OPTIMAL CONDITION FOR CHROMOSOMAL IMBALANCES ANALYSIS OF CANCER GENOME BY COMPARATIVE GENOMIC HYBRIDIZATION

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ABSTRACT

Comparative Genomic Hybridization (CGH) is a technique for analysis of copy number changes throughout the genome based on the hybridization of differentially labeled tumor (test DNA) and normal DNA (reference DNA) to normal human metaphase chromosome (target DNA). In this report, we suggested some critical steps such as metaphase preparation and an average fragment length of DNA probe which obtains a uniform and intense hybridization signal. CGH technique will show gain and loss of DNA sequence regions in cancer genome. Those regions may harbor candidate genes such as oncogenes and tumor suppressor genes playing important roles in the initiation and progression of cancer.

Keywords: chromosome imbalances; comparative genomic hybridization; cancer genome

1. INTRODUCTION

Comparative genomic hybridization (CGH) is a molecular cytogenetic technique first described by Kallioniemi and coworkers [1] which scans the entire genome in a tumor sample. It provides an overview on chromosomal imbalances or DNA sequence copy number changes such as gains and losses (corresponding to putative oncogenes and tumor suppressor genes) within the whole tumor cell genome without any prior information about the chromosomal aberration in question. In a CGH experiment, based on fluorescence *in situ* hybridization, two genomic DNA populations, one derived from the tissue to be analyzed (test DNA) and one from a karyotypic normal reference (reference DNA) are compared. Test and reference DNA are differentially labeled with fluorochromes (direct labeling) or haptens (indirect labeling) and then simultaneously hybridized *in situ* to normal metaphase spreads. During co-hybridization, test and reference DNA compete for the same targets on the metaphase chromosomes. The intensity ratio of the two fluorescence signals gives a measure for the copy number ratio between the two genomic DNA samples. The region of chromosomal imbalances can be detected by analyzing the ratio of test fluorescence to reference fluorescence (green/red) along the target chromosome using digital image analysis. A brief outline of the CGH technique is shown in Fig 1.

Hundreds of CGH studies have been published reporting chromosomal imbalances in a large variety of cancers and the results were recently reviewed [2]. However, little

information about chromosomal imbalance occurring in cancers of Thai sample is available. Therefor, we investigate the Thai cancer samples via CGH technique to provide information regarding the analysis of candidate genes involved in the particular disease.

The overall result of a CGH experiment depends on the performance of each step. Every step in the preparation must be optimized in order to obtain reproducible results. The purpose of this study is to determine the optimal conditions of the CGH technique to investigate chromosomal imbalance in cancer samples. Thus, we describe basic approach to perform CGH experiments and discuss some detail suitable for genomic analysis such as metaphase preparation, points requiring special attention. Additionally, practical applications of CGH analysis in the colorectal cancer are discussed. It may serve as a guideline for starting CGH analysis or a troubleshooting guide for application of CGH in interested areas in Thailand.

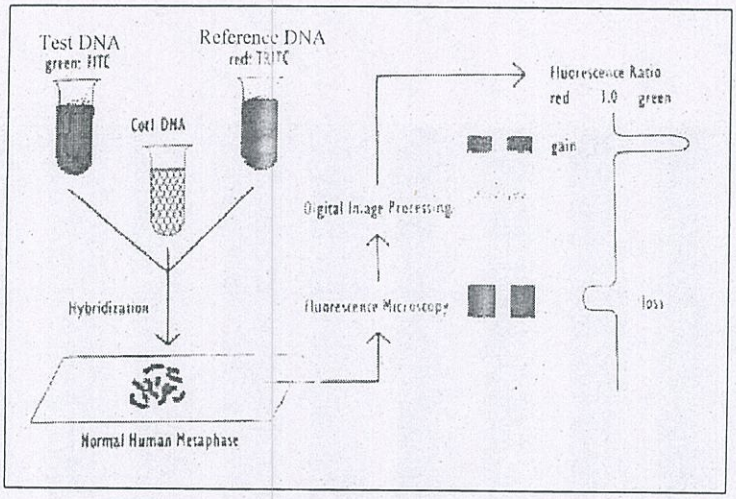

Figure 1. Schematic diagram of the CGH technique. Test and reference DNA are isolated and differentially labeled by digoxigenin (detected in fluorescent green: FITC) and biotin (detected in fluorescent red: TRITC), respectively. The CGH probe, consisting of a 1:1 mixture of both DNAs supplemented with human Cot-1 DNA to block repetitive sequences, is co-hybridized onto normal metaphase spreads. After digital imaging, the green to red ratios are calculated for the individual chromosomes. Thresholds for gains and losses of genetic material of the test DNA were set at 1.25 and 0.75, respectively. (From Hermsen et al.) [3]

