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Review Article

TOWARDS ANALYSING COMPARATIVE GENOMIC HYBRIDISATION METHOD

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ABSTRACT

Human DNA has 6 million nucleotides packaged into 2 sets of Chromosomes. Mutations can change the sequence of nucleotides. Mutations can be defined as any change in the DNA sequence of a cell. Mutations may be caused by exposure of DNA to DNA damaging agents in the environment like ultraviolet rays, chemical carcinogens (eg: aflatoxin) etc., or by mistakes during cell divisions. COPY - NUMBER VARIATIONS (CNVs) are alterations of a genome that results in the cell having an abnormal number of copies of one or more sections of the DNA. The molecular - cytogenetic method for the analysis of copy number changes in the DNA content of a given subject's DNA and often in tumor cells is known as comparative genomic hybridization. Comparative genomic hybridisation is used for studying chromosomal changes in cancer. In human pathology, analysis of genetic abnormalities which causes tumours and cancers is increasingly important. Many techniques are available to fulfill this purpose such as DNA cytometry, tumor cytogenetics, fluorescence in situ hybridization (FISH) and microsatellite assay. But all these techniques have certain advantages and disadvantages. They may be sometime expensive (DNA cytometry) and more laborious. So, the scientists developed a molecular cytogenetic technique called comparative genomic hybridization.

Keywords: Copy Number Variations, Test DNA, Reference DNA, Fluorescence in-situ hybridization, Array CGH

1. INTRODUCTION

Copy number variations can be analyzed by a cytogenetic method called Comparative genomic hybridization (CGH).In CGH two types of DNA are compared, that is, a test DNA and a reference DNA. There are many techniques which are used for analyzing copy number variations but, CGH is widely used because it does not involve culturing of cells. In this technique two genomic samples are compared, a tumour DNA (test DNA) and a normal DNA (reference DNA).Both are taken from the same individual. This technique gives us information about the gains and losses of a whole chromosome or a part of a chromosome [1].

Kallioniemi and colleagues analyzed CGH for the first time in 1992 at the University of California, San Francisco. They used CGH for the analysis of solid tumors. The solid tumors they used are the breast cancer tumors and primary bladder tumors. The cells of both the tumors were extracted and karyotyping was done. They identified 16 different regions of amplification [1].

There are other cytogenetic analysis techniques like, giemsa banding (it is a cytogenetic technique which is used to produce a visible karyotype by staining condensed chromosomes) and fluorescence in-situ hybridisation (FISH). But, CGH is a more improved technique because the former two techniques are limited by the resolution of the microscope. Moreover, giemsa banding involves staining of condensed chromosomes which might not give proper results [2, 3].

Fluorescence in situ hybridization (FISH) is a technique which is used to detect a specific DNA sequence on chromosome by using a probe (a small segment of DNA that is synthesized in the lab and labeled with fluorescence dye). This specific DNA sequence on the chromosome is the abnormal or diseased chromosome. The principle involves isolation of the cells from the patient and incubating them with the labeled probe. This process is called hybridization. The probe binds with the complementary sequence on the chromosome. The hybrid DNA is then detected by using fluorescence microscope. It is to be noted that the probe binds (the sequence on) the chromosome. We do not extract genomic DNA from the cells.

In 1993 du Manoir et al. described the same methodology [4]. Two different fluorophores in different proportions were used to label the individual human

chromosomes from a DNA library to test the technique. The authors also applied CGH to the genomic DNA of the patients suffering from Down's syndrome or T-cell prolymphocytic leukaemia and also the cells of a renal papillary carcinoma cell line. They concluded that the fluorescence ratios which they got were accurate and that differences between genomic DNA from different cell types were detectable. Therefore, CGH was considered a highly useful cytogenetic analysis tool [4].

The principle of CGH involves the use of different colored fluorochromes, like, FITC (fluorescein isothiocyanate) and TRITC(tetramethyl rhodamine).It then involves the isolation of DNA from the same individual but from two different cells which are to be compared, most commonly a test and reference source. Each DNA sample is then labeled with the fluorochromes. Then, the DNA is denatured so that it is single stranded. The next step is hybridization between the test and the reference DNA. The last step is the usage of fluorescence microscope and computer software to compare the differentially colored fluorescent signals along the length of each chromosome for identification of gains or losses of chromosomes between the two sources. If there is a higher intensity of the test sample color, it indicates gain or duplication of a specific region of a chromosome, while a higher intensity of the reference sample color indicates the deletion of a chromosome. If the chromosomes of both the DNA are same, that is, no gains or losses, then a neutral color (yellow) is observed [2,3].

