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Comparison of Fluorescein Isothiocyanate- and Texas Red-Conjugated Nucleotides for Direct Labeling in Comparative Genomic Hybridization

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In this study, we investigated whether fluorescein isothiocyanate (FITC)-labeling of test DNA and Texas-red (TR) labeling of reference DNA in comparative genomic hybridization (CGH) experiments cause the results to differ from those obtained using the opposite combination (reverse labeling). Analysis was performed on a total of 20 DNA specimens consisting of 13 frozen bone marrow aspirates from patients with acute myeloid leukemia, and fresh peripheral blood samples from seven healthy donors. For CGH, one aliquot from each test DNA sample was labeled using nick-translation with FITC-dUTP and another with TR-dUTP. Afterwards, the FITC-dUTP and TR-dUTP-labeled test DNAs were hybridized to TR-dUTP- and FITC-dUTP-labeled normal reference DNAs, respectively. The results using the two combinations were compared with each other and with the results of G-banding karyotype analysis. Karyotype data was used to detect artifacts known to occur in some chromosome regions in CGH analysis. The control DNAs labeled with FITC or TR showed no DNA copy number changes. Regardless of the fluorochrome employed for labeling, no DNA copy number changes were detected using CGH in patients with normal karyotypes, nor in patients whose karyotype aberrations were present in less than 40% of cells. In the remaining patients, CGH revealed DNA copy number changes that coincided with the results of the G-banding analysis. Hybridization artifacts known to occur in CGH experiments affecting chromosome regions 1p33-pter, 16p, 17p, 19, and 22 were observed in 15–23% of the tumor samples labeled with FITC, but not in samples labeled with TR. In addition, other previously unreported overrepresentations affecting 7q21, 9q34, 16q, 17q, and chromosome 20 were observed at very low frequencies in up to 10% of the samples when FITC was used to label test DNA. However, when TR was used, overrepresentations were observed at 4q13–q21, 11q21–q23, 13q21-qter, and Xq21–q22, whereas 19p was underrepresented. The results demonstrate that TR-labeling confirms abnormalities detected using FITC-labeling and reduces hybridization artifacts in the known problematic regions of the human genome. Cytometry 31:174–179, 1998.

Key terms: CGH methodology; fluorescein isothiocyanate; Texas-red; reverse labeling

Since the development of the comparative genomic hybridization (CGH) methodology presented by Kallioniemi and collaborators in 1992 (6), the procedure has been beset with technical limitations and difficulties. Among them, DNA copy number changes in 1p, 16p, 17p, and in the whole of chromosomes 19 and 22 are considered difficult to interpret, leading to false-positive observations (7,8,18). It has been suggested that this drawback can be overcome by using fluorochrome-conjugated nucleotides during the nick translation procedure (direct method), i.e., labeling with fluorescein isothiocyanate (FITC) or Texas-red (TR)(7,18). In addition, a very uniform hybridization is achieved with nucleotide-conjugated fluorochromes, which

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favors the interpretation and resolution of the ratio profiles. Accordingly, the direct method for visualizing hybridized DNAs in CGH protocols has been adopted in many recent studies (2–5,9,10,13,14,18,19). Interchanging the labels between the test and reference DNAs (reverse labeling) has been suggested as a method to confirm CGH results obtained using FITC (7,13,16-18).

In this study, we investigated whether FITC-labeling for test DNA and TR-labeling for reference DNA in CGH experiments cause the results to differ from those obtained using reverse labeling. A total of 20 samples (bone marrow from 13 patients with acute myeloid leukemia (AML) or peripheral blood from seven normal donors) were analyzed using both combinations and the results were compared with each other and with those obtained from conventional karyotype analysis. The karyotypic data were used to clarify the unresolved issue of hybridization artifacts encountered in specific regions of the human genome.

MATERIALS AND METHODS

DNA Specimens

The material consisted of frozen bone marrow aspirates from 13 patients with AML obtained from the Department of Medicine (Helsinki University Central Hospital, Helsinki). In addition, samples of peripheral blood from seven normal donors (controls) were included in the study. DNA was extracted using standard procedures.

Conventional Cytogenetic Analysis

Bone marrow aspirates from the AML patients and peripheral blood samples from the controls were studied using standard G-banding methods (11).

