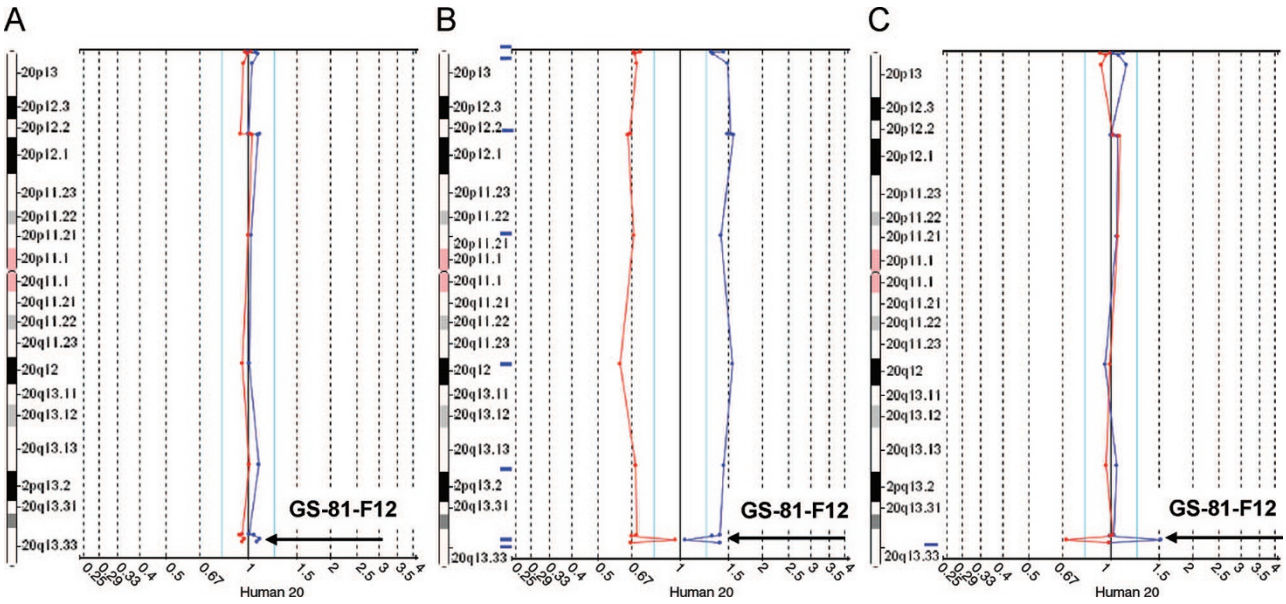

Fig. 2. Determination that the incorrect DNA was spotted on the array for the terminal subtelomeric clone on chromosome 20q. (A) Failure to detect the 20q subtelomere abnormality in sample 59. (B) GS-81-F12 does not deviate in a POC specimen with trisomy 20. (C) POC specimen with trisomy 12 demonstrating duplication of GS-81-F12 on chromosome 20q. POC, products of conception.

Table 2
Mismapped clones

Clone	Expected chromosomal location	Expected distance from telomere (kbp)[a]	Expected clone position (relative to subtelomere)	Actual chromosomal location
RP11-2I16	5qter	3115	2nd clone	11q
GS-57-H24	6qter	172	2nd clone	2p
RP11-11B21	7qter	857	4th clone	5p
RP11-324E12	7qter	424	3rd clone	2p
RP11-1260E13	17pter	91	4th clone	8
GS-202-L17	17pter	10[b]	Terminal clone	17p[c]
GS-81-F12	20qter	43	Terminal clone	12p

[a]Distance from the telomere to the distal end of the clone.
[b]Based on STS marker.[4]
[c]Actual clone location was demonstrated to be 5 Mbp proximal to the 17p subtelomere (data not shown).

FISH studies and/or array experiments with POC specimens harboring appropriate trisomies to clarify the anomalous array results. Table 2 lists additional subtelomeric region clones where the DNA spotted on the array is actually derived from a clone on a different chromosome. In total, DNA from at least seven clones spotted on the array did not represent the actual clone or chromosomal location that was designated for this chip.

