JHKMTA 1999/2000; 8: 7-12

Comparative Genomic Hybridization: a companion to conventional cytogenetics

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Abstract

Comparative genomic hybridization (CGH) is a recently described molecular-cytogenetic technique that globally assays for chromosomal gains and losses throughout the entire genome. Normal metaphase chromosomes are competitively hybridized with two differentially fluorochrome-labeled genomic DNAs (test and reference). Using fluorescence microscopy and digital image analysis, regions of gain or loss of DNA sequences in the test genome are reflected in the ratio of the intensities of the two fluorochromes along the target chromosomes. CGH has proved to be a valuable tool in tumour genetics. Moreover, the efficacy of CGH in revealing aneuploidies is also a useful adjunct in screening for prenatal and neonatal chromosomal aberrations.

Key words: Comparative genomic hybridization. Cytogenetics. Tumour genetics. Aneuploid

Introduction

Conventional cytogenetics (or karyotyping) has identified many chromosomal aberrations in human cancer cell lines and primary culture of haematological malignancies. The information has facilitated the identification of a number of important genes associated with tumourigenesis. Loss of chromosomal material on 13q has led to the identification of the tumour suppressor gene *RB1*[1]. Karyotyping involves the culture of fresh tumour samples. Mitotic cells are then stained to produce banding pattern on the chromosomes for their identification. Microscopic examination reveals information on the aneuploidy and structural changes including deletions, translocations, inversions, and duplications. However, it has a number of inherent limitations[2]. Firstly, conventional karyotyping is limited to dividing cells and thus excludes a large number of non-dividing cells. It is possible that dividing cells represent only a subclone of the main neoplastic population. Secondly, normal cells may sometimes outgrow the tumour cells, thus giving normal metaphases. Thirdly, chromosomal banding is difficult in solid tumours owing to the complex nature of chromosome changes and the poor morphology of the metaphases. Finally, karyotypic analysis depends on the availability of fresh tissues and cannot be carried out in archival materials. Therefore, in contrast to leukaemias, karyotyping of solid tumours has not been routinely performed.

Comparative genomic hybridization (CGH) is a novel molecular cytogenetic tool that permits evaluation of genetic changes throughout the whole genome of tumours in a single hybridization experiment using a small amount of DNA[3]. A relative copy number karyotype is generated for a tumour by the comparison of DNAs from malignant and normal cells, thus enabling the identification of regions of DNA gain or loss. In the assay, tumour DNA (labeled green) and normal reference DNA (labeled red) are competitively hybridized to normal human metaphase spread. The reference DNA serves as a control for local variations in the ability to hybridize to target chromosomes. The relative amounts of tumour and reference DNA bound at a given chromosome are dependent on the relative abundance of those sequences in the two DNA samples. Digital image analysis gives a measurement of the ratio of green-to-red fluorescence and is presented as a relative copy number karyotype. Gene amplification or chromosomal duplication in the tumour DNA produces an elevated green-to-red ratio, and deletions or chromosomal loss has a reduced ratio. If the absolute copy number of any region in a tumour genome is known, relative numbers can be converted to actual copy numbers at all loci. CGH can be

performed on DNA extracted from fresh or frozen materials, or even formalin-fixed paraffin-embedded specimens, sometimes after whole genome amplification if the DNA yield is too low[4,5]. It is thus especially helpful in the investigation of genetic changes underlying solid tumours.

CGH: the technique

The methodology of CGH has been fully described[6,7,8]. A schematic overview of the technique is shown in Fig. 1.

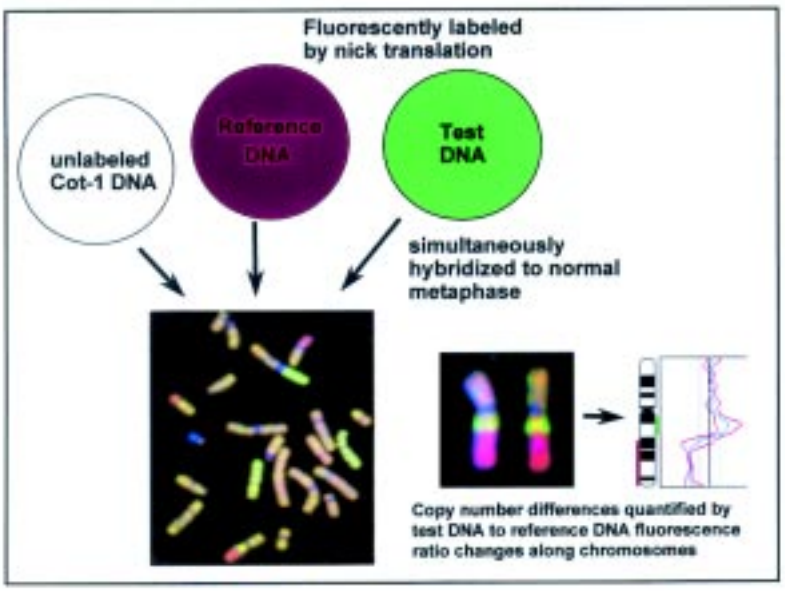

Figure 1. The diagram summarizes the procedures of CGH, from nicking of test and reference DNA to the fluorescence ratio generated after digital image analysis. Reproduced with permission from Vysis, Inc.