Comparative Genomic Hybridization

Comparative genomic hybridization (CGH) was performed using direct fluorochrome-conjugated DNAs for all samples according to a recently published protocol with minor modifications (7). Briefly, the tumor, control and reference DNAs were labeled by nick translation with FITC-dUTP (DuPont, Boston, MA) and TR-dUTP (DuPont) to obtain DNA fragments of 600–2,000 base pairs (bp). Each DNA sample was divided in two aliquots: one labeled with FITC-dUTP and the other with TR-dUTP. The hybridization mixture consisted of 400 ng of labeled tumor or control DNA, 400 ng of differently labeled reference DNA, and 10 µg of unlabeled human Cot-1 DNA (Gibco-BRL, Life Technologies, Gaithersburg, MD) dissolved in 10 µl of hybridization buffer (50% formamide, 10% dextran sulfate, 2× SSC). The mixture was denatured at 75°C for 5 min, then hybridized onto normal metaphases denatured in 70% formamide/2× SSC (65°C, 2 min). Hybridization was carried out at 37°C for 48 h. The slides were then washed three times in 50% formamide/2× SSC (pH 7.0), twice in 2× SSC and once in 0.1× SSC (45°C, 10 min each), followed by washes in 2× SSC, in a buffer containing 0.1 M NaH_2PO_4 and 0.1M Na_2HPO_4 (pH 8.0), and in distilled water (room temperature, 10 min each). The slides were counterstained with 4′,6-diamidino-2-phenylindole-dihydrochloride (DAPI) and mounted with Vectashield® antifading buffer (Vector Laboratories, Burlingame, CA).

Nick-translation, precipitation, hybridization, and washings were carried out simultaneously for each pair of FITC- and TR-labeled DNA samples from the same specimen, to minimize intra-experiment variation.

Digital Image Analysis

The hybridizations were analyzed using an Olympus fluorescence microscope and the ISIS digital image analysis system (MetaSystems GmbH, Altlussheim, Germany), which comprises an integrated high-sensitivity monochrome CCD camera and automated CGH analysis software. Three-color images, green (FITC), red (TR) and blue (DAPI for the chromosome counterstaining) were obtained from 12-20 metaphases in each hybridization. Chromosome regions were interpreted as overrepresented if the corresponding color ratio was higher than 1.17 (gains) or 1.5 (high-level amplifications), and underrepresented if the ratio was lower than 0.85 (losses). These cutoff values were derived from analyses of negative controls whereby two differently labeled normal DNAs were hybridized against each other.

All results were confirmed within a 99% confidence interval. Briefly, intra-experimental standard deviations for all positions of the CGH ratio profile were calculated from the variation of the ratio values of all homologous chromosomes within the experiment. Confidence intervals for the ratio profiles were then computed by combining them with an empirical inter-experiment standard deviation and estimating the error probabilities based on the t-distribution.

RESULTS

G-banding analysis revealed that all our controls had normal karyotypes and that 11 out of 13 AML patients had various karyotypic aberrations (Table 1). CGH did not reveal any DNA copy number changes in the control samples (donors 14-20) labeled with FITC or TR (Table 1) using cutoff values of 0.85 for losses and 1.17 for gains (Table 1).

Eleven tumor samples (donors 1, 2, 4-8, and 10–13) showed complex chromosomal aberrations in the banding analysis, and DNA copy number changes were detected by CGH using both labeling systems in all but one of the samples (donor 11) (Table 1). Chromosomal regions with an increase or decrease in the DNA sequence copy number are summarized in Figure 1. Changes in DNA copy number were not observed in one patient whose karyotype was normal (donor 9), nor in one patient (donor 11) who had chromosomal imbalances present in 33% of the metaphases from which the karyotype was determined. For some patients (donors 4, and 6–8), the karyotype analysis revealed marker chromosomes and monosomies of chromosomes 2, 4, 5, 6, 7, 12, 16, 17, 20, and 22. In the CGH analysis, some of these chromosomes were either

Table 1
Cytogenetic Characteristics and DNA Copy Number Changes in Tumor and Control Specimens Analyzed by Comparative Genomic Hybridization

