Classical and Molecular Cytogenetics: The Principle and Application of Involved Techniques

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Abstract. Cytogenetics is the study of chromosomes. The involved techniques have an indispensable role in chromosome aberration detection. Taking the invention of in situ hybridization (ISH) as a dividing line, all techniques are categorized into classical and molecular cytogenetics. Typical techniques in classical cytogenetics are chromosome banding and flow karyotyping. Branches of ISH, such as fluorescence in situ hybridization, spectral karyotyping, and comparative genomic hybridization, are often classified as molecular cytogenetics. All of these techniques have provided researchers with either quantitative or qualitative data to aid in the analysis of chromosomes and have made significant contributions to the fields of prenatal detection, prognostic, diagnostics, oncology, etc.

Keywords: chromosome, karyotype, cytogenetics, chromosome banding, flow karyotyping, fluorescence in situ hybridization, spectral karyotyping, comparative genomic hybridization.

1. Introduction

Prior to the dawn of cytogenetic techniques, little was known about chromosome-related diseases. Research and analysis can only be done by observing the approximate length, size, and centromere position of metaphase chromosomes through a microscope. As a result, a formidable barrier was built in diagnosing chromosomal disorders due to the causes and characteristics of many abnormalities that were not understood then. Back to the present, the development of classical and molecular cytology has established a relatively complete system for chromosomal analysis in both quantitative and qualitative means, which has effectively assistance the diagnosis of chromosomal aberrations and the understanding of the mechanism of tumor development. In the following paper, the principle and application of typical techniques involved in classical and molecular cytogenetics are introduced.

2. Classical Cytogenetics

2.1. Chromosome Banding

Chromosome banding is a technique used to stain chromosomes for karyotyping and abnormality identification. Before the development of the banding technique, chromosomes were classified according to their size and the location of the centromere. This often leads to confusion when abnormalities occur in chromosomes. For example, Chromosome 1 with a deletion at the end of the short arm could be mistaken for Chromosome 2. Such a problem was solved after the advent of the chromosome banding technique. This technique demonstrates the unique banding patterns of human 23 pairs of chromosomes and assists in classifying these chromosomes. Furthermore, the banding technique helps detect chromosome aberrations by comparing the bands on abnormal chromosomes with those on normal. Several types of chromosome banding techniques were developed for different circumstances. They are, for example, Q-, G-, R-, and High-resolution banding.

2.1.1. Q-banding

Before staining chromosomes, cells must be proliferated by adding mitogen to initiate cell division [1]. One of the excellent cell sources is peripheral blood lymphocytes due to their rapid dividing speed and easy accessibility, which can be obtained from blood.

The extraction of the most condensed chromo-some from metaphase was needed for banding techniques to proceed. This is achieved by applying agents that interfere with the formation of mitotic spindle apparatus, such as colcemid or colchicine, to the cultured cells[2]. The resultant cells were further cultivated with a hypotonic solution and acetic acid (fixative) in order to preserve the chromosomes and spread them out for better labelling when the turgid cells burst inside a microscope slide [3].

In Q-banding, chromosomes were stained with a fluorochrome called Quinacrine mustard[4]. When viewed under a fluorescence microscope, the chromosomes exhibit alternating glowing (positive) bands on AT (adenine and thymine) -rich regions. Q-banding is especially helpful in distinguishing the human Y chromosome and identifying X-Y or Y-autosome translocation [5]. However, as fluorescence gets quenched easily, timely photos are required for karyotyping after banding [6].

2.1.2. G-banding

The pretreatment of G-banding is very similar to that of Q-banding. The main difference is the spread chromosomes were stained with Giemsa and were treated with trypsin before staining [7]. On chromosomes, alternating dark and light bands form, with the dark (positive) bands in the AT-rich region and the light (negative) bands in the GC-rich region. From the position of staining, G-banding is similar to Q-banding. Yet, the former is more commonly used nowadays because due to its absence of fluorescence, stains can be preserved for a more extended period and do not necessarily need a fluorescence microscope to observe [8]. Furthermore, G-banding exhibits more distinct bands than Q-banding does.

G-banding is widely applied in karyotyping and prenatal detection. It contributes to identifying aneuploidy and structural abnormalities such as deletion, insertion, and translocations [9].

