Fluorescence In-situ Hybridization (FISH) and Its Application in Oncology

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Abstract. Fluorescence in situ hybridization (FISH) technique was developed in early 1970s for DNA sequence identification. FISH has gained wide application in cytogenetic research, thanks for its high sensitivity and specificity. FISH analysis has also become a powerful tool for cancer research and treatment, through detecting genetic aberrations in cancer cells. Compared to other cytogenetic tests, FISH analysis is fast and compatible with various cell types. It is particularly useful under clinical settings, by providing crucial information of chromosomal abnormalities, such as gene microdeletion, amplification, and translocation, to guide cancer therapy selection, and monitor effectiveness of the treatment. For example, FISH is used as a companion diagnostic tool for non-small-cell lung cancer (NSCLC) patients with ALK or ROS1 gene rearrangement. It's also a confirmative test for breast cancer patients with HER2 abnormality. Through combination with other novel technologies, newer versions of FISH have been developed to improve the throughput and sensitivity, which will further expand the potential of FISH analysis in fighting cancer.

Keywords: Fluorescence in situ hybridization, FISH, M-FISH, SKY, CGH, ALK, ROS1, HER2.

1. Introduction

Cancer represents a group of diseases characterized by uncontrollable cell growth and division. In its later stage, the abnormal cells can invade neighboring parts of the body, spread to other organs, and eventually cause death. The abnormal growth and multiplication of cancer cells are often attributed to their gene defections, which can be inherited from parents, caused by errors during cell replication, or damaged by harmful substances in the environment. Regardless of the origin, understanding of genetic abnormality would be essential for cancer diagnosis, treatment, and development of novel cancer therapies.

Fluorescence in situ hybridization (FISH) is a molecular cytogenetic technique developed in the late 1970s for localization and identification of presence of a specific DNA sequence on cell chromosomes [1]. Compared to conventional method of cytogenetic metaphase karyotype analysis, FISH is less time-consuming. It has been widely applied in genetic diseases detection, gene mapping, and identification of chromosomal abnormalities. In oncology area, FISH was applied to detect the genetic mistakes of cancer cells, such as chromosomal microdeletion, amplification, and subsequently, translocation. This information is used for cancer diagnosis, prognosis, predicting response to therapy, and surveillance. This article aims to provide overview of FISH's mechanism, discuss its expanding usage in oncology, and highlight some new progress of this technique.

2. Fluorescence in situ Hybridization (FISH) Analysis

2.1. Mechanism and procedure of FISH

Fluorescence in situ hybridization (FISH) is a macromolecule recognition technique based on the complementary nature of DNA or DNA/RNA double strands which was revealed by James Watson and Francis Crick in 1953 [2]. Through the extensive hydrogen bonding effect between the nucleotides, the two antiparallel strands of DNA are held together to form stable helix. If treated with heat or certain chemicals, the double strands DNA can be broken apart into two single stand DNAs. However, as the condition becomes favorable, the double helix can re-form from two single strand molecules with appropriate base-paring. This cross-binding process is called molecular hybridization.

Through hybridization, a labeled DNA or RNA sequence can be used to not only identify presence of the complementary sequence in a biological sample, but also provide its location information at the same time. This idea was first demonstrated in 1969 [3]. By using radioactive labelled ribosomal DNA as the probe, the complementary DNA sequence was identified in the nucleus of a frog egg. However, application of isotopic hybridization is quite difficult due to following factors. The isotope decays along time so the specific activity of the labeled probe is constantly decreasing. Radiography is generally known for high sensitivity but low special resolution. In addition, radiography film exposure is time-consuming, and handle of radio-active material must follow complicated safety measures to comply with stringent regulation. These intrinsic drawbacks of isotopic labeling encouraged exploration of new techniques. Soon after that, helped by commercialization of fluorescence microscopes and development of confocal microscopy technology, fluorescent labels [4] quickly take the lead position among hybridization labels because they are safe, stable, convenient for handling, and easy for detection. Inserted with fluorophore-coupled nucleotides, specific DNA/RNA sequence can be used as probes in hybridization, after which the complementary counterparts in tested cells and tissues can be visualized under a fluorescence microscope.

FISH analysis requires appropriately probe molecules, which are short complementary DNA or RNA sequences of the targeted gene, labeled with fluorophores. Three approaches, including PCR labeling, nick translation, and random oligo primed synthesis are commonly used for labeling of the probes. PCR labeling is relatively straight forward, achieved by using a labeled dNTP during PCR. Nick translation is a technique enabling direct modification of a specific DNA sequence. Utilizing DNase, I, Nicks, which are single strand breaks, are introduced to the DNA backbone. The following treatment of DNA polymerase I removes nucleotides and replaces them with modified dNTPs, giving the new DNA sequence as a labeled probe. Random oligo primed synthesis goes through three steps. First, DNA is broken into single strands, and then hybridized with random hexamer oligonucleotides to form a double strands DNA with many breaks, to where finally labeled nucleotides are introduced with the help of DNA polymerase.

