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Application of Ki-67 Immunofluorescence in Tumors

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Abstract: A tumor is an abnormal mass of tissue resulting from uncontrolled cell division, and their ability to continue to proliferate is the key to determining their biological behavior and prognosis. Therefore, assessing cell proliferation status is of great significance in tumor research and clinical diagnosis. Ki-67, a nuclear protein closely related to cell proliferation, is expressed in the G1, S, G2, and M phases of the cell cycle, but is absent in the quiescent G0 phase. This characteristic makes it an ideal molecular marker reflecting cell proliferation activity. Immunofluorescence (IF) is a commonly used method for detecting and localizing Ki-67. In this study, we performed Ki-67 IF on hepatocellular carcinoma (HCC) and paracancerous tissues to assess proliferation capacity. The results showed that the proportion of Ki-67-positive cells in HCC tissues was significantly higher than that in paracancerous tissues, and most of them were located in the cell nuclei; only a few cell nuclei in paracancerous tissues were weakly positive. Then we explored the feasibility of applying Ki-67 IF in oncology research and discussed limitations in advancing cancer research.

1. Introduction

Cancer is a disease characterized by abnormal cell proliferation that can occur in any organ or tissue of the body. Normal cells are strictly regulated in terms of growth, division, and death, but cancer cells often lose this regulatory ability and not only continue to proliferate but also evade normal programmed cell death (such as apoptosis) [1]. This uncontrolled growth causes cancer cells to cause double damage to the body: on the one hand, they invade normal tissues and destroy their original structure and function; on the other hand, cancer cells consume a large amount of nutrients and energy in a high metabolic state, competing with normal cells for resources, causing the latter to be energy-deficient and their mitochondrial function to be impaired, ultimately further inhibiting their survival and proliferation. This imbalance will drive the continued expansion of cancer cells, forming a vicious cycle that is difficult to contain [2]. Because of the characteristic, assessing their proliferation status has become a key step in understanding tumor biology, assisting in tumor classification, and predicting prognosis. To better understand and evaluate this abnormal proliferation, reliable molecular markers are essential. Ki-67 is one such marker that reflects the proliferative activity of tumor cells.

Ki-67 was first identified as an antigen that is specifically localized in the cell nucleus in a study

of cell cycle-related proteins. Its name comes from the antibody number originally used ("Ki" stands for the University of Kiel, Germany, and "67" stands for the clone number). Early studies found that Ki-67 is highly expressed in proliferating cells and is almost undetectable in quiescent cells. Therefore, it is considered an ideal marker for identifying the state of cell proliferation [3]. Subsequent functional studies further revealed that Ki-67 is not just a passive cell cycle indicator protein, but plays a direct role in chromosome dynamics. During mitosis, Ki-67 is evenly coated on the outer layer of chromosomes, forming a structural barrier similar to a "biosurfactant" that helps maintain the physical separation between chromosomes and prevents them from entanglement and fusion. This role is crucial for ensuring accurate separation of chromosomes in the late stage. Once Ki-67 is lost or underexpressed, cells will experience chromosome condensation defects, sister chromatid separation errors, and other phenomena, leading to genomic instability and impaired cell proliferation [3]. In terms of cell cycle distribution, the expression of Ki-67 protein shows highly specific phase characteristics: it can be detected in the G1, S, G2 and M phases, but is completely absent in the quiescent G0 phase [4]. The G0 phase is a dormant phase in which cells are in a non-dividing state. Some cells in normal tissues maintain tissue homeostasis and functional reserve by entering the G0 phase, while tumor cells often enter the G0 phase less frequently due to the loss of cycle regulation ability, thus showing a higher replication frequency and sustained proliferation drive [4]. This difference makes Ki-67 not only an important molecular marker for distinguishing the proliferation state of tumor cells from normal cells, but also able to reflect the replication activity level of the cell population to a certain extent. In addition, the Ki-67 expression index (Ki-67 labeling index) is often used as an indicator to measure the proportion of cell populations in the proliferation phase. By combining the cell cycle duration, the cell division rate and proliferation potential can be indirectly inferred [5]. This characteristic has led to the widespread application of Ki-67 in many fields, including tumor pathological diagnosis, prognosis assessment, and treatment response monitoring, and it is closely related to clinical outcomes in a variety of malignant tumors [5].