Table 3 lists clones that did not perform appropriately in some or all array experiments. In each case, the clones in question harbor significant LCR sequences that likely dampen the ability of these clones to detect copy number differences and contribute to their poor performance in the array experiments. FISH experiments performed for this study and reported in previous studies indicate that six of these eight clones cross-hybridize to other regions of the genome.[4] For example, the chromosome 16 array plot for sample 49 (Table 1) demonstrated a large 16p duplication (Fig. 3, A). However, the distal clone on this chromosome (RP11-568F1) did not show the duplication. Follow-up FISH studies with DNA from this clone demonstrated that this clone hybridized as expected to the appropriate location on both normal chromosomes 16 and to the derivative chromosome 8. However, this clone also hybridized to numerous other subtelomeric loci (Fig. 3, B). The significant proportion of LCR sequence contained in this clone likely explains the observed cross-hybridization signals (Fig. 3, C).

Copy number variants (CNVs) are very common in the human genome and can present interpretive difficulties if included on an array CGH chip.[10,11] Several CNVs were detected in the 77 individuals tested in this study (Table 4) and their frequencies ranged from 2.6% to 35.1%. All but one of these CNVs have been reported as variants.[8,12–15]

Table 3
Poor performing clones

Clone	Chromosomal location	Samples in which clone did not perform appropriately	Samples in which clone performed appropriately	FISH results	Approximate LCR content	Relative clone position (distance from telomere)	Comment
GS-963-K6	4qter	16 (del), 27 (dup), 34 (del), 44 (del), 62 (del)		Confirmed deletion on sample 16 and duplication on sample 27	30%	Terminal clone	Did not deviate in POC specimen with trisomy 4
RP1-191N21	6qter	19 (del)	33 (dup)	Confirmed deletion on sample 19; numerous cross-hybridization signals	50%	Terminal clone	
GS-580-L5	8pter	5 (dup), 57 (del)	14 (del), 49 (del)	Confirmed duplication on sample 5 and deletion on sample 57; cross-hybridizes to 1p	80%	Terminal clone	Noted to cross-hybridize in another study[4]
RP11-424E7	9qter	6 (dup)	17 (del)	Confirmed duplication on sample 6	40%	2nd clone	
GS-112-N13	9qter	6 (dup)	17 (del)	Confirmed duplication on sample 6	30%	Terminal clone	Noted to cross-hybridize in another study[4]
GS-124-O5	15qter	44 (dup)	59 (del)	Confirmed duplication on sample 44	40%	Terminal clone	Noted to cross-hybridize in another study[4]
RP11-568F1	16pter	49 (dup), 77 (del)		Confirmed duplication on sample 49 and deletion on sample 77; numerous cross-hybridization signals	30%	Terminal clone	
GS-202-L17	17pter	46 (del)		Confirmed deletion on sample 46; cross-hybridizes to 12p	40%	Terminal clone	Did not deviate in POC specimen with trisomy 17; Noted to cross-hybridize in another study[4]; also mismapped (Table 2)

FISH, fluorescence in situ hybridization; LCR, low copy repeat; POC, products of conception.

DISCUSSION

Publications on the validation of commercially available array CGH products for use in clinical laboratories have been essentially nonexistent in the literature. In this study, we attempted to validate the Spectral Genomics Constitutional Chip 3.0 Human BAC array for use as a replacement for subtelomere FISH analysis in clinical testing. Sixty-seven patients with abnormalities defined by chromosome analysis and a subtelomere FISH panel and 10 normal controls were selected for a blinded analysis to determine the efficacy of this array platform. In total, 72 of the 74 subtelomeric abnormalities tested on the chip were detected by the array yielding an overall analytical sensitivity of 97%. No false-positive results were obtained. The array also detected a 9pter deletion that was not observed by clinical FISH testing, indicating that neither platform is 100% sensitive for the detection of subtelomeric rearrangements.