DNA microarray is a recently developed technology and widely used in cancer research. The basic principle of DNA microarray is nucleic acid hybridisation. By conjugating DNA microarrays along with CGH techniques, a more specific form of CGH called array CGH (aCGH) has been developed. This is an improved technique which will measure copy number variations locus-by-locus with increased resolution as low as 100 kilo base pairs [5, 6].

2. METHODOLOGY

2.1. Metaphase slide preparation

The DNA is taken on the slide as a reference sample and is thus obtained from a karyotypically normal man or woman. Female DNA is preferentially use as they possess two X chromosomes which contain far more genetic information than the Y chromosome present in the male. Peripheral blood lymphocytes which are used is stimulated by phytohemagglutinin. 1mL of heparinised blood is added to 10ml of culture medium and incubated for 72 hours at 37°C in an atmosphere containing 5% CO_2. Colchicine is used to arrest the cells in mitosis, the cells are then harvested and treated with hypotonic potassium chloride and fixed in 3:1methanol/acetic acid [3].

Only one drop of the cell suspension should then be dropped onto a slide cleaned with ethanol from a distance of about 30 cm, optimally all this process should be carried out at room temperature where humidity levels should be 60-70%. Minimal cytoplasm can be observed by visualization of slide under the phase contrast microscope. Chromosomes should not be overlapping, and it should be 400-550 bands long with no separated chromatids. Then finally it should appear dark rather than shiny. Then air dry the slide overnight at room temperature, and any further storage should be in groups of four at -20°C with either silica beads or nitrogen which is basically present to maintain dryness. Because of the variations present in the hybridization, the donors should be tested. Commercially available slides can be used, but it should always be tested first [3].

Only metaphase of the mitosis is used, because in that phase only, the chromosomes are aligned at the centre of the cell, and it becomes easy to observe the chromosomes in metaphase.

2.2. Isolation of DNA from test and reference tissue

To obtain DNA from test or reference (karyotypically from normal individual) tissue, Standard phenol extraction method is used. This method involves the combination of Tris-Ethylenediaminetetraacetic acid and phenol with aqueous DNA in equal amounts. This is followed by agitation and centrifugation for separation, after that the aqueous layer is removed and then it is further treated using ether and finally ethanol precipitation is used to concentrate the DNA [3].

DNA isolation can be done using DNA isolation kits available commercially which are based onaffinity column [3].

Preferentially, DNA should be extracted from fresh or frozen tissue because it gives the highest quality. For the CGH experiment, 0.5-1 μg of DNA is sufficient, though if the desired amount is not obtained DOP-PCR may be applied to amplify the DNA [3].

2.3. DNA labelling

Nick Translation is used to label the DNA and it involves making a nick (Gape) in single strand of the double stranded DNA without removal of any nucleotide base pair. This method involves the substituting of nucleotides which labeled with fluorophores or chromogenic substances (direct labeling) or biotin or oxigenin to get fluorophore conjugated antibodies (indirect labeling). It is then important to check fragment lengths of both test and reference DNA, sogel electrophoresis is used, as they should be within the range of 500kb-1500kb for optimum hybridization [3].

2.4. Blocking

The placental DNA enriched with repetitive sequences of length 50bp-100bp is known as Cot-1 DNA. This Cot-1 DNA is added. It blocks normal repetitive DNA sequences, particularly at telomeres and centromeres. If this repetitive sequence is detected, it may decrease the fluorescence ratio and cause losses or gains to disturb the detection [3].

2.5. Hybridization

Mix the 8-12µl of each of labeled test and reference DNA. Then add 40 µg Cot-1 DNA. Because of this cot-1 DNA, labeled test and reference DNA precipitates. Then subsequently dissolved it in 6µl of hybridization mixture. In order to decrease DNA melting temperature, this hybridization mix contains 50% formamide and contains 10% dextran sulphate to increase the effective probe concentration in a saline sodium citrate (SSC) solution at a pH of 7[3].