In a typical FISH procedure, sample cells, such as formalin-fixed paraffin-embedded (FFPE) or frozen tissue sections, are fixed on the glass slide through treatment of formaldehyde or paraformaldehyde solutions. After fixation, the sample cells are treated with mild detergent to enhance tissue permeability for hybridization reagents. Next, both the labeled probe and the specimen DNA are denatured through treatment under proper temperature, pH, and salt concentration. Then the probe and the sample are mixed together and cooled down to allow the probe to hybridize specifically to its complementary DNA sequences on the chromosome. After that, all unhybridized probes are washed away and the processed sample is visualized under the fluorescence microscope. The probe sequence can be labeled either directly or indirectly. In contrast to direct labeling using nucleotides containing a fluorophore, indirect labeling strategy involves modified nucleotides not fluorescence active but with a moiety to which a fluorophore can be attached afterwards, usually through an enzymatic or immunological process. Finally, the site of hybridization can be detected under the microscope.

2.2. Advantages and Limitations of FISH

Due to its significant advantages, FISH technology has greatly contributed to cytogenetic analysis and pathology laboratory's growing potential [5, 6]. FISH analysis has high sensitivity, specificity and reproducibility, so the result interpretation relies less on subjective judgement. It is often applied as a useful tool supporting interpretation of results from karyotyping and arrays. Compared to other techniques, it has shorter procession time from 4-24 h with high efficiency. This provides critical cytogenetic pattern information of tumor cells in a time frame short enough for clinical decision making.

Another particular advantage of FISH technique relies on its capability to obtain cytogenetic information from nondividing or terminally differentiated cells. Interphase FISH can be performed on FFPE slides from tumor material, providing information on fusions, amplification and deletions

without the need for cell culture. FISH can retrieve cytogenetic information from relatively inadequate specimen, such as post-treatment samples or hypocellular samples, which may contain insufficient cells for routine cytogenetic analysis. It also allows direct correlation of cytogenetic and morphological features, and enable simultaneous assessment of genetic aberrations, cell phenotype, and microstructure of the sample. In addition, FISH can detect whole genome ploidy changes and mosaicism.

There are also limitations of FISH can't be ignored [5, 6]. Most notably, as a targeted test, its application is restricted to the abnormalities that are detectable with currently available probes, which limits the utilization as a screening tool for cytogenetically heterogeneous diseases. Meanwhile, in one experiment only limited number of probes can be used with only two or three colors possible. In addition, FISH is demonstrated to be highly sensitive of detecting chromosome gain but less sensitive for chromosome loss or deletion. On the technical side, it is labor intensive and experienced analysts are required for high quality image interpretation. It also requires expensive fluorescence microscopy system and image analysis software.

2.3. Advanced Modifications of FISH

To overcome the throughput limitation of traditional FISH, Spectral Karyotyping (SKY) [7] and Multiplx-FISH (M-FISH) [8] were independently developed. SKY and M-FISH use different methods for image acquisition and analysis, but both techniques utilize 24-color probe sets for unique colorization of each chromosome, enabling distinct visualizations of chromosomes. The mixtures of chromosome-specific probes are used in hybridization with metaphase chromosome spreads. Chromosome translocations are easily observed when a different color shows on a chromosome, and the second color can direct the source chromosome participated in the translocation. However, due to the same mechanism, SKY and M-FISH are not designed to detect intrachromosomal rearrangement.

Another breakthrough of FISH technique is the introduction of comparative genomic hybridization (CGH) [9], which expand the capability to perform genome-wide screening. Unlike standard FISH, CGH requires isolation and fragmentation of DNA from both the specimen and a control sample. The fragmented control DNA is dyed in green, and the fragmented experimental DNA is dyed in red. Equivalence amount of each DNA samples are then fused and co-hybridized with normal chromosomes. The resulting different green: red fluorescence ratio indicates difference in DNA content from specimen and control sample. For chromosomal regions without genetic changes, the green and red probes should have the equal chance to bind, then the observed fluorescence would be an equal blend of red and green, showing an orange/yellow color. If there are chromosomal deletion on the DNA copy from the specimen, that region would be greener under the microscope. On the contrary, if there are chromosomal amplifications on the specimen DNA, that region would appear redder under the microscope. Following this rule and with digital image analysis, genomic alterations can be identified through comparing the color difference along the length of the chromosomes.