Donor No. (sex, age)[a]	Code	Specimen type[b]	Chromosome study[c]	DNA copy number changes CGH results[d] Losses	Gains
1 (M, 23)	890581	AML	47,XY,+8,t(8;12)(p21;p11), del(12)(p11p1?3) [9]	F: 12p12 T: No	F: 8 T: 8
2 (F, 40)	890827	AML	46,XX,del(7)(q31.2) or del(7)(q22q34),inv(16)(p13q22) [11]/46,XX[1]	F: 7q31-qter T: 7q31-qter	F: 17p12-q24 T: No
3 (M, 21)	900417	AML	46,XY[20]	F: No T: No	F: No T: No
4 (M, 50)	910986	AML	43-45,XY,add(2)(p?21),i(5)(p10), -7,+8,-12,-16,-17,t(17;?) (p11.2;?),+1-3mar,inc [cp17]	F: 5q13-qter, 7, 16, 18q T: 5q14-qter,7p15-qter, 16	F: No T: No
5 (F, 39)	911322	AML	46,XX,del(5)(q13q33), del(7)(q?21.1q?34), del(12)(p11.2 or p13) [15]	F: 5q14-q3l, 7q21-q32, 12p12 T: 5q15-q31, 7cen-q31	F: 17 T: No
6 (F, 47)	920391	AML	45,XX,del(3)(q21q26),-6,7, -22,+mar1,+mar2[12]	F: 6q16-q21, 7p, 7q22-qter T: 6q16-q21, 7p, 7q22-qter	F: No T: No
7 (M, 66)	940819	AML	45,XY,-5,-17,-20,+2 mar,inc[3]/ 42,XY,idem,-4,-5,-7,add(11) (p11),-16,+3-4 mar, inc[7]/88,idemx2,inc[3]	F: 4p, 4q23-qter,5cen-q13, 5q21-q33, 7p12-q11.2, 7q31-qter, 12q22-qter, 16, 17 T: 4p, 4q31-qter,5q22-q31, 7p13-q11.2, 7q32-qter, 12q22-qter, 16, 17, 19p	F: 7q21, 8 T: 4q13-q21, 8, 11q21-q23, 13q21-qter
8 (M, 47)	950392	AML	46,XY,-2,der(3)?x2,der(5)t(1;5) (p11;q35)del(5)(q13q35),der(7) t(3;7)(q25;q22),i(8)(q10),+1-2 mar,inc[10]	F: 3p21-pter, 3cen-q21, 5q14-q31, 7q32-qter, 8cen-p12 T: 3p21-pter, 5q21-q31, 7q32-qter, 8p	F: 1p22-pter, 2p13-p23, 8q T: 1p31-p32, 2p15-p23, 8q, Xq21-q22
9 (M, 50)	950979	AML	46,XY[20]	F: No T: No	F: 1p33-pter, 20,22 T: No
10 (M, 49)	870087	AML	46,XY,del(9)(q3?4)[4]/46, XY[13]	F: No T: No	F: 16p12-qter, 19 T: No
11 (M, 34)	910327	AML	45, XY,del(9)(p22),-20[6]/ 46,XY[12]	F: No T: No	F: No T: No
12 (M, 51)	930132	AML	46,XY,t(9;12)(q34;q11),t(12;14) (q?14;q32),inc[14]	F: No T: No	F: 1p33-pter, 17q12-qter 22 T: 17q24-qter
13 (M, 64)	950689	AML	46,XY,del(7)(q22q36)[6]/ 46,XY[10]	F: No T: No	F: 1p33-pter, 17q12-qter, 22 T: No
14-20 (3M, 4F), (25-43)		ND	46,XY or 46,XX	F: No T: No	F: No T: No

[a]F, female; M, male; age in years at diagnosis.
[b]AML, acute myeloid leukemia; ND, normal donor.
[c]After G-banding analysis.
[d]F, FITC-labeled DNA; T, Texas red-labeled test DNA.

normal or had only partial deletions, suggesting a rearrangement of the remaining DNA material in the marker chromosomes. In one patient (donor 10), CGH did not reveal the del(9)(q34) found in 31% of cells studied for karyotype analysis. However, CGH revealed some DNA copy number changes that were not observed in G-banding analysis (donors 2–5, 7–10, 12, and 13) (Table 1, Figure 1). Moreover, some DNA copy number losses, which did not match any chromosomal aberrations seen in the G-banding analysis, were observed in donors 4 and 7 (Table 1, Fig. 1).

Figure 1 illustrates that, of the aberrations detected by both fluorochromes, 42% of the gains or losses observed in FITC-labeled DNA extended into a wider chromosomal area than that revealed by TR-labeling. Overrepresentation in 1p33-pter, 16p, 17p, 19, and 22 were observed in 15–23% of the tumor samples labeled with FITC, but not in those labeled with TR.

