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Technical Advance

Analysis of Changes in DNA Sequence Copy Number by Comparative Genomic Hybridization in Archival Paraffin-Embedded Tumor Samples

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Analysis of previously unknown genetic aberrations in solid tumors has become possible through the use of comparative genomic hybridization (CGH), which is based on competitive binding of tumor and control DNA to normal metaphase chromosomes. CGH allows detection of DNA sequence copy number changes (deletions, gains, and amplifications) on a genome-wide scale in a single hybridization. We describe here an improved CGH technique, which enables reliable detection of copy number changes in archival formalin-fixed paraffin-embedded tumor samples. The technique includes a modified DNA extraction protocol, which produces high molecular weight DNA which is necessary for high quality CGH. The DNA extraction includes a 3-day digestion with proteinase K, which remarkably improves the yield of high molecular weight DNA. Labeling of the test DNA with a directly fluorescein-conjugated nucleotide (instead of biotin labeling) improved significantly the quality of hybridization. Using the paraffin-block technique, we could analyze 70 to 90% of paraffin blocks, including very old samples as well as samples taken at autopsy. CGH from paraffin blocks was highly concordant (95%) with analyses done from matched freshly frozen tumor samples (n = 5 sample pairs; κ coefficient = 0.83). The method described here has wide applicability in tumor pathology, allowing large retrospective prognostic studies of genetic aberrations as well as studies on genetic pathogenesis of solid tumors, inasmuch as premalignant lesions and primary and metastatic tumors can be analyzed by using archival paraffin-embedded samples. ***(Am J Pathol 1994, 145:1301–1308)***

Our newly developed molecular cytogenetic technique, comparative genomic hybridization (CGH), provides a unique approach to detect and map clonal DNA sequence copy number changes across the genome in a single hybridization.[1,2] CGH is based on a competitive *in situ* hybridization of differentially fluorescently labeled whole genomic DNA probes, one from the tumor and another from a normal reference, which are hybridized to normal metaphase chromosome preparations. Regions of altered DNA sequence copy number (deletions, gains, and amplifications) in the tumor are highlighted and quantitated as color ratio changes along each metaphase chromosome. CGH has been used in the analysis of DNA sequence copy number changes in a number of different solid tumors, such as breast, lung, and ovarian tumors,[3–5] that typically are very difficult to analyze by using conventional cytogenetic methods.

The clinical value of CGH is currently not well established. Our pilot prognostic study of archival frozen samples from 48 node-negative breast cancer

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patients indicated that the total number of deletions per tumor significantly predicted clinical outcome, suggesting that CGH might possess important prognostic potential by determining how genetically advanced the tumors are (Isola et al, submitted for publication). Evaluation of the prognostic and diagnostic significance of genetic aberrations would be facilitated by analyzing archival paraffin-embedded tumor samples from patients whose disease outcome is already known.

We describe here a modification of CGH that allows analysis of genetic aberrations from archival formalin-fixed, paraffin-embedded tissue samples. The use of paraffin blocks as starting material required a modified DNA extraction method and optimization of DNA labeling. The method is validated by comparing the results from fresh tissues with those from the same samples after formalin fixation and embedding in paraffin. The method has been reproducible in our hands and has proved effective for analysis of samples from many different pathology laboratories, often after extensive (>48 hours) formalin fixation.

Materials and Methods

Tumor Samples

Selected archival paraffin block samples from breast cancers were obtained from four different pathology laboratories. Matched pairs of freshly frozen and archival paraffin-embedded tumor specimens were obtained from five breast cancer patients from the University of California, San Francisco. Paraffin-embedded tumors had been routinely fixed in 4% neutral buffered formalin, usually for more than 12 hours. A colon carcinoma sample was fixed for 4, 24, and 72 hours in buffered 4% formalin to test the effect of fixation. Histologically normal kidney was used as a paraffin-embedded normal tissue control.