Normal metaphase slide

Normal metaphase slides are commercially available. They are prepared from phytohaemagglutinin-stimulated peripheral blood lymphocyte cultures of normal male individual.

DNA preparation

Reference DNA, matching the sex of the tumour DNA, is obtained from karyotyped normal individuals. Using standard nick translation technique, both the tumour and test DNA (1 μg each) are labeled directly with green and red fluorochromes respectively. The fragment lengths of the labeled probes should range from 500 to 2000 base pair. In contrast to the use of biotinylated- / digoxigenin-conjugated deoxynucleotides, directly fluorochrome-conjugated deoxynucleotides avoids the use of post-hybridization detection step and thus improves the quality of the hybridization signals. Labeled tumour and normal DNA (450 ng each) are then ethanol precipitated in the presence of 36 μg Cot-1 DNA, dried and resuspended in hybridization buffer containing dextran sulphate and deionized formamide. Cot-1 DNA consists of purified repetitive sequences and is added to block the highly polymorphic repeat sequences in the tumour and reference DNA.

Alternatively, tumour DNA and reference DNA can be reversely labeled with red and green fluorochrome respectively[9]. This 'inverse' labelling CGH serves as control for the differences in hybridization between the green- and red-labeled probes. This would help to correct for minor fluctuations of the green-to-red fluorescence ratio, making interpretation of genetic changes with borderline amplitude much more reliable[10].

Hybridization

The hybridization mixture is denatured at 72°C for 5 minutes and allowed to preanneal at 37°C for 30 minutes to compete out hybridization signal due to repeat sequences. Before hybridization, normal

metaphase spreads are denatured in 70% formamide / 2x SSC at 72°C for 5 minutes and dehydrated in ice-cold 70%, 85% and 100% ethanol. Hybridization is performed in a light-tight humid chamber at 37°C for 3 - 5 days. The slides are washed in 0.4x SSC / 0.3% NP40 at 73°C for 2 minutes, followed by a 1-minute wash in 2x SSC / 0.1% NP40 at room temperature. The slides are briefly dehydrated in 70% and 85% ethanol for 30 seconds each, then left to dry in the dark before counterstained with 125 ng/ml 4',6-diamidino-2-phenylindole (DAPI) in anti-fade solution.

Digital Image Analysis

Metaphases that showed uniform and intense hybridization and contained well-separated chromosomes are captured with a cooled charge-coupled device (CCD) camera mounted on a fluorescence microscope. A 100X Plan fluotar objective (NA 1.30, oil) is used for capturing the images. Three-colour digital images (green for the tumour DNA, red for the normal reference DNA and blue for the DAPI-counterstained chromosome) are acquired from at least 10 metaphases per hybridization using a filter-wheel containing excitation filters appropriate for the green, red and DAPI fluorochromes. The filter-wheel allows visualization of all three fluorochromes without any registration shifts between images. Chromosomes are identified on the reverse DAPI banding images. Digital imaging system is used for calculation of the green-to-red fluorescence ratio for each chromosome. Ratio profiles from the analyzed metaphases are combined to improve signal to noise ratio. The calculated average ratios are plotted along ideograms of their corresponding chromosomes in a relative copy number karyotype. Gain of DNA sequences in the test DNA relative to the reference DNA are visualized as increased fluorescent ratio values on the chromosomal region from which the sequences are derived. Similarly, losses of DNA sequences are detected as decreased fluorescent ratio values. The ratio values of 1.25 and 0.75 are used as upper and lower thresholds for the identification of chromosomal imbalances. The Cot-1 DNA included in the hybridization inhibited binding of the labeled DNA to the centromeric and heterochromatic regions, and thus these regions are not analyzed. Chromosomes that are heavily bent, overlapping, or with overlying artifacts are excluded from the analysis. Cautious interpretation should be taken in the analysis of telomeric and heterochromatic regions, 1p32- pter, 16p, 19 and 22 as recommended by Kallioniemi et al[6]. Fig. 2 shows a typical CGH profile.