2. MATERIALS AND METHODS

In order to refine the CGH technique, we have optimized some conditions and provide suggestion for some steps. The technique was slightly from that reported by Kallioniemi et al. [4] as described in the followings: (1) metaphase preparation that serve as targets DNA for hybridization is prepared from macrotechnique of phytohemagglutinin-stimulated peripheral blood lymphocyte cultures. (2) isolation of high molecular weight genomic DNA (reference DNA and test DNA) that the DNA was extracted by using DNA extraction kit (Neucleospin) and standard phenol-chloroform protocol (3) labeling of the test DNA and reference DNA with different hapten is compared between random primer labeling and nick translation method (4) *in situ* hybridization of the labeled DNA to a normal metaphase, the hybridization condition has been tested for the amounts of DNA probes and denaturation time (5) digital image analysis used to facilitate the identification of chromosomal regions with abnormal fluorescence ratio as described in Kallionimie et al. [4]

3. RESULTS AND DISCUSSION

CGH technique is more demanding than most other molecular cytogenetic techniques because it is based on the quantification of ratios of two fluorochromes along every chromosome within a normal metaphase. The accuracy and variability of the CGH technique depends to a large extent of the hybridization properties of the metaphase spreads. Careful attention to all the steps of the CGH procedure, including DNA labeling, metaphase preparation, hybridization, microscopy, image analysis, and interpretation of the data is therefore necessary to derive optimal benefit from the analysis. [5, 6]

Peripheral blood lymphocytes from healthy person were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. Phytohemagglutinin (0.1 mg/40 mL medium) was added to stimulate growth of the lymphocytes. After 3 days of cell culture at 37° C, mitotic cells were arrested by colchicine incubation followed with special care by ice-cold hypotonic treatment (0.075M KCl) and subsequent fixation in methanol-acetic glacial acid (3:1) on ice. After repeated washing with ice-cold fixative solution, the cells were dropped onto slides. The quality of CGH analysis that is adequate for whole chromosome probe with minimize cytoplasm debris and non-overlapping chromosome is extremely dependent on the characteristics of the metaphase spreads. A large number of slides is prepared once that store at -20°C and should be quality tested for the hybridization characteristics using labeled DNA from normal cells and the control cell line. Besides adequate hybridization quality, the chromosome morphology also needs identification to reveal the optimized condition of denature temparature and time for each batch of slides. The slides that were prepared from macrotechnique culture not only increase the best morphology of metaphase spreads but also decrease background. So, we suggest that CGH technique should use metaphase slide from macrotechnique culture.

Normal DNA as a reference DNA can be taken from blood lymphocytes from any healthy male individual and test DNA can be obtained by any type of interested cancer sample. DNA extraction from both standard method and DNA extraction kit can give good yield of high molecular weight genomic DNA. However, the DNA from the commercial kit yielded better high molecular weight DNA compared to the standard method. For efficient yield of test DNA from tumor tissue, lysis buffer containing proteinase K was added to the cell lysate and incubated at 56°C overnight. The extracted DNA quantity is estimated by spectrophotometric measurement and appropriate quality is evaluated by representative fragments of DNA by agarose gel electrophoresis. The standard method usually requires a large amount of tissues (300 mg/sample) and results in a variety of DNA fragment lengths that are hard to be controlled while labeling. On the other hand, the DNA extraction kit needs little amount of tissue (25-50 mg/sample). So, the DNA extraction kit is suitable for CGH. The test and reference DNA extraction should be prepared from the same method. Contamination with normal cells significantly dilute the ability to elucidate copy number changes (discuss in CGH analysis). Nacrotic and inflammation tissue should be avoided because they contained degraded DNA which was carried out successfully by lack of or weak signal.