Extraction of DNA from Paraffin Blocks

Twenty to thirty 5-μ sections were deparaffinized in eppendorf tubes (2 × 1 ml xylene for 10 minutes each and 2 × 1 ml 100% ethanol for 10 minutes each). After air drying at room temperature, samples were suspended in 1 ml DNA extraction buffer (0.3 mg/ml proteinase K (Sigma, St. Louis, MO), 100 mmol/L NaCl, 10 mmol/L Tris-HCl pH 8, 25 mmol/L EDTA pH 8, and 0.5% sodium dodecyl sulfate) and were incubated with shaking at 55 C overnight. Additional proteinase-K (10 μl from 20 mg/ml stock solution) was added 24 hours and 48 hours later for a total incubation time of 72 hours. DNA was extracted by using a phenol-chloroform-isoamyl alcohol method. A 500-μl sample mixed with 500 μl phenol chloroform isoamyl (Amresco, Solon, OH) was incubated at room temperature for 10 minutes and centrifuged. DNA in the top layer was collected and precipitated with 250 μl of 7.5 mol/L ammonium acetate and 1 ml of ice-cold 100% ethanol. DNA was pelleted by centrifugation (14,000 rpm for 20 minutes). Glycogen (0.1 mg/ml; Sigma) was added before centrifugation as a carrier to increase the volume of the pellet. DNA was dissolved overnight in 20 to 40 μl of TE buffer (10 mmol/L Tris, 1 mmol/L EDTA) and DNA size was estimated after denaturation (5 minutes at 70 C) by agarose gel electrophoresis with ethidium bromide staining. DNA concentration was determined with a fluorometer (Hoefer Scientific, San Francisco, CA). For comparative experiments, DNA was extracted from freshly frozen tumor tissues as described previously.[3]

Labeling of DNA with Nick Translation

One microgram of tumor DNA (per 50 μl of reaction mixture, adjusted by distilled water) was mixed with 5 μl of 10 × A4 dNTP mixture (200 μmol/L dATP, dCTP, and dGTP; 0.5 mmol/L Tris (pH 7.2); 0.2 mmol/L $MgCl_2$; 100 mmol/L b-mercaptoethanol; 100 μg/ml bovine serum albumin), 1 μl (1 nmol) fluorescein-12dUTP (DuPont, Boston, MA), 5 μl of DNA polymerase I/DNAse I (0.4 U/μl and 40 pg/μl, GIBCO BRL, Gaithersburg, MD) and 1 μl (10 U) DNA polymerase 1 (GIBCO)). The reaction time (45 to 60 minutes) and the amount of DNA polymerase I/DNAse I were adjusted to obtain a probe fragment size ranging from 300 to 3000 bp (detected by nondenaturing agarose gels stained with ethidium bromide). Reaction was stopped by a 10-minute incubation at 70 C. Normal reference DNA was extracted from mononuclear cells obtained from peripheral blood of healthy female volunteers,[3] and labeled as described above with Texas Red-5-dUTP (DuPont).

Hybridization

Genomic DNA probes were hybridized onto normal lymphocyte metaphase preparations essentially as described elsewhere for frozen tissue DNA.[3,6] In brief, 100 ng of fluorescein-labeled tumor DNA, 100 ng of Texas Red-labeled reference DNA, and 10 μg of unlabeled human Cot-1 DNA (GIBCO) were precipitated with 0.3 mol/L sodium acetate and two final volumes of 100% ethanol. The DNAs were dissolved in 10 μl of hybridization mixture (70% formamide, 10%

dextran sulfate and 2X SSC, pH 7.0) and denatured (5 minutes at 70 C) immediately before applying onto slides. Slides were denatured in 70% formamide and 2X SSC (pH 7.0) at 74 C for 3 minutes, dehydrated in graded ethanols, digested with proteinase K (0.1 µg/ml in 20 mmol/L Tris and 2 mmol/L $CaCl_2$, pH 7.5) at room temperature for 7.5 minutes, and dehydrated again. Hybridization was done under sealed coverslips for 2 to 3 days at 37 C in a moist chamber. After hybridization, the slides were washed three times in a washing solution (50% formamide in 2X SSC, pH 7.0) at 45 C, twice in 2X SSC (pH 7.0) at 45 C, once in PN buffer (0.1 mol/L NaH_2PO_4, 0.1 mol/L Na_2HPO_4, and 0.1% Nonidet P40, pH 8.0), and once in distilled water (both 10 minutes at room temperature). Samples were counterstained with 4,6-diamino-2-phenylindole in an anti-fade solution.