Figure 2. Example of CGH profile. The central line represents a ratio value of 1.0. Green lines to the right indicate ratio values of 1.25 and 1.5, while red lines to the left indicate ratio values of 0.75 and 0.5. The chromosome numbers are given below the individual ideograms (n = number of homologues examined). The CGH profile suggests loss of region 6q16-q25 and whole chromosome X.

Quality control

Validity of the test procedure and the CGH results should be checked, especially with each new batch of metaphase slide and reagent. A good negative control includes hybridization of a green-labeled normal male DNA against a red-labeled normal female DNA and monitoring of the green-to-red ratio of the X chromosome in relation to that of the autosomes. This will help to examine the dynamic range of the hybridization. In addition, tumor cell line with known genetic abnormality should be included as positive control. Genetic alterations should be detected unequivocally.

Advantages of CGH

CGH has distinct advantages in the analysis of genetic alterations in a tumour genome. CGH requires no prejudgement as to what genomic regions are likely to be involved, and does not require chromosome preparations from the tissue to be studied. Unlike fluorescence in situ hybridization or restriction fragment length polymorphism analysis which target only one locus at a time, detailed information on DNA gains and losses throughout the whole genome can be obtained in a single hybridization experiment. The technique is particularly helpful in the analysis of genetic changes underlying solid tumour, which are relatively inaccessible to cytogenetic analysis. Thus, CGH is a valuable research method for genome-wide scanning for regions of genomic amplification or deletion. A small amount of fresh or frozen tumour DNA is sufficient for the assay. Moreover, successful hybridization has been reported from DNA extracted from formalin-fixed paraffin-embedded tissue and after whole genome amplification using degenerate oligonucleotide primed polymerase chain reaction. This application allows retrospective analysis of many tumours that are pathologically well-characterized.

Limitations of CGH

CGH is not capable of detecting balanced chromosomal translocations, when there is no change in the relative DNA copy number. Gain of DNA (amplicon size times level of amplification) has to be at least 2 Mb for it to become detectable by CGH. The smallest size of deletions detectable is expected to be around 3-5 Mb[6]. Thus, low level DNA amplification or small deleted regions may be missed. Hybridization to an array of mapped sequences instead of metaphase chromosomes (array CGH) should provide better resolution[11]. Sensitivity of the CGH assay is hampered by contamination of tumour with normal cells. Kallioniemi et al[6] have stated that 50% of nontumourous cells in the test sample is the maximum limit for the detection of monosomies or trisomies. However, the admixture of nontumour cells in the test sample can be reduced using microdissection technique on histological samples[12]. The sensitivity for deletion detection is also dependent on ploidy level. It is more difficult to detect a loss of one chromosome homologue in a tetraploid cell line than in a diploid one.

Application of CGH in tumour genetics and clinical genetics

Since the introduction in 1992, more than 280 publications has applied the CGH technique and provided an enormous amount of data that describe a number of recurrent DNA gains and losses in different malignancies[13,14]. These included leukaemias and lymphomas, common tumours (colon, breast and lung), gender specific tumours (ovarian, cervix, testicular and prostate), paediatric tumours (neuroblastoma, rhabdomyosarcoma) and less common tumours (brain, renal, uveal melanoma). Visakorpi et al[15] were the first to apply CGH to the search for novel cancer genes. They found that the androgen receptor gene was amplified in prostate cancers that had recurred during androgen deprivation therapy. Moreover, CGH has led to the discovery of the tumour suppressor gene STH11/ LKB1 (serine/threonine kinase) important in the Peutz-Jeghers syndrome[16]. Application of CGH to archival specimens would result in collections of copy number karyotypes for all tumour types, from premalignant to metastatic lesions. This should lead to better understanding of the pathogenesis of different tumours.

In view of the fact that developmental abnormalities, such as Down's, Prader-Willi, Angelman and Cri du Chat syndromes often result from gain or loss of one copy of a chromosome or chromosomal region, CGH has been applied to detection and characterization of whole and partial aneuploidies in prenatal and neonatal diagnosis[17]. Recently, CGH has been used to characterize cytogenetic aberrations in fetoplacental tissues in cases of recurrent abortions[18,19].

Conclusion

The development of CGH has provided the technology to identify many new areas of genomic alteration which were not previously recognized to be involved in tumourigenesis. CGH has expanded and identified chromosomal regions that are important for further molecular genetic studies. Gene amplification (gain of DNA) is an essential mechanism of oncogene activation. In contrast, detection of commonly deleted regions within specific tumour types by CGH would signpost chromosomal regions that need to be analyzed by loss of heterozygosity studies and would potentially result in the isolation of novel tumour suppressor genes. Thus, CGH is a powerful technique, complementary to conventional cytogenetics, in identifying chromosomal aberrations.

Acknowledgement

The authors thank P. Moldenhawer, and Vysis, Inc for permission to reproduce Figure 1.

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