The test and reference DNA can be labeled with different hapten by indirect labeling or with different fluorochrome by direct labeling to distinguish the hybridization ratio in with the two genomes. Normally, the test DNA is detected with FITC which produces a green fluorescence and the reference DNA with TRITC which produces a red fluorescence. Our method used indirect method to label test DNA with biotin-16-dUTP (Roche, Mannheim, Germany) and reference DNA with digoxigenin-11-dUTP (Roche) by random primer labeling and standard nick translation. A standard labeling is used to label 1 g of DNA which produces labeled fragments of approximately 500–3,000 bp. Fragment lengths of genomic probes are estimated by electrophoresis in 2% agarose gels and compared with a DNA molecular weight marker. Determining the probe size is an essential part of the CGH experiments. In this study, we compared labeling method by nick translation and random primer labeling. Nick translation labeling is used to cut DNA to small fragments by adjusting the ratio of DNase to DNA polymerase concentration when incubation that an average of 500–1,000 bp (smear from

500 to 1000 bp) gives best result to obtain a uniform and intense hybridization signal. In random primer labeling method, DNA were cut into small fragments by heating in an autoclave at 100°C for 15 mins and DNA were adjusted by spectrophotometric measurement. From this labeling method, variety of DNA fragment length that the quality of the hybridization is often poor.

For *in situ* hybridization condition, approximately 300-400 ng of both labeled test and reference DNA are mixed with excess unlabeled of human Cot-1 DNA (GibcoBRL Life Technologies) and ethanol precipitation. Cot-1 DNA, consists of purified repetitive sequences, was added to block the highly polymorphic repeat sequences in the DNA. The pellet was resuspended in hybridization buffer and co-hybridized onto normal denatured metaphase chromosomes.These probe mixtures and the metaphase slides are denatured separately using 10 µl hybridization buffer containing 50% deionized formamide/20% dextran sulfate in 2XSSC at 75°C for 8 mins and prehybridized at 37°C for 30 min for the probes, and 70% formamide/2XSSC at 70-72°C for 1-2 mins for metaphase slides that optimal denaturation time and temparature required before this step. The hybridization was performed in a humidity incubator for 3 days at 37°C. After hybridization, The slides were then washed extensively and blocked with bovine serum albumin (BSA) solution. Detection of the differentially labeled genomic DNA was done by incubation with mouse α-biotin and sheep α-DIG antibody and followed by α-mouse/TRITC and α-sheep IgG/FITC and counterstain with 1 mg/ml of 4.6-diamino-2-phenylindole (DAPI) to produce a banding pattern that enables chromosome identification and standard karyotyping.

Differential fluorescent signals representing gains and losses of the tumor DNA relative to the normal DNA are shown by the graphs. In order to determine whether a chromosomal loss or gain occurred, the green and red fluorescence intensities on the target DNA are calculated using fluorescence microscopy and digital image analysis. Digital images of metaphase chromosomes slides were obtained with a charge-coupled device (CCD) camera on microscope using filter-set for DAPI, FITC, and TRITC. Three digital black and white images are captured for each metaphase. Images were analyzed with Cytovision software (Applied Imaging) by karyotyping and the calculation of the fluorescence ratio of each chromosome. Usually, the average ratio for each case was calculated for 5 or more metaphases of highest quality. Poorly or inhomogeneously stained metaphases were excluded from analysis, as well as heavily bent or overlapping chromosomes. In regions of normal sequence copy number the average green to red ratio should be around 1.0. In 100% of the cancer cells, in the case of trisomy, the factor would be expected to increase to 1.5 and in the case of monosomy, the factor would be expected to decrease to 0.5. So, Under the assumption that 50 % of the cancer cells in a diploid tumor cell population carry a monosomy the theoretical ratios are 0.75 and for a trisomy 1.25. For identification of chromosome imbalances, threshold values for green-to-red ratio profiles were set on 0.75 and 1.25, respectively, to indicate significant losses and gains of DNA sequence copy number in the test sample. Gains of chromosomal material surpassing the threshold of 1.5 were defined as high-level amplifications.

For example, we experimented a total of 28 Thai colorectal cancer samples. Genetic changes such as gains of chromosome 20q (50%: 14 cases from total of 28), 8q (32.14%), 19q (25%), 12q (21.42%) and 17q (21.42%) and losses of chromosome 18q (17.85%), 4q (14.28%), and 4p (10.71%) were found. The most high-level amplification was found on 20q that has been shown graphically in Fig 2. The most frequent alteration in previous CGH analyses of primary colorectal cancer was gains of chromosome arm 20q [7, 8]. This aberration was observed with similar frequency in our study, it was suggested that the gain in 20q was closely related to colorectal cancer. This region is the location of the gene for matrix metalloprotease-9 (MMP9). We should therefore examine a larger number of cases with CGH and investigate the gains and losses in chromosome by other methods such as FISH, RT-PCR and sequenceing.