Furthermore, it is evident from Figure 1 that, in addition to those chromosomal regions reported to have false-positive results, chromosomal imbalances not supported

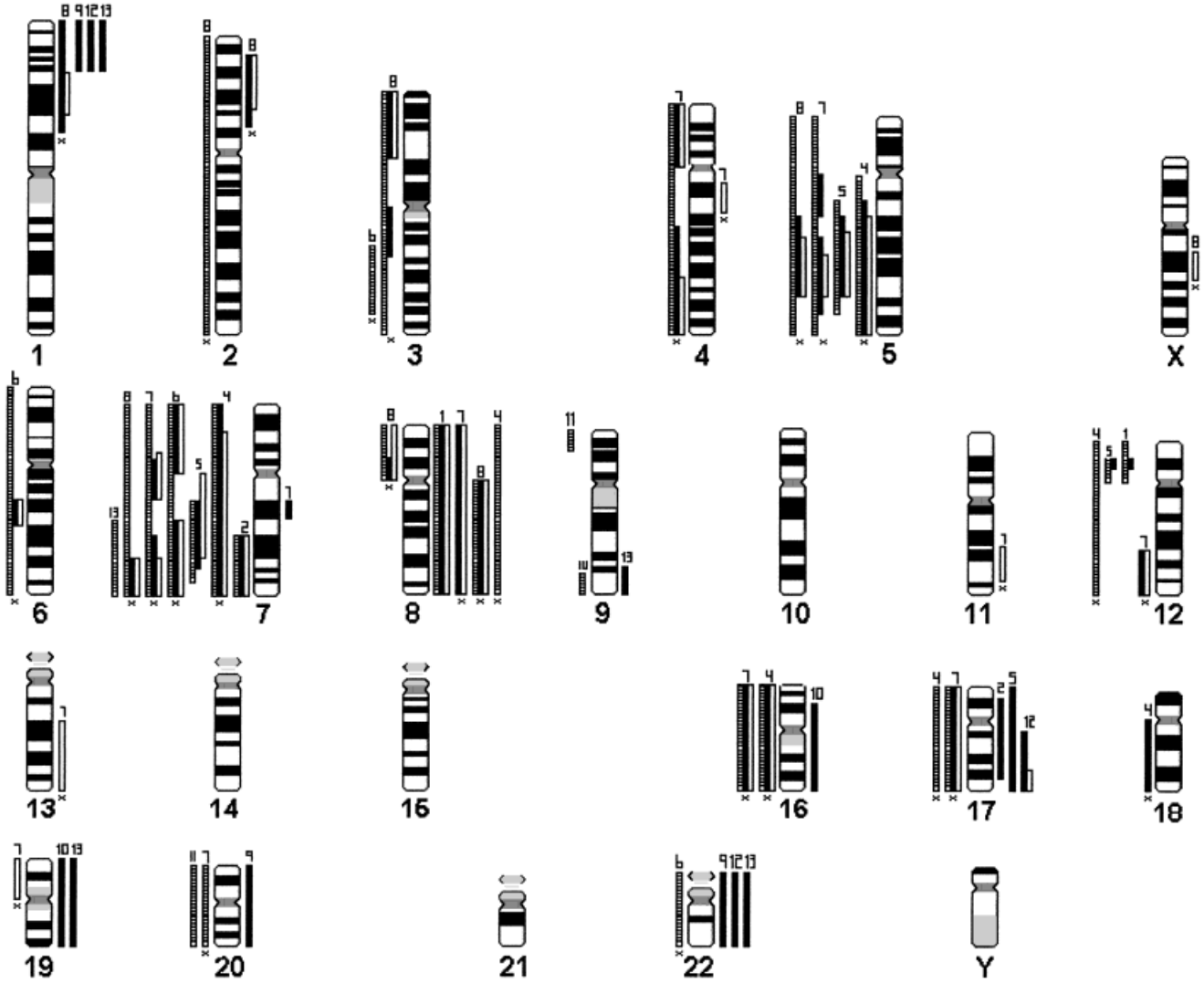

FIG. 1. Summary of gains and losses in DNA sequence copy number in 13 acute myeloid leukemia samples analyzed by comparative genomic hybridization. Losses are shown on the left side of each chromosome and gains on the right. *Single and double bars*, genetic aberration seen in one sample; *solid bars*, DNA copy number changes observed in FITC-labeled DNA; *empty bars*, DNA copy number changes observed using Texas red; *hatched bars*, karyotyping results. Figures above the bar(s) denote donor number. All cases with marker chromosomes are indicated by X under the corresponding bar.

by karyotyping also occur at very low frequencies in regions not previously reported. When test-DNA was labeled with FITC, overrepresentation of DNA copy number was seen in 7q21 (5%), 9q34 (5%), 16q (5%), and 17q (10%) and in chromosome 20 (5%), whereas underrepresentation was only observed in chromosome 18 (5%). When TR was used for labeling of test DNA, overrepresentation was observed at 4q13–q21 (5%), 11q21–q23 (5%), 13q 21-qter (5%), and Xq21–q22 (5%), but 19p (5%) was found to be underrepresented (Fig. 1).