Controls

Validation of CGH by comparisons with karyotyping and LOH analyses has been described elsewhere.[6] Each CGH experiment included a fluorescein isothiocyanate (FITC)-labeled female mononuclear cell DNA as a negative (normal cell) control sample, and a FITC-labeled MCF-7 breast cancer cell line DNA as a positive control. Selected tumor samples were also labeled with Texas Red and hybridized with FITC-labeled normal reference DNA to control for differences in hybridization of FITC versus Texas Red-labeled probes ("inverse" labeling CGH). Comparison between these two hybridizations confirmed the presence of copy number aberrations at almost all loci.

Digital Image Analysis

CGH hybridizations were analyzed using a Zeiss Axioplan microscope equipped with a 63x Neo-Fluar objective (Zeiss, Germany). Images were captured to the digital image analysis system with a black and white cooled CCD camera (Microimager 1400, Xillix Technologies, Vancouver, Canada). The digital image analysis system developed specifically for CGH has been described previously.[7] In brief, green and red fluorescence images were captured and analyzed as separate images. The fluorescence intensities were determined from p-telomere to q-telomere by integrating intensities (gray values in images) at one-pixel intervals along the chromosome medial axis. After background correction, normalization of the green-to-red ratio for the entire metaphase to 1.0, green-to-red fluorescence intensity ratio profiles were calculated for each chromosome. A whole chromosome arm loss or subregional deletion was defined as having a green-to-red ratio less than 0.85. Copy number increase was defined as having green-to-red ratios greater than 1.2.

Results

In initial experiments using the same DNA extraction protocol as was used for frozen tissues, only a very low yield (less than 1 to 2 µg, usually undetectable by agarose gels) of low molecular weight (<2000 bp) DNA was obtained. This material could not be used successfully for CGH. High quality CGH was obtained from ethanol-fixed paraffin blocks (data not shown) suggesting specifically that formalin fixation led to poor yield of DNA. A test tumor with samples fixed for variable times showed specifically that degradation of extracted DNA was inversely proportional to the length of formalin fixation (Figure 1). A short fixation (4 hours) yields relatively unfragmented DNA (a sharp lane at about 20 kb, which is identical to what is obtained from fresh tissues) whereas a degradation is clearly evident with increasing fixation time. The yield of DNA was also similarly proportional to formalin fixation. The sample fixed for 72 hours had only 15% of the amount of DNA obtained from the sample fixed for 4 hours (corrected by cell counts in adjacent H&E-stained sections).

To improve the DNA extraction, we tried several published protocols. Isolation of entire nuclei from 50 µ sections, as is done for DNA flow cytometry,[8] pretreatment with 1 mol/L sodium isothiocyanate,[9] or

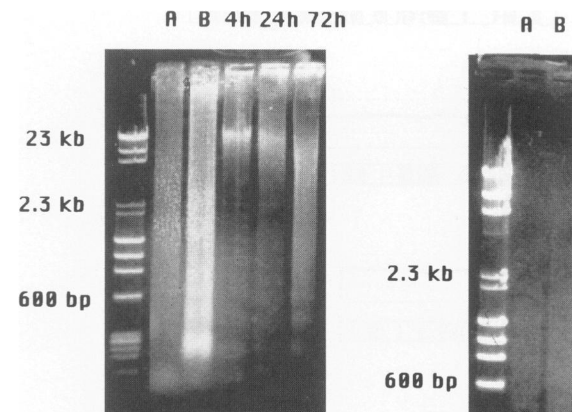

Figure 1. *An ethidium bromide-stained 1% agarose gel showing the size distribution of genomic DNAs extracted from the paraffin blocks. In the* **left panel, lanes A** *and* **B** *represent archival breast cancer samples showing a wide DNA size distribution from over 23 kb to less than 600 bp. The following three lanes illustrate the inverse dependence of DNA size and the length of formalin fixation (fixation for 4, 24, and 72 hours, all from the same colon carcinoma sample). The* **right panel** *shows the size distribution of nick-translated DNA probes of samples A and B. The majority of the labeled DNA has an optimal size for CGH, between 600 bp 2.3 kb.*