2.1.3. R-banding

R-banding gives the reverse result of G-banding. The dark bands produced by R-banding are located in the GC-rich region, and the light bands are located in the AT-rich region. This is due to the different pretreatment of the two banding techniques. In R-banding, chromosomes were treated with hot buffer solution before being stained with Giemsa. Because the melting point of the AT regions is lower than that of the GC regions, denaturation of these regions occurs during incubation [10]. This results in the AT regions of chromosomes having lighter bands.

R-banding is commonly used to determine abnormalities related to telomeres because telomeres are abundant in GC and emphasized as dark bands after staining. Chromosomal aberrations like *Jacobsen syndrome* (11q terminal deletion disorder) can be detected through this technique.

2.1.4. High-resolution banding and others

High-resolution banding uses Wright stain to mark chromosomes extracted from late prophase and prometaphase where they are less supercoiled [11]. High-resolution banding allows more bands to appear on chromosomes than other banding techniques. This enables the examination of more subtle chromosomal structural aberrations like *Prader-Willi syndrome* (submicroscopic 15q11-q13 deletions) [12].

The four banding techniques introduced above are either typical or most commonly used. Other than these, there are also C-banding, T-banding, NOR banding, DAPI staining, etc. Each of them has a different principle and specialized applications. Detailed information for these techniques is summarized by Wegner in *Diagnostic Cytogenetics* [10]**.**

2.2. Flow Karyotyping

While chromosome banding techniques provide visual comparisons between bands labelled on the chromosomes, the flow karyotyping technique produces qualitative data and graphs for genome analysis.

Supercoiled chromosomes in metaphase were the analyte of this technique, usually extracted from cultured lymphocyte cells. Similar to G-banding, flow karyotyping also incubated cells with trypsin and colcemid to hinder the formation of spindle apparatus for obtaining metaphase cells[13]. Chromosomes were liberated from these cells using a syringe after the incubated cells were cultured inside a hypotonic chromosome isolation buffer and lysed by *Triton X-100*. HO and CA3 staining were performed on the freed chromosomes. The sample had sodium citrate added to enhance the resolution of the flow karyotype [14].

Two laser beams, one of which is 458nm and the other is ultraviolet, hit the stained chromosomes to excite CA3 and OH, respectively. Two kinds of fluorescence were released in this process and were shot onto two different photomultipliers. The result was amplified, digitalized, and then integrated into a graph, namely HO-versus-CA3 flow karyotype [15].

Flow karyotyping is applied in prenatal detection to identify chromosomal aberrations. Abnormalities can be detected by analysing both peak volume and peak location of chromosomes shown in the HO-versus-CA3 flow karyotype [16]. For example, a patient with trisomy 21 would have a peak volume of chromosome 21 approximately 50% greater than the normal chromosome 21. New peak locations appeared on the flow karyotype when translocation occurred and created derivative chromosomes.

However, flow karyotyping is incapable of sensing minor aberrations in chromosomes and has a relatively high false-positive rate if nuclear debris is not thoroughly filtered during the chromosome extraction process [16].

3. Classical Cytogenetics

3.1. Fluorescence in situ hybridization

The limited resolution has always been an obstacle for classical cytogenetics to diagnose submicroscopic aberrations of chromosomes. An advance in viewing chromosomes at a molecular level is achieved by the introduction of *in situ hybridization* (ISH) [17]. *Fluorescence in situ hybridization* (FISH), a variant of ISH, was then developed with the same basic principle, except FISH replaced radioactive isotopes with fluorescence probes as a marker to label chromosomes [18]. Its safety and high resolution allow it to be used in the diagnosis of various chromosomal aberrations.

The basic principle of FISH begins with the preparation of the slide. Fresh tissue is affixed to a microscope slide, and acetic acid or methanol is added to the sample to preserve morphology. The proteins embedded in the DNA sequences are extracted by treating with HCl, which enhances the infiltration of the probe and decreases the background noise. Denaturation of the sample DNA is conducted on the slide at approximately 70°C. Probes with either fluorescein (for direct labelling) or hapten (for indirect labelling) are added and hybridized with the sample DNA at 37 °C for 6–12 h [19]. The slide is washed to remove unbound probes and mounted in an antifade solution. Labelled chromosomes can be observed with a fluorescence microscope [20].

