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BRIEF METHODS

Optimization of Comparative Genomic Hybridization Using Fluorochrome Conjugated to dCTP and dUTP Nucleotides

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Using a mixture of fluorochrome-dCTP and -dUTP during nick translation for DNA labeling, we performed comparative genomic hybridization (CGH) analyses on 46 samples from various tissues to assess the possibility of reducing comparative genomic hybridization artifacts in the problematic areas of the genome (1p33-pter, 16p, 17p, 19, and 22). One part of each DNA sample was labeled only with fluorochrome-dUTP for comparison with the standard procedure. Both labeling systems gave essentially the same information about DNA copy number changes, although the use of the fluorochrome-dCTP and -dUTP mixture for labeling significantly reduced the number of changes in the problematic areas. The frequency of changes in 1p33-pter was reduced from 20% to 4%, in 16p from 11% to 2%, in 17p from 17% to 3%, in chromosome 19 from 26% to 9%, and in chromosome 22 from 20% to 7%. The karyotype information available for six of these cases did not support the changes detected by the fluorochrome-dUTP system alone. However, in two cases, the karyotype information confirmed the changes that were detected by both the modified method and the standard procedure. Moreover, the hybridizations were smoother and aberrations were more clearly seen using the new procedure.

Comparative genomic hybridization is a powerful tool used in the study of DNA copy number changes in soft tissue and solid tumors (El-Rifai et al, 1996; Kallioniemi et al, 1994a; Ried et al, 1996). Kallioniemi et al (1994b) suggested that labeling DNA directly with fluorochrome-conjugated nucleotides improves the results obtained in CGH experiments; since then, this method has been used in several studies. Changes in DNA copy numbers due to hybridization artifacts have been observed in 1p33-pter, 16p, 17p, 19, and 22, which are therefore considered problematic areas for CGH (Kallioniemi et al, 1994b). Thus far, all reported CGH studies using the direct method have used fluorochromes conjugated to dUTP, which label the A bases in the genome but leave the GC-rich areas unlabeled.

In the present study we report, for the first time, DNA labeling using fluorochromes conjugated to a mixture of dCTP and dUTP for standard nick translation. The method was tested on 46 samples: 9 frozen bone marrow samples, 18 frozen tumors, 12 paraffin-embedded tumors, and 7 peripheral blood samples from healthy donors. Standard karyotype analysis was performed on 16 samples. CGH was performed using fluorochrome-conjugated DNA for direct labeling as described previously (Kallioniemi et al, 1994b) with the following modifications. A mixture of fluorescein isothiocyanate (FITC) -dCTP and FITC-dUTP (1:1; DuPont, Boston, Massachusetts) was prepared for labeling the tumor DNA. A similar mixture was prepared using Texas red for labeling the reference DNA. We prepared a buffer that contained unlabeled deoxynucleotide triphosphates (dNTPs) to be included in the nick translation procedure. The mixture of unlabeled dNTPs consisted of 5 ul dATP (10 mM; GIBCO-BRL, Gaithersburg, Maryland), 5 ul dGTP (10 mM, GIBCO-BRL), 1.5 ul dTTP (10 mM, GIBCO-BRL), 1.5 ul dCTP (10 mM, GIBCO-BRL), 1.7 ul mercaptoethanol (14.7 M), 2.5 ul BSA (10 mg/ml, GIBCO-BRL), 125 ul Tris HCl (1 M), 12.5 ul MgCl2 (1 M), and double-distilled water to adjust the volume to 250 ul. To label 1 ug of DNA, we used 5 ul of the buffer, 1.5 ul of the fluorochrome-conjugated nucleotide mixture, 1.1 ul DNA polymerase I (Promega, Madison, Wisconsin), and 7 to 12 ul DNA polymerase I/DNase I (GIBCO-BRL), all of which were mixed with water to obtain a total volume of 50 ul; the mixture was then incubated at 15°C for 40 to 75 minutes to obtain DNA fragments ranging in size from 600 to 2000 base pairs. To compare the results of our modified method with those yielded by the standard direct procedure, which uses fluorochrome-dUTP, each sample labeled using our modified method was also labeled with FITC-dUTP and Texas red-dUTP for the test sample and reference DNA, respectively. To minimize interexperimental variations, all nick translations, hybridizations, and washes were carried out simultaneously for the samples labeled using the modified method and the standard procedure. Hybridizations, washings, and ISIS digital image analysis (Metasystems GmbH, Altlussheim, Germany) were performed as described previously (El-Rifai et al, 1996). Based on our earlier reports, we used 1.17 and 0.85 as cut-off levels for gains and losses, respectively.