increase of the concentration of proteinase K up to 3 mg/ml[10] proved only partially effective and did not sufficiently improve the yield of DNA. The use of multiple (20 to 30) thin (5-μ) paraffin sections instead of one to three 50-μ sections (routine for DNA flow cytometry) and a prolonged (3 days) digestion of proteins with proteinase K dramatically increased the yield of high molecular weight DNA. Estimated by the cell counts on adjacent H&E-stained sections (assuming 9.6 pg of DNA per cell), the efficiency of the modified DNA extraction protocol was 10 to 30%. The DNA extraction protocol has been successful in 80 to 90% of paraffin blocks attempted. Two examples of DNA from archival paraffin blocks demonstrate that partially degraded DNA is typically obtained (DNA fragment size varies from 200 bp to over 20 kbp; Figure 1).

A prerequisite for high quality CGH is that the majority of the nick-translated DNA be between 0.5 and 3 kb. In our experience, this can only be achieved when the starting material (extracted genomic DNA) has a significant proportion of high molecular weight DNA fragments (4 to 20 kb; Figure 1). Using this material, we obtained optimally sized CGH probes after labeling with nick translation, during which DNA is typically further fragmented (Figure 1B).

Another important step for optimization of our CGH protocol was changing the DNA labels. The use of directly fluorescein- and Texas Red-labeled probes (instead of previously used biotin and digoxigenin labeling and detection with avidin-FITC and anti-digoxigenin rhodamin) improved significantly the quality of hybridization. Variation between different observations (as evaluated by standard deviation of four observations of each chromosome) was reduced approximately by half. More importantly, loci often showing green-to-red fluorescence ratio reductions below 1.0 with indirect labeling (at chromosomes 1p32-ter, 16p, 19, and 22; see Reference 6 for detail) did not show these false positive deletions with direct labeling (Figure 2). Normal-to-normal DNA hybridizations also showed the equal quality of hybridizations done from frozen and paraffin-embedded samples (Figure 2). As a measure of the quality of hybridization, a low degree of variation between each observation was obtained (coefficients of variation of