Fluorescence in situ hybridization has an indispensable role in the field of prognostic and cancer cytogenetics. With centromeric enumeration probes (CEP), FISH is appliable to demonstrate aneuploidy abnormalities such as trisomy 21 (Down syndrome) and monosomy X (Turner syndrome) [21]. Moreover, since there is no need to culture cells, FISH can be used to analyse cells that are difficult to culture, like solid tumor cells. Cancer, for instance, HER2 breast cancer, can be identified via hybridizing erythroblastic leukemia viral oncogene with fluorescence probes [22].

3.2. Spectral karyotyping

Spectral karyotyping, abbreviated SKY, is a technique of labelling all 24 human chromosomes with different colors. A theoretical number of 31 colors can be synthesized among the five fluorescent dyes, *Spectrum Green*, *Texas Red*, *Cy 2*, *Cy5.5*, and *Cy5***.** After removing the combination of similar colors and colors within the infrared wavelength, the remaining 24 colors were individually made as fluorescence probes. Each probe is responsible for attaching to one of the normal 24 human

chromosomes only [23]. Metaphase chromosomes were hybridized with these probes in the dark. The spectral karyotype (image of all colored chromosomes) was then captured with a two-dimensional imaging spectroscopy system [24].

With spectral karyotype, SKY has dramatically improved the efficiency of discriminating different chromosomes and has been examined to be extremely helpful in identifying chromosomal aberrations such as substitution and translocations where the derivative chromosome appears in two or more different colors [25]. However, any mutations that occur without interaction between different chromosomes, for example, inversion and duplication, are difficult to be identified visually through color because they result in no color change of the chromosomes [26].

3.3. Comparative genomic hybridization

Due to the fact that spectral karyotyping requires the cultivation of metaphase cells and is insensitive to some aberrations like duplication, a new technique named comparative genomic hybridization (CGH) was developed and overcame these limitations.

The basic principle of CGH is to identify copy number changes in chromosomes by comparing the fluorescence intensity of tumor DNA and normal DNA [27]. The tumor DNA is extracted from the patient and is labelled with green fluorochrome. It is then hybridized with normal DNA obtained from a health volunteer labelled with red fluorochrome. After this process, two different fluorescence colors, green and red, are emitted in a ratio. If the ratio is greater than 1, more probes have been bound to the tumor DNA compared with the normal DNA. It indicates that duplication occurs in the chromosomes. On the contrary, if the ratio is smaller than 1, fewer probes are bound to the tumor DNA, suggesting that the patient suffers from a deletion in chromosomes[27-29].

CGH has made substantial contributions to the analysis of solid tumors, especially malignant cells because no metaphase cell culturing is needed in this technique. The screening of chromosome aberrations has helped for a better understanding of subsets of cancers and tumor progression [30]. Yet, the limitation of CGH is that it cannot detect abnormalities in which the copy number of DNA sequence is not altered, such as balanced translocation and inversion [31].

4. Conclusion

Over the course of six decades, cytogenetics has evolved from the rough analysis of chromosomes monochromatically to the extent to which aberrations can now be detected at the molecular level. The advent of classical chromosome banding techniques has visually assisted in chromosome classification and simple aberrations detection. Flow karyotyping is another technique used in classical genetics, which examines abnormal chromosomes by qualitatively comparing the peak location and peak volume of both normal and abnormal chromosomes. Its advantage is its fast-detecting speed, but it also has a relatively high false-positive rate. With the development of in situ hybridization, molecular cytogenetics has made it easier to diagnose chromosomal aberrations at the submicroscopic level. Variants of ISH like FISH, SKY, and CGH shares the basic principle of hybridizing the target DNA sequence with safe fluorescence probes, yet they provide outcomes in either visual or digital means. For instance, SKY diagnoses abnormalities by observing the colors of the chromosomes, while CGH compares the fluorescence intensity emitted from normal and abnormal DNA sequences.

It is worth noting that the techniques presented in this paper are far from the complete picture of cytogenetics. Some researchers have developed C-banding and DAPI staining techniques based on chromosome banding, and some researchers have combined the advantages of classical and molecular cytogenetics for use in specific circumstances. Nevertheless, each of these technologies contributes greatly to prenatal testing, prognostic, diagnostics, oncology and become an indispensable presence in these fields. As more safe, efficient, high-resolution technologies continue to be developed, the mysteries of chromosomes and tumors are going to be unravel.

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