The capability of CGH was further expanded through the combination with microarray technique [10]. Instead of using metaphase chromosomes, array CGH method uses a microchip covered by arrays containing a large number of DNA fragments of human genome. Each spot of the microarray contains an individual DNA fragment, corresponding to a specific DNA sequence which has been well identified and located on human chromosome. Similar as standard CGH protocol, two sets of fragmented DNA probes, control and specimen, are labeled with green and red fluorescence respectively, and the used in co-hybridization with the micro array. The microchip is finally scanned with an automated system and the image is analyzed though a software. Same color-code as the standard CGH is applied for the array version. Orange/yellow color is resulted from equal contribution of green and red probes, representing an unaltered chromosome region. Meanwhile, red color suggests an amplified region, and green color means a deleted region on the specimen chromosome. Like running thousands of standard FISH experiments simultaneously, array CGH enables the researchers to achieve high throughput and high resolution to identify gene amplification

or missing. This strength makes array CGH as a perfect partner for SKY or M-Fish, which is most capable in defining gene translocation.

3. Application of FISH in Oncology

Thank for the high specificity and sensitivity, chromosomal aberration detected by FISH can provide powerful guidance for precision cancer treatment. A few examples of FISH application are summarized below.

3.1. ALK FISH for NSCLC

Lung cancer is the leading cause of cancer-related deaths worldwide. The American Cancer Society predicts 127,070 deaths from lung cancer in US for 2023 [11]. Non-small cell lung cancer (NSCLC) accounts for 85% of all lung cancers, and about 3-7% of NSCLC patients carry anaplastic large-cell lymphoma kinase (ALK) rearrangement. While the medical community obtaining more molecular mechanistic understanding in the linkage between NSCLC and ALK fusion, ALK inhibitors, such as crizotinib, certinib, alectinib and brigatinib, were developed as personalized medicines for this class of cancer. It worth to mention that, when Crizotinib, the first target drug for ALK rearranged NSCLC was approved by the United States Food and Drug Administration (FDA) in 2011, the Vysis ALK FISH Probe Kit (by Abbott Molecular) was approved concurrently to diagnose potential patients for crizotinib therapy. This combined treatment has been standard protocol in ALK rearrangements identification, but recently a broader molecular profiling, including IHC, real time PCR, and NGS are recommended for their higher diagnose frequency and precision in ALK fusion identification [12].

3.2. ROS1 FISH for NSCLC

ROS1 gene is another distinct molecular target in NSCLC diagnosis and treatment. Genomic rearrangements of ROS1 represent about 2% of NSCLC cases [13]. A transmembrane receptor protein encoded by ROS1 expressed traits homologous to the intracellular kinase domain and ATP binding site of ALK. The structural similarity encouraged development and approval of using crizotinib, a known ALK inhibitor, to treat ROS1 positive NSCLC patients. In FISH assay for ROS1 gene rearrangement, separation of a break-apart probe is triggered by splitting ROS1 gene, causing the probes to display distinct colors. Patient is classified as ROS1 positive when ROS1 gene rearrangement is observed in at least 15% of the analyzed population of nuclei. According to NCCN recommendation, the patient should take ROS1 rearrangement test before the corresponding kinase inhibitors are prescribed [14]. ROS1 FISH is used as the second line test to confirm a ROS1 rearrangement suggested by ROS1 over expression on IHC studies.

3.3. HER2 FISH for Breast Cancer

According to WHO, breast cancer caused 685000 deaths globally in 2020 [15]. In 20% to 30% of human breast carcinomas, the level of human epidermal growth factor receptor 2 (HER2) is higher than normal. As a tyrosine kinase, the HER2 protein has important function in normal cell growth and differentiation. Over expression of HER2 in breast can cause uncontrollable cell growth and multiplication, and finally lead to tumor. Trastuzumab is a humanized monoclonal antibody approved by the FDA in 1998 for HER2 positive breast cancer therapy. In such personalized medicine approach for breast cancer clinical management, HER2 status becomes an important predictive factor. Evaluation for HER2 condition in routine clinical specimen involves immunohistochemistry (IHC) for protein expression and FISH for gene amplification. In clinical, IHC is usually applied as the primary test for HER2. However, samples from breast cancer patients with 2+ IHC scores are recommended to be additionally examined with FISH to improve accuracy and reliability for HER2 assessment [16].

4. Conclusion

In summary, since its origination in 1970s, FISH has grown to be a powerful tool for the detection of chromosomal abnormality and changes in gene expression. Thanks for the high sensitivity to identify specific genetic aberrations, FISH has demonstrated its value in both research and diagnostic applications for oncology. In particular, FISH analysis of interphase cells enables the direct detection of recurrent chromosomal abnormalities and clonal evolution, thus providing critical information to classify different type of tumors, select proper therapies, and monitor effectiveness of the treatment. Through integration of microarray technique, super-resolution imaging system, and more powerful data analysis software, newer versions of FISH become more efficient and disease specific. In combination with other cytogenic analysis, FISH will continue to play a crucial role to battle with cancer.

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