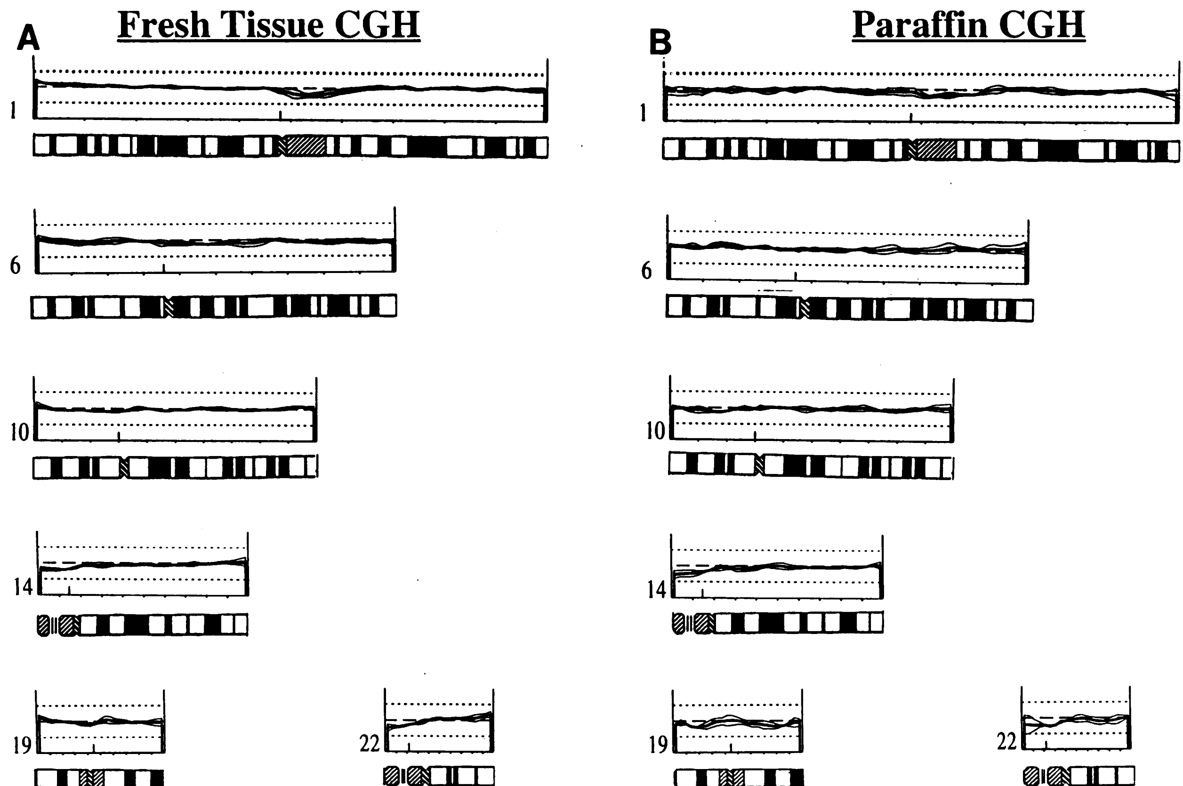

Figure 2. *Green-to-red ratio profiles from a control experiment comparing fluorescein-labeled fresh* (A) *or paraffin-embedded* (B) *DNA from histologically normal tissues against Texas Red-labeled normal reference DNA. The mean of four experiments (thick line) and one standard deviation (thin lines below and above) of the green-to-red fluorescence ratio profiles are shown for selected chromosomes (indicated on the p arm). No ratio changes are seen along any of the chromosomes indicating an even hybridization of the two differentially labeled DNAs (heterochromatic areas and centromeres are ignored from analysis). Dashed horizontal lines correspond to a green-to-red ratio of 1.0, and dotted lines correspond to ratios of 0.5 and 1.5.*

green-to-red ratios were 3.2% and 5.7% for fresh and paraffin embedded samples, respectively).

After successful DNA extraction and labeling with 12dATP-fluorescein, CGH was carried out without any other modifications to the fresh tissue CGH protocol. The principle of detecting green and red color ratios and examples of copy number karyotype profiles are shown in Figure 3, which describes the DNA copy number aberrations in a 30-year-old paraffin block sample. In this example, genetic aberrations can be seen in the pseudocolored digitized image (Figure 3A) and verified in corresponding green-to-red ratio profiles obtained by using an image analysis program (Figure 3B).

Another example highlighting the usefulness of the technique is illustrated in Figure 4. The use of paraffin block samples made possible the comparison of genetic aberrations of a primary breast cancer and its later appearing distant metastasis (the latter sample taken at autopsy). The clonal nature of genetic aberrations was clearly seen in this pair of samples. Interestingly, some of the genetic changes seen in the primary tumor were not present in the metastasis.

We validated paraffin block CGH by using five breast cancers from which frozen and paraffin-embedded DNA samples were analyzed separately. A high concordance (95%) was found between the two assays to detect the copy number aberrations (kappa coefficient, 0.83; 95% confidence interval, 0.69 to 0.98). Some of the discordances were obviously due to normal cell contamination of the frozen samples (frozen samples were not histologically controlled before DNA extraction), inasmuch as similar patterns of copy number profile were sometimes seen, but their amplitude was too low to fulfill the criteria used for interpreting CGH results. Finally, aberrations found in paraffin samples were verified in two cases by using inverse labeling CGH as a control, in which possible differences in hybridization of fluorescein-*versus* Texas Red-labeled probes were controlled (data not shown).