The results of this blinded study demonstrated that the Spectral Genomics Constitutional Chip 3.0 Human BAC array system was technically capable of producing robust, reliable, and consistent data for the detection of subtelomeric abnormalities. However, several of the clones representing the subtelomeric regions on the array were identified as problematic and could have led to false-negative results or misleading clinical interpretations.

In the process of completing this study, seven clones were identified in which the DNA spotted on the array did not represent the correct clone (Table 2). These spots represent DNA from unknown clones in completely different chromosomal locations. In addition to the mismapped clones, eight clones performed poorly in samples containing abnormalities in the regions represented by the clones (Table 3). All of these clones contain significant amounts (>30%) of LCR sequences. The LCR sequences within these BAC clones are highly homologous to other regions of the genome, which dampens the ability of these clones to detect copy number differences and contributes to their suboptimal performance. The LCR sequences also result in significant cross-hybridization signals in FISH experiments, complicating FISH verification of abnormal results. These results demonstrate that clones used for array CGH should be carefully selected to avoid significant LCR sequences that mask bona fide abnormalities and contribute to interpretive difficulties.

Mismapped or poorly performing clones on the array could lead to false-negative results or misinterpretation of the abnormalities they are intended to detect. For the terminal clones on the chromosome, the results may be misinterpreted as an interstitial deletion rather than a terminal deletion or sufficiently small abnormalities could be missed altogether. The mis-