Denatured formamide/2xSSC for 5-10 minutes at 72°C, while the probes were denatured by immersion in a water bath of 80°C for 10 minutes. These are immediately added to the metaphase slide preparation. Then cover this whole reaction with a coverslip and keep it in humid chamber for two to four days at 40°C [3].

Then remove the cover slip, and apply the 5-minute washes using 2xSSC for three times at room temperature, one at 45°C with 0.1xSSC and one time using TNT at room temperature. Then preincubated this reaction for 10 minutes then subsequently followed by a 60-minute, 37°C incubation. Then wash this slide with TNT for three time, then one time with 2xSSC at room temperature. Then dry this slide using an ethanol series of 70%/96%/100%. Then, counterstaining the slide with DAPI (0.35 µg/ml) for chromosome identification, and then sealing the slide with a cover slip [3].

2.6. Fluorescence visualisation and imaging

A fluorescence microscope with the appropriate filters for the DAPI stain and it utilised the two fluorophores for visualization, and these filters should also minimize the crosstalk between the fluorophores, such as narrow band pass filters. Uniform illumination without chromatic variation must be provided by the microscope [3]. Camera is used to capture the pictures with spatial resolution at least 0.1 µm at the specimen level and give an image of at least 600x600 pixels. The camera must be able to conjugate the image for at least 5-10 seconds, with a minimum photometric resolution of 8 bit [3].

For the image processing step, Dedicated CGH software is commercially available, and this software is also required to subtract background noise, remove materials not of chromosomal origin, and it also requires to normalize the fluorescence ratio. A "relative copy number karyotype" is present in chromosomal areas of deletions or amplifications. This can be generated by averaging the ratios of several high-quality metaphases and plotting them along with an ideogram (a diagram identifying chromosomes based on banding patterns). By using fixed or statistical thresholds, interpretation of the ratio profiles can be conducted (confidence intervals). By using confidence intervals, gains or losses are identified when 95% of the fluorescence ratio does not contain 1.0 [3].

It is require to take extreme care to avoid contamination of any step involving DNA, especially with the test DNA, because contamination of the sample with normal DNA will skew results closer to 1.0, which causes the abnormalities to undergo undetected. FISH, PCR and FLOW CYTOMETRY experiments maybe employed to confirm results [5, 7].

2.6.1. Array Comparative Genomic Hybridisation

Array comparative genomic hybridization is one type of conventional CGH technique which is also known as microarray-based comparative genomic hybridization, matrix CGH, array CGH and aCGH. This technique is a molecular cytogenetic technique which is used for the detection of copy number changes in chromosome on a wide genome and high-resolution scale [8]. Array CGH compares the reference genome with a patient's genome and then it identifies the differences between the two genomes, and thus it locates regions of genomic

imbalances (insertion and deletion in the chromosome) in the patient. Like a traditional CGH, array CGH also utilizes the same principles of competitive fluorescence in situ hybridization (FISH).

To solve the main limitation of conventional CGH which is low resolution, scientists have developed the technique named Array CGH which gives the high resolution. In array CGH, the metaphase chromosomes which are used in conventional CGH are replaced by cloned DNA fragments having a size around +100–200 kb. The exact chromosomal location of this cloned DNA is generally known. This technique allows the detection of mutations present inthe sequence of DNA in detail and, it also makes possible to mapping the DNA sequence to get the changes directly onto the genomic sequence [9].

Array CGH is more, sensitive, fast and high through put technique, with many considerable advantages as compared to other methods which used for the analysis of DNA copy number changes like conventional CGH, and it is more manageable technique for the diagnostic applications. Copy number changes at a level of 5–10 kilobases of DNA sequences can be easily detected by using this method [10]. The another type of the array CGH which is high resolution CGH (HR-CGH) (2006). This technique is accurate to detect structural variations (SV) at resolution of 200 bp [11]. This method allows identifying the new chromosome changes such as microdeletions and duplications in human conditions such as birth defects and cancer due to chromosome aberrations.

2.6.1.1. Methodology

Array CGH has same principle as conventional CGH. In array CGH technique, DNA from a test sample (or patient) and DNA from a reference sample are differentially labeled with two different fluorophores by using the method nick translation. And this labelled DNA is used as probes that are hybridized competitively with nucleic acid targets. In traditional CGH, the target is a reference sample in which chromosomes are present in its metaphase. In array CGH, these targets are genomic (DNA) fragments which are cloned in different types of vectors such as BACs or plasmids, oligonucleotides, or cDNAs [12].