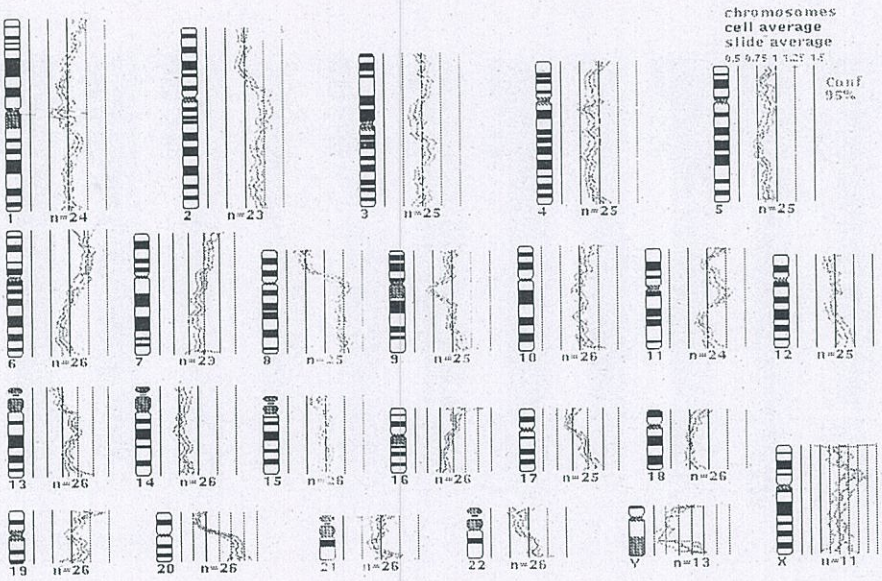

Figure 2. An example of CGH ratio profile in one case of a colorectal cancer. There was gains in 2q, 6p, 8q, 17q and 20q and losses in 5q, 8p and 18q. The baseline (middle) ratio is 1.0. The left-side shift indicates a ratio of 0.75 while right-side shift indicates ratio of 1.25. The bold line to the left and right of indicate loss and gain of DNA sequence, respectively. The n number underneath each profile indicates the number of chromosome counted.

CGH have some limitation as the balanced chromosomal rearrangements as inversions, reciprocal translocations, or imbalances affecting only smaller regions or single mutations are not detectable by means of CGH, some regions that have high repetitive sequences and addition GC rich areas that are associated with the non-uniform denaturation and hybridization properties [5]. So in our laboratories, the centromeric regions, the short arm of the acrocentric chromosomes, the heterochromatin blocks on 1q12, 9q12, 16q11.2, and Y chromosome were excluded from CGH profile analysis. Additionaly, sensitivity of the CGH analysis can be hampered by contamination of the tumor with normal cells. For example, CGH profile from to the colorectal cancer sample that contained cancer cells > 50% demonstrated more sensitive ratio profiles of chromosomal gains and losses than sample that normal cells contamination (Fig 3).

So, CGH permits the rapid detection and mapping of DNA sequence copy number used for the study of chromosomal imbalances in a single experiment. CGH technique was applied to genome-wide screening for gains and losses of DNA sequence regions in cancer genome. Those regions may harbor candidate genes playing important roles in the development and progression of cancer. Further investigation on those regions via specific techniques may lead to the identification of tumor suppressor genes and oncogenes that play important roles in tumorigenesis and contributes to our understanding of cancer biology in Thai samples.

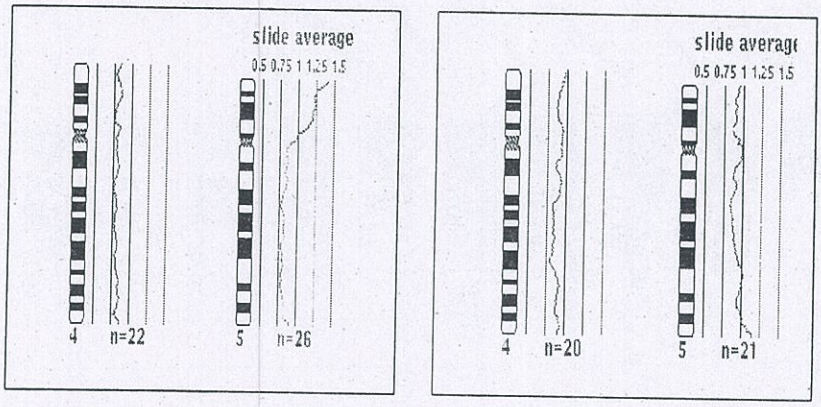

Figure 3. Ratio profile (A) from sample that contained cancer cells > 50% demonstrates more significant ratio profiles with detection of a loss region at chromosome 4 and 5 in comparison with (B) normal cells contamination.

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