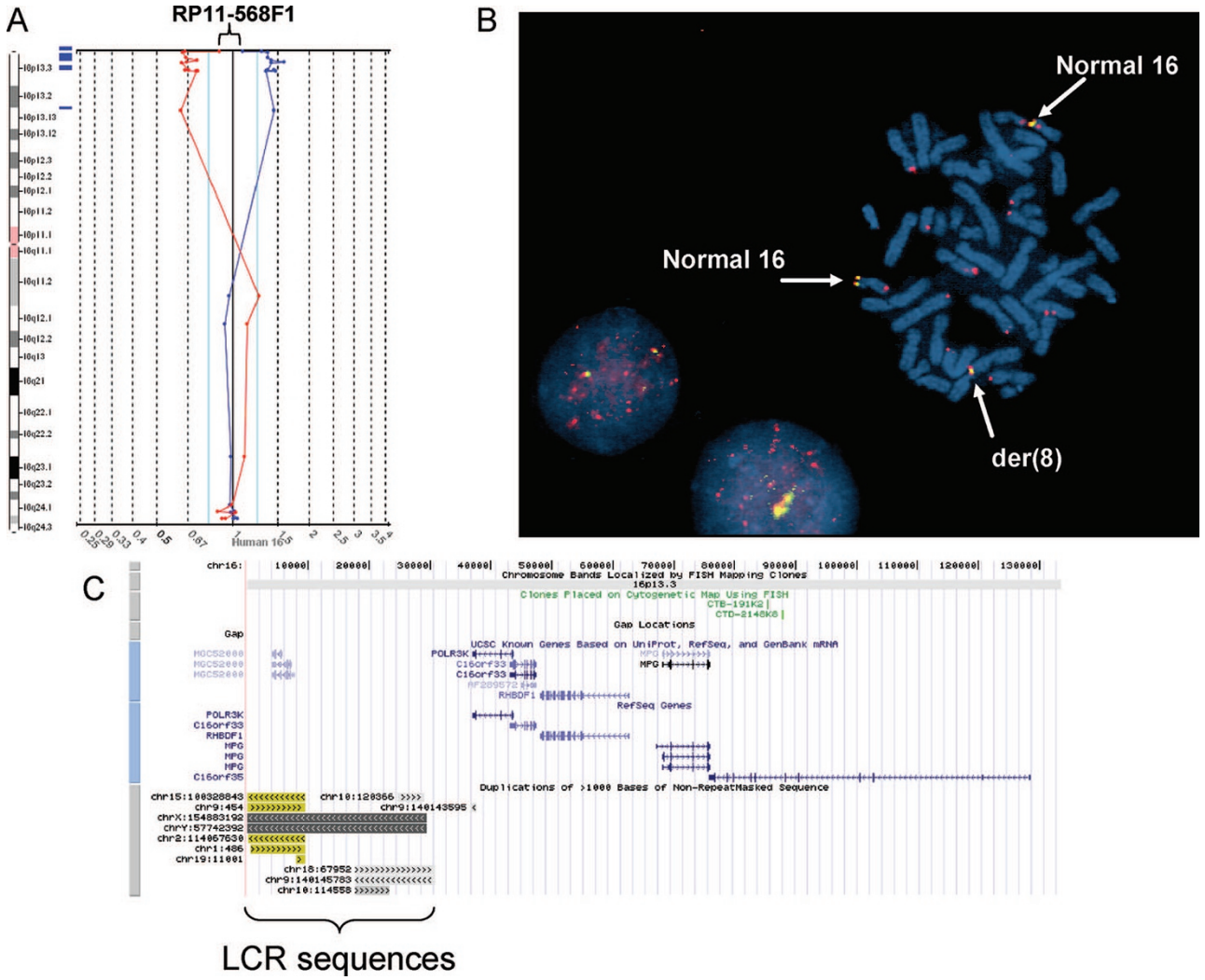

Fig. 3. Example of a poorly performing clone and the effect of low copy repeat (LCR) structure on the ability of array comparative genomic hybridization (CGH) to detect copy number changes. (A) Array CGH plot of sample 49 showing 16pter duplication. The terminal subtelomere clone RP11-568F1 does not demonstrate duplication. (B) Fluorescence in situ hybridization using clones RP11-568F1 (labeled in red) and GS-121-I4 (labeled in green) on 16pter to produce yellow fusion signals in cells from Patient 49. Arrows indicate fusion signals on the two normal chromosomes 16 and the derivative chromosome 8. Significant cross-hybridization of the RP11-568F1 (red) probe to multiple additional chromosomal locations in both metaphase and interphase was present. (C) UCSC Genome Browser representation of the genomic region covered by clone RP11-568F1. Brackets indicate LCR sequences present within this clone and on chromosomes 1, 2, 9, 15, 19, X, and Y.

Table 4
Observed CNVs in 77 patient specimens

BAC clone(s)	Cytogenetic location	Loss	Gain	Known CNV?
GS-1011-O17/ RP11-341N2	2q37.3	2/77 (2.6%)		Yes (Ballif et al., 2000[16])
RP11-463J17	4q35	4/77 (5.2%)		Yes (Redon et al., 2006[14])
RP4-764O12	7q36.3		4/77 (5.2%)	No
RP11-122N11	8p23.1	22/77 (28.6%)	5/77 (6.5%)	Yes (Sharp et al., 2005[13])
GS-221-K18	12q24.33	4/77 (5.2%)	5/77 (6.5%)	Yes (Redon et al., 2006[14])
RP11-80H14/RP11-289D12/RP11-26F2	15q11.2	1/77 (1.3%)	1/77 (1.3%)	Yes (Sebat et al., 2004[12])
RP11-483M24	Xp22.31		2/77 (2.6%)	Yes (Shaw-Smith et al., 2004[8])

BAC, bacterial artificial chromosome; CNV, copy number variant.

mapped clone at 20qter (GS-81-F12) led to the two false-negative results detected in this study. Based on the relative positions of the 20q subtelomere clones on the array, 20q subtelomere abnormalities less than approximately 1 Mb in size will not be detected by this array. In a clinical setting, the use of this array without appropriate 20q subtelomere coverage could lead to a completely negative result (as in sample 47) or to the interpretation of an unbalanced translocation as a

deletion (as in sample 59). The second situation is particularly concerning in a clinical setting. The parents of a child with an unbalanced translocation would likely be screened for a balanced form of the translocation, the results of which would have implications for recurrence risks in future pregnancies, whereas the parents of a child with a deletion may not have follow-up studies due to relatively low recurrence risks.