DNA from the test sample which we want to be compared is labeled with a red fluorophore (cyanine 5) and a reference DNA sample is labeled with green fluorophore (Cyanine 3). Then take both the sample in equal quantities and mixed them. After that hybridized this mixture with the DNA microarray of several thousand evenly spaced cloned DNA fragments or oligonucleotides, which shows the triplicate on the array. After hybridization process, to quantify and capture the relative fluorescence intensities of each of the hybridized fluorophores, the digital imaging systems are generally used [12]. The ratio of the fluorescence intensities is directly proportional to the ratio of the copy numbers of changes in DNA sequences in the reference and test genomes. If the intensities of the fluorophores generated are equal on probe, this region of the patient's genome is indicated as the mixture is having equal quantity of DNA in the test and reference samples; if there is some changes in the ratio of Cy3:Cy5, it shows a gain or loss in patient's DNA sequence at the specific genomic region [13].

2.6.1.2. Applications

1. Conventional

For the diagnosis and prognosis of cancer conventional CGH has been used. It is mainly used for the identification of chromosomal regions that are lost or gained in tumors [14]. This is also used to study chromosomal aberrations in foetal and neonatal genomes. Moreover, for detecting chromosomal aberrations this technique is used and can diagnose complex abnormalities [9].

2. In cancer research

Some data from CGH reports indicates that a non-random genetic aberrations occurs which is followed by the accurate pattern. Some of these changes are common to the various types of malignant tumour and others belong to the specific type of tumour [15].

For example: The region of chromosome which have the gain of 1q , 3q and 8q and the losses of regions 8p , 13q, 16q and 17p leads to a common types of tumours like prostate, renal, bladder cancer. There are specific tumours which need unique forces during the treatment of cancer development in different organs. These unique forces are needed if there is a gain of 12p a 14q in testicular cancer, gain of 13q and loss of 9q in bladder cancer, loss of 14q in renal cancer and loss of Xp in ovarian cancer [15].

3. Chromosomal aberrations

The partial deletions of the short arm of chromosome leads to a syndrome called Cri du chat. For detecting the

deletions and other chromosomal alterations, the conventional CGH plays a suitable role.

For example: A cat-like cry was reported with an infant Levy et al in 2002[16].

This cat-like cry was due to the indistinct karyotype of chromosomes. The loss of chromosomal material from 5p 15.3 was diagnosed by the Conventional CGH. From this it is proved that the conventional CGH is trustable for analysing the structural aberrations as well as many complex abnormalities in the chromosomes [16].

4. Genomic abnormalities in cancer

The alterations and rearrangement of genes frequently occur in cancer and leads to the pathogenesis. For detecting such alterations the array CGH is used which gives the information about the location of genes which cause cancer and for the classification and prognostication of cancer.

All genetic material losses do not lead to the pathogenetic. Some DNA material is lost physiologically during the immunoglobulin gene rearrangement.

Recently the chromosomal aberrations are identified in many mouse models which were subjected to the breast cancer by the array CGH technique.

It has been detected that the cooperating genes are involved during myc induced oncogenesis. The array CGH is not only applied for detecting the abnormalities in chromosomes but also in research studies in diagnosing the development of tumour [17].

5. Sub-microscopic aberrations

The array CGH is a sensitive technique in diagnosing the sub-microscopic aberrations. Chromosomal imbalances, especially when associated with additional malformations or developmental delay in chromosomes leads to the congenital heart defects. Such aberrations can be identified with high resolution cytogenetic test like array CGH [18].

6. Prenatal genetic diagnosis

The pre-implantation genetic screening is becoming a popular idea which uses the array CGH as a diagnosing tool. The aneuploidy in eggs, sperms and embryos are detected by the array CGH. It reduces the life altering conditions and also improves the rates of successful IVF treatments [7, 19, 20].

2.7. Limitations of CGH and array CGH

CGH's inability to detect structural chromosomal aberrations without copy number changes, such as balanced chromosomal translocations, mosaicism, and inversions made it limited to use. Relative to ploidy level, CGH can only detect gains and losses [21].Also, as chromosomal regions are highly variable between the individuals due to the presence of short repetitive DNA sequences, they can interfere with CGH analysis [9]. So, to avoid this interference, repetitive regions of DNA like telomeres and centromeres are blocked with Cot1 DNA or any other unlabelled repetitive DNA and can be excluded from screening [22].