Discussion

Comparative genomic hybridization is a new technique that allows the detection and mapping of rela-

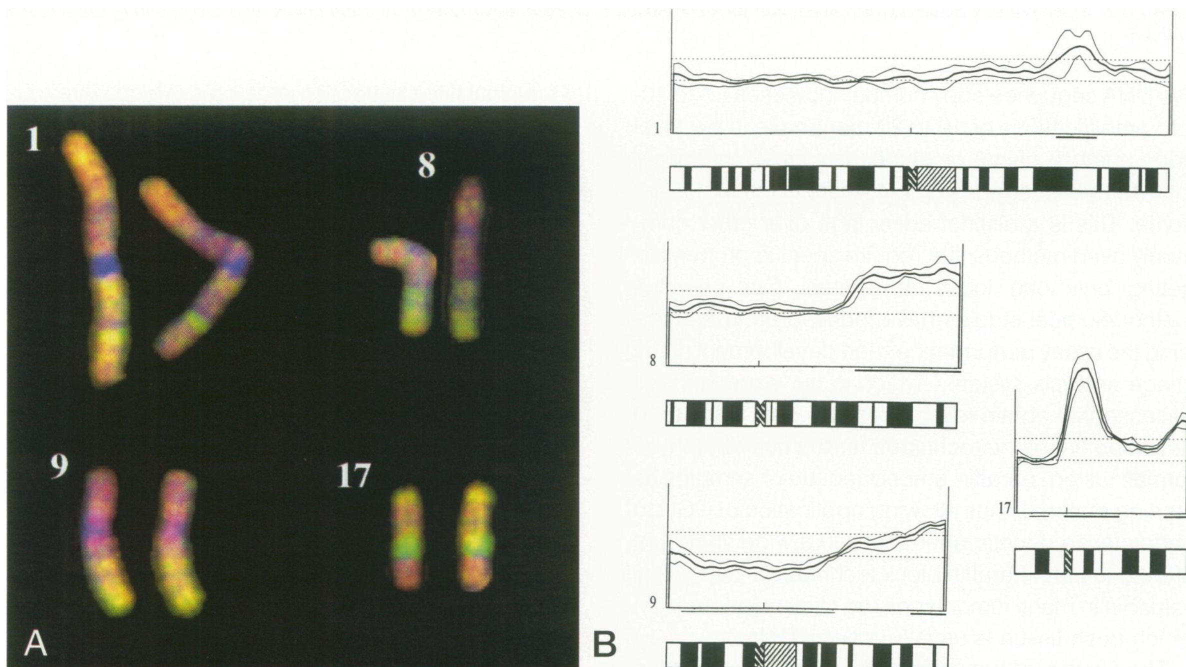

Figure 3. *An example of a digital image of a CGH experiment showing genetic aberrations found in a 30-year-old paraffin block of a breast carcinoma* (**A**). *DNA extracted from the paraffin block (labeled in green) and normal reference DNA (labeled in red) were hybridized to a normal metaphase chromosome (counterstained blue with DAPI). Chromosomal regions that were over-represented in the tumor are visualized as a predominantly green color, whereas regions having deletions in the tumor are seen as predominantly red color (ie, relative lack of green). For this figure, three different black and white exposures of the same metaphase were made (matching 4,6-diamino-2-phenylindole, fluorescein, and Texas Red stains). The images were overlaid in pseudo-colors, color-balanced, and contrast-stretched for display of the color differences.*[8] **B** *shows the corresponding green-to-red ratio profiles obtained from the image analysis program,*[8] *which verify the genetic aberrations of this sample: a subregional amplification at chromosome 1q31–32, gain of 8q22-ter, deletion in 9p and a terminal gain in 9q, deletion in 17p, high level amplification of 17q12 (locus for c-erbB2 oncogene), and a distal amplification at 17q (aberrations marked by bars under x axes). Dotted lines show the normal range (green-to-red ratio 0.85 to 1.2).*