A second example highlights another clinically significant subtelomere abnormality that would be missed by this array. The two most distal clones at 6qter on the chip are mismapped (GS-57-H24, Table 2) or LCR rich and perform poorly (RP1-191N21, Table 3). Based on the positions of these clones, deletions or duplications smaller than approximately 1.7 Mb would appear normal on the array. This presents a significant diagnostic problem because patients with 6qter deletions smaller than 1.7 Mb have a clinical phenotype.[16]

Finally, abnormalities involving mismapped or poorly performing clones that are proximal to the most terminal clones could be misinterpreted as noncontiguous deletions or duplications. For example, the third and fourth clones from the 7q subtelomere (RP11-324E12 and RP11-11B21, Table 2) are both mismapped, leading to confusing and potentially misleading results.

The results of these studies indicate that although this array CGH platform is capable of producing very robust data, additional work needs to be done to implement more effective clone selection, quality control, and validation processes. The selection of clones for inclusion on an array CGH platform should include a process to exclude clones containing LCR sequences using available online resources (http://genome.ucsc.edu/; http://humanparalogy.gs.washington.edu/structuralvariation/; http://projects.tcag.ca/humandup/). Our data demonstrate that clones with as little as 30% LCR can have significant effects on clone performance. Therefore, clones with even minimal LCR content have the potential to be affected, and we suggest using only clones with $<10\%$ LCR content for inclusion on an array CGH platform. Quality control processes to ensure that the appropriate DNA is spotted on the slides are also critical for optimal array performance and appropriate clinical interpretation. At a minimum, DNA from each clone should be end-sequenced, PCR verified, or FISH verified before spotting on the chip to eliminate mismapped clones. Finally, initial array CGH experiments to verify that clones are sensitive to dose changes should be performed. The use of specimens with monosomy or trisomy of various chromosomes was very helpful in this regard (POCs with autosomal trisomies and patients with Turner, Klinefelter, or XYY syndrome). In summary, the appropriate experiments should be performed to identify and remove clones that do not perform appropriately or do not hybridize to the appropriate genomic locations.

Few, if any, studies have been published validating the performance of commercially available array CGH platforms. This presents particular difficulties in the context of the Standards and Guidelines for Clinical Genetic Laboratories document regarding microarray analysis for constitutional cytogenetic abnormalities (Shaffer et al., see page 654 in this issue). These recommendations by the Laboratory Quality Assurance Committee of the American College of Medical Genetics provide specific recommendations for clinical laboratories beginning to use array CGH technology. The guidelines suggest that for an investigational use only product intended for use as an adjunct to traditional cytogenetic analysis (such as the platform evaluated in this study), each laboratory should validate the array platform with a series of normal and abnormal specimens to address the regions of the genome represented on the array. A minimum of 30 abnormal specimens is suggested for this validation. However, these guidelines do not address initial and, in our view, critical comprehensive validation studies that the platform and the probes included on the platform perform appropriately, particularly those products that are commercially available and labeled as investigational use only. These guidelines also do not discuss the roles and responsibilities of the manufacturer providing these platforms before the validation studies suggested by the guidelines.

We propose two levels of validation before clinical implementation of any array CGH platform is considered. First, the company providing the array performs initial validation that includes (1) verification that each probe on the chip represents the intended genomic region (using end-sequence, polymerase chain reaction, or FISH techniques) and provides these data to the end users and (2) comprehensive verification of probe performance through the analysis of normal and abnormal specimens. It is recognized that companies marketing array CGH chips may not have formal affiliations with CLIA (Clinical Laboratory Improvement Amendments) approved clinical laboratories and may not have access to appropriate clinical samples for validation of probe performance. In this case, a consortium of clinical laboratories may be necessary to appropriately validate array platforms since a single laboratory may not have access to all the necessary specimens. These specimens may include samples with known aneuploidy, syndrome-specific, or subtelomeric abnormalities and peer-reviewed publication of the initial validation of probe performance so that all laboratories can access and rely on these data.

The second level of preclinical validation involves the laboratory intending to use a particular array CGH platform for clinical testing. This laboratory should perform additional validation, as outlined in the ACMG guidelines, primarily to demonstrate performance characteristics and proficiency with the particular platform being implemented. These guidelines include running a series of normal and abnormal controls, blinded sample exchange with another laboratory, and various other aspects of analytic standards and quality control.