No DNA copy number changes were seen in the seven healthy-donor samples using the fluorochrome-dCTP and -dUTP mixture. However, when using fluorochrome-dUTP alone, one showed a gain at 1p33-pter and of chromosome 22 as a hybridization artefact. In the genome areas that are known to display hybridization artefacts, the frequency of aberrations remained unchanged regardless of the tissue

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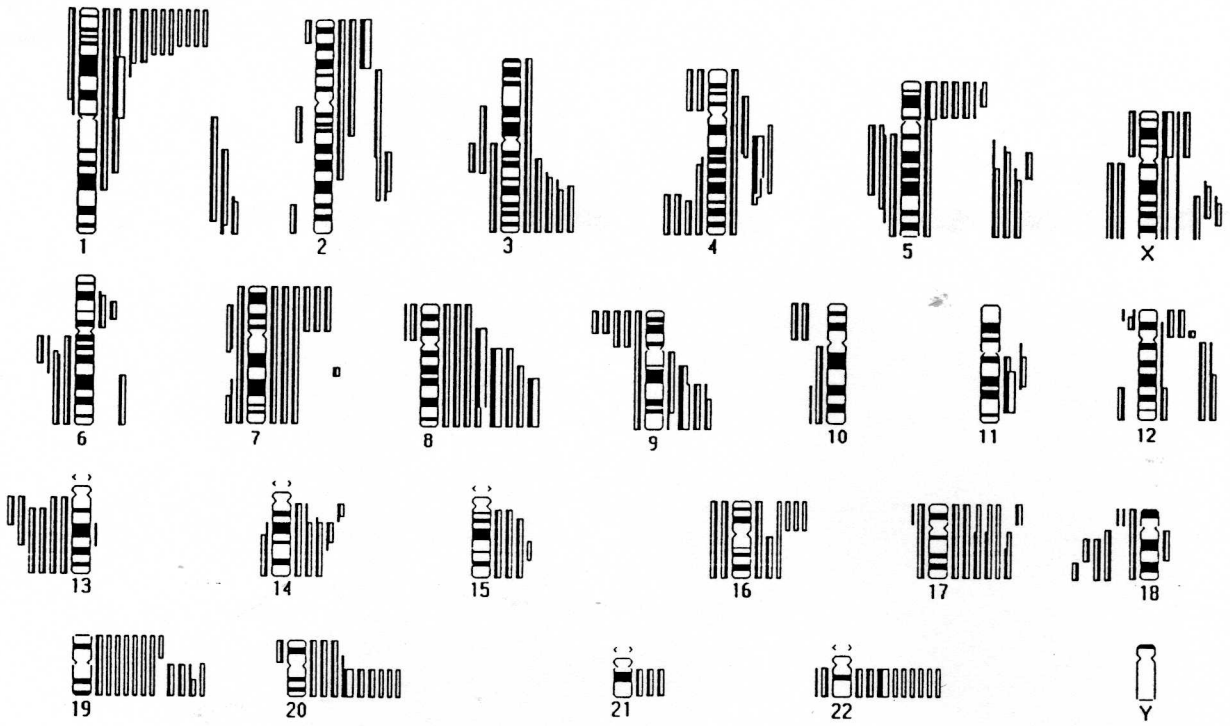

Figure 1.

Summary of DNA copy number gains and losses detected by comparative genomic hybridization (CGH). Each *bar* represents one patient. The *solid bar* shows the results of the CGH analysis using a mixture of fluorochrome-dCTP and -dUTP. The *empty bar* shows the results of the CGH analysis using only fluorochrome-dUTP for the same case. Gains are on the *right*, and losses are on the *left*. High-level amplifications are represented by *wide bars*.

origin. However, the modified method showed fewer changes in the problematic areas than did the standard procedure. In 1p33-pter, the frequency was reduced from 20% to 4%, in 16p from 11% to 2%, in 17p from 13% to 7%, in chromosome 19 from 26% to 9%, and in chromosome 22 from 20% to 7%. The karyotype information available for six of these cases did not support the extra changes found using the standard system. However, in two cases, the karyotype information available showed gains in 16p and 17p, which were detected by both the standard and the modified method. We can therefore conclude that the fluorochrome-dCTP and -dUTP mixture significantly decreases the occurrence of hybridization artefacts in these problematic areas.

From a total of 150 aberrations detected in other chromosomal regions (excluding the above-mentioned problematic areas), 140 aberrations (93%) were revealed by both systems. Of the 10 aberrations that were seen only when using one system, 8 were shown by the modified method, but no karyotype was available for confirmation in these cases. In 10 samples, the aberrations were still visible at the cut-off values of 1.22 and 0.80 (for gains and losses, respectively) using the modified method but not the standard procedure, thus reflecting the improved sensitivity of the new method. These results are summarized in Figure 1.

The areas that have been reported to display hybridization artefacts when using the standard fluorochrome-dUTP system are also known to be GC-rich regions and to contain the highest concentration of genes in the genome (Saccone et al, 1992). Our modified method ensures labeling of GC as well as AT sequences in the genome and, compared to dUTP labeling, yields a more uniform hybridization with high color intensities, which is especially important for analyzing samples from paraffin-embedded tissues. Therefore, our new method for labeling DNA in CGH analyses not only produces fewer hybridization artefacts, but also yields higher quality results and more representative labeling of the genome.

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