Moreover, because of the limited resolution of conventional CGH, its clinical applications are limited. Even due to the limited resolution of conventionalCGH, it is a reliable and useful technique in the diagnostics and research of both human genetic disorder and cancer, but, it involves bulky aberrations. If aberrations are smaller than 5-10 Mb, they cannot be detected by conventional CGH.

As the conventional CGH cannot detect such aberrations, array CGH, which is a high-resolution technique can overcome many such limitations. The major advantage of array CGH is its high resolution as compared to conventional CGH. Array CGH has a standard resolution between 1 and 5 Mb, but, if the array is supplemented with extra clones, the resolution can be increased up to 40 kb. However, like conventional CGH, array CGH has the inability to detect aberrations that do not result in copy number changes and is limited in its ability to detectmosaicism[9].It is the sensitivity and spatial resolution of clones on the basis of which the level of mosaicism can be detected. At present, the detection limit is 50% of the rearrangements present in cells. To detect such aberrations, other techniques, such as FISH or SKY (Spectral karyotyping) have to be still used[23].

3. DISCUSSION

Observation

The test sample and reference sample are visualised under the fluorescence microscope to detect the gain and loss of nucleotides in the test sample as the compare of reference sample. By using the commercial dedicated CGH software, the gain and loss of nucleotides from the test sample can be detected in the form of graph. It shows two different colours of line which are red and green.

The ratio of green to red fluorescence value is used to quantitate genetic imbalances in test (tumour) sample.

CGH analysis software measures the fluorescence intensity values along the length of the chromosomes and translates the ratios into chromosome profiles.

If the amount of test DNA and reference DNA is same and if this is perfect hybridization, then hybridization of resultant sample will be in the ratio of 1:1.

If there is any duplication of nucleotide sequence in the test sample, then CGH software shows the high concentration of test sample then the reference sample. So that the intensity of the green fluorescence will increase towards the 1.25.

If there is any deletion of nucleotide sequence in the test sample, then CGH software shows the low concentration of test sample then the reference sample. So that the intensity of the red fluorescence will decrease towards the 0.75.

4. CONCLUSION

The conclusion we can draw from CGH is, it is a widely used technique in the field of cancer and chromosomal aberrations. In cancer research CGH technique is used to determine the chromosomal gains and losses. For example, gains of chromosomal regions lq, 3q and 8q, as well as losses of 8p, 13q, 16q and 17p, are common for a various type of tumor, such as breast, ovarian, prostate, renal and bladder cancer. Other examples are testicular cancer in which 12p and Xp chromosomes are gained, 13q gain 9q loss in bladder cancer, 14q loss in renal cancer and Xp loss in ovarian cancer [15]. In research and diagnostics of B cell malignancies, Array CGH is also used, such as chronic lymphocytic leukaemia. Chromosomal aberrations like Cri du chat, Prader-Willi syndrome, Wolf-Hirschhorn syndrome etc, can also be detected by this technique. The partial deletion of the short arm of chromosome 5 causes Cri du chat (CdC). The deletion of the short arm of chromosome 4 causes Wolf-Hirschhorn syndrome. The loss of some functional genes from chromosome 15 is lost. This leads to Prader-Willi syndrome.

For Prenatal genetic diagnosis conventional CGH is not used much. Instead, a more specific and advanced technique called array CGHfor pre-implantation genetic screening. Copy number variations and aneuploidy in sperms, eggs or embryos lead to failure of successful implantations or chromosomal aberrations like Down's syndrome. Array CGH has the ability to detect such CNVs and aneuploidy. This can improve success rates of IVF attempts.

If CGH and FISH are to be compared then the former is widely used because it saves labour and expense.

Though CGH has many advantages, it has many limitations as well. CGH has a limited resolution that limits its clinical applications. Chromosomal aberrations smaller than 5-10 Mb cannot be detected using by CGH. A major disadvantage of CGH is that it is not able to detect structural chromosomal aberrations without copy number changes, such as balanced chromosomal translocations, inversion and mosaicism.

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