DISCUSSION

Our results showed that CGH did not reveal any DNA copy number changes in control samples using either FITC- or TR-labeling. However, artifacts known to occur in chromosomes 1, 16, 17, 19, and 22 were observed in 15–23% of the tumor DNAs when labeled with FITC, but not when labeled with TR. In addition, overrepresentation at 7q21, 9q34, 16q, 17q and in chromosome 20, as well as losses of DNA sequence copy number in chromosome 20 were observed in FITC-labeled test DNA, whereas TR-labeled tumor DNAs showed overrepresentation at 4q13–21, 11q21–q23, 13q21-qter, and Xq21–q22, and underrepresentation at 19p. Since these regions of gains and losses were not supported by karyotyping, they are most probably artifacts caused by the use of the fluorochromes. In agreement with our observations are previous findings reporting discrepancies in the results depending on the fluorochromes used to label DNA (1,21). It has to be noted that the tumor DNA was obtained from frozen material,

whereas the control DNA was from fresh blood samples. Differences in the DNA preparation and/or storage therefore existed and could have been one factor responsible for the hybridization artifacts seen only in the tumor samples. It would be ideal to extract the reference DNA and tumor DNA from tissue of the same origin and under the same laboratory conditions. However, this may be difficult to accomplish during routine laboratory work.

Although the precise mechanism(s) underlying labeling and hybridization in these problematic areas of the genome are unknown, it has been suggested that the reverse labeling system using TR could be one way to confirm the results for those areas known to be affected by hybridization artifacts (7,13,17,18). We found that reverse labeling of the tumor DNA with TR essentially confirmed the CGH results obtained using FITC-labeling, and suppressed simultaneously the hybridization artifacts affecting chromosomes 1, 16, 17, 19, and 22. Nevertheless, the analysis showed other artifacts inherent to TR-labeling, such as gains in up to 5% of the samples at 4q, 11q, 13q, and Xq, or losses of DNA sequence copy number at 19p. So far, we have no explanation for this observation. However, it might reflect an underlying difference in the way FITC and TR molecules bind to DNA molecules, or it could be related to a differential affinity of the fluorochromes to certain types of DNA families not homogeneously distributed in the genome, such as the CG rich areas or small interspersed repeated sequence elements. It is known that the areas reported to yield false positive results in CGH studies correspond accurately to some of the most CG-rich areas in the genome (20) and that some DNA molecules are known to be highly enriched in interspersed repeated sequences such as the SINEs, Alu family that is dominant in reverse positive bands, and the LINEs, L1 family elements that are dominant in Giemsa- or Quinacrine-positive bands (12,15). Alu comprises 56% G-C and L1 is 58% A-T; each may comprise 13–18% of the total DNA in a chromosome band (12). Therefore, with the exception of some telomeric and other chromosomal regions of simple sequence DNA, the distribution of SINEs and LINEs is precisely inverse, suggesting an inverse functional relationship.

Comparison of the CGH data with the data obtained by banding analysis showed that regardless of the fluorochrome employed for labeling, patients with normal karyotypes (donors 3 and 9) did not have DNA copy number changes, nor did the patients whose karyotype aberrations were present in less than 40% of their bone marrow cells (donors 10, 11, and 13) nor the patient whose sole aberrations were balanced translocations (donor 12). In the remaining patients, the DNA copy number changes revealed in CGH analysis, using both FITC- and TR-conjugated nucleotides for labeling the tumor DNA, coincided with the results of the G-banding analysis. However, 42% of the gains or losses observed in FITC-labeled DNA extended into a wider chromosomal area than that revealed by TR-labeling. Moreover, CGH provided important information about net gains and losses of DNA sequences in AML patients with complex karyotypes (donors 4, 6, 7, and 8). Instead of complete monosomy, CGH showed that the deletions are small and interstitial in most cases, suggesting that the marker chromosomes in these AML cases contained DNA material from the deleted chromosomes.

In conclusion, TR-labeling of tumor DNA can be used to confirm results obtained by FITC-labeling, especially in samples that have DNA copy number changes in the problematic regions of the genome. Further studies are needed to explain the differences observed in the behavior of these fluorochromes.

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