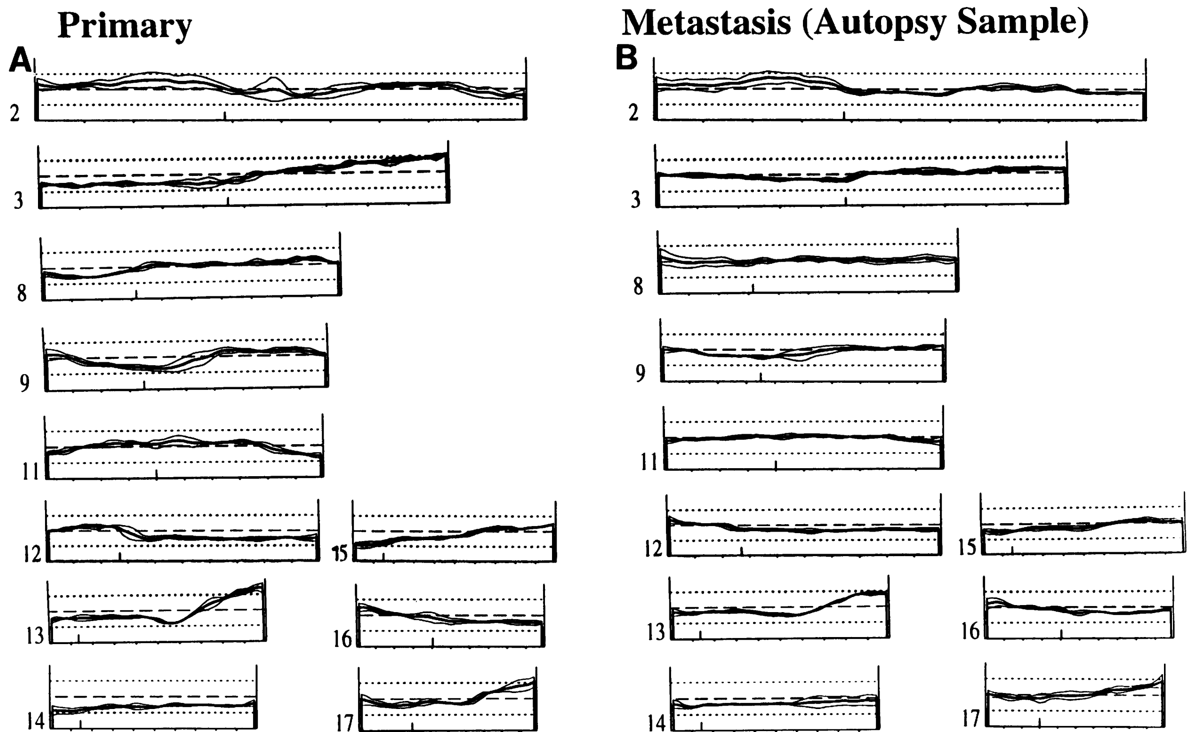

Figure 4. *Green-to-red ratio profiles (thick line-mean, thin lines-+/-standard deviation) for chromosomes showing genetic aberrations in a paraffin-embedded primary breast carcinoma sample and its abdominal metastasis found at autopsy (both samples from paraffin blocks). Most of the genetic aberrations (2p+, 9p–, 12q–, 13q22+, 14–, 15q–, 16q, 17q22-24+) were found in both samples, whereas 3p–, 3q+, and 8p– appeared only in the primary tumor. Dashed horizontal lines correspond to a green-to-red ratio of 1.0, and dotted lines correspond to ratios of 0.5 and 1.5.*

tive DNA sequence copy number increases (5- to 10-fold amplifications or 10 to 20 megabase lower level copy number gains) or decreases (deletions larger than 10 to 20 megabases) anywhere in the tumor genome. This is a distinct advantage over other commonly used methods that require specific probes targeting only one locus at a time. Our previous methodological studies have focused on characterizing the assay performance[6] and development of the image analysis system,[7] which in our experience is necessary to obtain reliable results. Here we extend development of the technique for the use of archival formalin-fixed, paraffin-embedded tumor samples as starting material, thus allowing application of CGH to characterize genetic aberrations in a wide spectrum of solid tumors. Paraffin block technique is especially valuable in many tumor types (eg skin melanomas) in which fresh tissue is usually not available.