In summary, the utility of array CGH has been demonstrated in multiple publications and is rapidly being implemented for routine use by clinical laboratories. However, guidelines for the appropriate validation of array CGH platforms have lagged behind this technology. The results presented in this study demonstrate that although the platform tested produces reproducible and robust data, there are clone selection and platform validation problems that should be addressed to avoid false-negative or misleading results in the clinical setting. These data also highlight potential deficiencies in the American College of Medical Genetics guidelines for array

CGH platform validation. Appropriate manufacturer validation of these highly complex assays is necessary to ensure the highest quality of clinical testing, a problem that will only become more complex as whole-genome platforms based on high-density oligonucleotides begin to be used as a replacement for established cytogenetic and FISH testing.

References

1. Shaffer LG, Bejjani BA. Medical applications of array CGH and the transformation of clinical cytogenetics. *Cytogenet Genome Res* 2006;115:303–309.
2. Veltman JA, De Vries BB. Diagnostic genome profiling: unbiased whole genome or targeted analysis? *J Mol Diagn* 2006;8:534–537.
3. Ravnan JB, Tepperberg JH, Papenhausen P, Lamb AN, et al. Subtelomere FISH analysis of 11688 cases: an evaluation of the frequency and pattern of subtelomere rearrangements in individuals with developmental disabilities. *J Med Genet* 2006;43: 478–489.
4. Knight SJ, Lese CM, Precht KS, Kuc J, et al. An optimized set of human telomere clones for studying telomere integrity and architecture. *Am J Hum Genet* 2000;67: 320–332.
5. Bejjani BA, Saleki R, Ballif BC, Rorem EA, et al. Use of targeted array-based CGH for the clinical diagnosis of chromosomal imbalance: is less more? *Am J Med Genet Part A* 2005;134:259–267.
6. Wong A, Lese Martin C, Heretis K, Ruffalo T, et al. Detection and calibration of microdeletions and microduplications by array-based comparative genomic hybridization and its applicability to clinical genetic testing. *Genet Med* 2005;7:264–271.
7. Veltman JA, Schoenmakers EF, Eussen BH, Janssen I, et al. High-throughput analysis of subtelomeric chromosome rearrangements by use of array-based comparative genomic hybridization. *Am J Hum Genet* 2002;70:1269–1276.
8. Shaw-Smith C, Redon R, Rickman L, Rio M, et al. Microarray based comparative genomic hybridisation (array-CGH) detects submicroscopic chromosomal deletions and duplications in patients with learning disability/mental retardation and dysmorphic features. *J Med Genet* 2004;41:241–248.
9. Karolchik D, Baertsch R, Diekhans M, Furey TS, et al. The UCSC Genome Browser Database. *Nucleic Acids Res* 2003;31:51–54.
10. Feuk L, Carson AR, Scherer SW. Structural variation in the human genome. *Nat Rev Gene* 2006;7:85–97.
11. Bejjani BA, Shaffer LG. Application of array-based comparative genomic hybridization to clinical diagnostics. *J Mol Diagn* 2006;8:528–533.
12. Sebat J, Lakshmi B, Troge J, Alexander J, et al. Large-scale copy number polymorphism in the human genome. *Science* 2004;305:525–528.
13. Sharp AJ, Locke DP, McGrath SD, Cheng Z, et al. Segmental duplications and copy-number variation in the human genome. *Am J Hum Genet* 2005;77:78–88.
14. Redon R, Ishikawa S, Fitch KR, Feuk L, et al. Global variation in copy number in the human genome. *Nature* 2006;444:444–454.
15. Ballif BC, Kashork CD, Shaffer LG. The promise and pitfalls of telomere region-specific probes. *Am J Hum Genet* 2000;67:1356–1359.
16. Eash D, Waggoner D, Chung J, Stevenson D, et al. Calibration of 6q subtelomere deletions to define genotype/phenotype correlations. *Clin Genet* 2005;67:396–403.