The fixation of tumor tissues in formalin results in extensive cross-linking of nuclear proteins, formation of tight complexes between proteins and DNA, as well as fragmentation of DNA. It is therefore not surprising that it is difficult to extract DNA from these fixed nuclei with compact chromatin. Our experience with freshly frozen material has indicated that high molecular weight DNA is needed to produce good quality CGH. Our initial DNA extraction protocols, which were routine for frozen tissues, gave only a low yield mostly of low molecular weight (<2 kb) DNA, which resulted in inadequate CGH hybridizations. Therefore, a modified DNA extraction procedure was developed. We did not find the DNA extraction protocol for CGH reported earlier by Speicher et al[10] to result in optimum sized DNA and high quality CGH when nick translation was used as a method for DNA labeling. Many previously described methods for DNA extraction from archival paraffin blocks have mainly been targeted to produce DNA for polymerase chain reaction studies, in which small amounts of low molecular weight DNA are sufficient for analysis (eg Reference 11). Some studies have reported the use of paraffin block DNA in Southern blot studies,[12–14] although this technique has not been widely applied in cancer research. DNA in these studies has been partially degraded, similar to the DNA after our extraction procedure.

We found that a prolonged digestion of multiple thin tissue sections in 0.3 mg/ml proteinase K at 55 C greatly improves the size and the total yield of DNA when compared with high concentration proteinase K digestion for shorter periods. However, even then the yield of DNA was dependent on the length of formalin

fixation, shown by using a test colon tumor fixed for varying times. The underlying mechanisms by which prolonged proteinase K digestion improves the DNA extraction efficiency remain unknown. The effect may be linked to partial reversal of fixation-induced cross-links in aqueous buffer during proteinase K digestion. As a result of the modified DNA extraction, we could extract 3 to 20 μg of DNA from surgical tissue samples having a typical diameter of 1 to 2 cm. The method described can be widely applied, inasmuch as samples studied so far have come from four different pathology laboratories all of which use variable tissue handling protocols, usually consisting of more than 12 to 24-hour fixation in neutral buffered formalin.

The performance of paraffin block CGH was shown to be highly concordant to that of frozen tissue CGH, as shown by normal-to-normal hybridizations and comparisons of pairs of paraffin and frozen samples taken from the same tumor. The most likely explanation for different results was the contamination of the malignant tumor cells with intra- or peritumoral non-malignant cells (lymphocytes and stromal cells). We have previously shown that CGH tolerates 30 to 50% dilution of the tumor cell DNA by normal DNA,[6] which makes histological controlling of all tissue samples to be analyzed necessary.

Many efforts have been made to validate CGH with other cytogenetic methods. Comparison of CGH with karyotyping fluorescence *in situ* hybridization, Southern blot hybridization, and restriction fragment length polymorphism analyses have clearly indicated that CGH reliably detects DNA sequence copy number aberrations of solid tumors (see Reference 6 for review). However, our experience from over 100 tumor samples indicates that reliable detection of deletions and chromosomal gains requires careful controlling of the quality of hybridization (coefficient of variation should be less than 10%). To further improve the accuracy of the CGH, we usually run inverse labeling experiments (see Materials and Methods), which control for the possible differential hybridization efficiency of differently labeled DNA probes. These experiments control for minor fluctuations of the green-to-red fluorescence ratio, making interpretation of genetic changes with borderline amplitude more reliable.

In conclusion, the method described here allows reliable DNA sequence copy number karyotype studies to be performed from extensively formalin-fixed tumor tissues. The ability to use archival paraffin block material makes it possible to correlate genetic aberrations with clinical outcome (prognosis or therapy response). The same retrospective approach has been successfully applied in hundreds of DNA flow cytometry reports over the past 5 to 10 years, which has produced clinically useful information for therapeutic decision-making.[15] In addition to clinically oriented studies, paraffin block CGH offers a powerful research tool to study pathogenesis of cancer by studying genetic progression from premalignant to fully malignant lesions, or by comparing genetic aberrations in primary tumors and their metastases.

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