Immunofluorescence (IF) staining is one of the main techniques used to detect Ki-67 protein expression. IF is a method that adds specific antibodies to tissue sections and uses fluorescent markers (such as fluorescein, rhodamine, or near-infrared fluorescent dyes) for visualization. The fluorescence signal can be directly observed by fluorescence microscopy, and the fluorescence intensity is closely related to the expression level of the target protein. Since Ki-67 is widely expressed in many tumors and can reflect the proliferation activity of tumor cells, Ki-67 IF has been widely used in pathology, oncology, and clinical research to assess tumor proliferation status and assist in formulating treatment plans [6]. The higher the Ki-67 index, the more active the tumor proliferation. Ki-67 is most commonly used in breast cancer and is an important biomarker for prognostic assessment and molecular typing of breast cancer patients [6]. In this study, hematoxylin and eosin (H&E) staining and Ki-67 IF staining were performed on hepatocellular carcinoma (HCC) and its adjacent tissues. H&E staining is used to observe and document basic features of tissue architecture and cellular morphology, providing morphological context for subsequent analysis; IF is used to label and quantitatively assess Ki-67 protein expression. We focused on comparing Ki-67 expression between cancerous tissue and adjacent tissues to explore its application value and significance in HCC research. We also discussed some limitations of this study.

2. Materials and Methods

2.1 Hematoxylin and Eosin staining

2.1.1 Paraffin Embedding, Slicing and slide baking

1) The process begins with fixing pathological tissues in polyformaldehyde for 18-24 hours. By

cross-linking proteins, polyformaldehyde rapidly inhibits intracellular enzyme activity, prevents tissue autolysis and decomposition, preserves cellular morphology and antigenicity, and hardens the tissue for subsequent slicing. Subsequent dehydration steps involve immersion in 80% ethanol (1 minute), 95% ethanol (1 minute), and absolute ethanol (1 minute). Tissue transparency is achieved by soaking in xylene to remove residual ethanol. Finally, melted paraffin is applied for permanent fixation. The tissue block is placed in an embedding mold with molten paraffin, cooled to solidify into a wax block.

2) A microtome is used to slice the wax block into continuous 5μm-thick sections.

3) The sections are floated and flattened in 40 ℃ water, then transferred onto glass slides for 2-hour baking in a 60℃ oven.

2.1.2 Deparaffinization and Rehydration

1) The paraffin in the paraffin slices will affect the penetration of the dye, so first you need to put the paraffin slices into xylene I for ten minutes, xylene II for ten minutes to remove the paraffin in the slices

2) After dewaxing, sections were sequentially placed in absolute ethanol I for five minutes, anhydrous ethanol II for five minutes, 95% ethanol for three minutes, 90% ethanol for three minutes, 85% ethanol for three minutes, 75% ethanol for three minutes, and distilled water for three minutes to hydrate the slices, so that they can be better stained.

2.1.3 Chromogenic reaction and observation

We first stain the tissue sections in hematoxylin for 30 seconds, then rinse them under running water for ten minutes. Next, we apply 0.5% eosin staining solution for ten seconds, followed by a ten-minute rinse under running water. We then dehydrate the samples through a graded series of ethanol solutions: 75% ethanol for 10 seconds, 80% ethanol for 20 seconds, 90% ethanol for 30 seconds, and 95% ethanol for 2 minutes. This is followed by two changes of absolute ethanol, each for 2 minutes. Subsequently, we clear the sections in two changes of xylene, each for 2 minutes. Finally, we mount the sections with neutral gum. It is important to emphasize that using reagents with gradually increasing concentrations is essential to minimize the risk of tissue damage.

2.2 IF

2.2.1 Paraffin Embedding, Slicing and slide baking

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2.2.3 Antigen Retrieval and Blocking

We perform antigen retrieval to reverse structural alterations caused by protein cross-linking and aldehyde groups introduced during paraformaldehyde fixation. This process helps restore native antigenic epitopes and improves detection sensitivity. Specifically, we apply a drop of sodium citrate retrieval solution to prevent antigen oxidation, then heat the slides in a microwave for six minutes. After heating, we let the samples cool naturally at room temperature for three minutes.

Next, we incubate the sections with 3% hydrogen peroxide at room temperature for ten minutes to quench endogenous peroxidase, alkaline phosphatase, or biotin activity, thereby reducing non-specific background staining.

We then treat the sections with PBS at room temperature for 10 to 30 minutes to block any remaining reactive sites and minimize non-specific antibody binding in subsequent steps.

2.2.4 Incubation of Primary and Secondary Antibodies

We begin by diluting the antibody using an appropriate stock solution. After removing the blocking buffer from the slides, we apply the primary antibody and incubate for 60 minutes. We then wash the slides three times with buffer, each wash lasting five minutes, to remove any unbound antibodies and reduce background.

We then incubate the slides with the secondary antibody at room temperature for 60 minutes. Finally, we perform three additional washes using washing buffer, each for five minutes, to eliminate any unbound secondary antibody.

2.2.5 Development and Observation

We apply a drop of DAB chromogenic solution to the tissue to allow color development. After staining, we cover the specimen with a coverslip and observe the results under a microscope.

3. Results

3.1 HE reveals disorganized HCC architecture

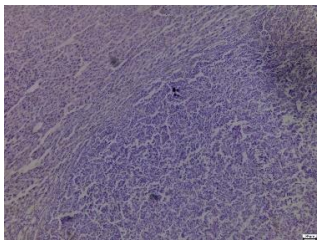

Figure 1 HE image of HCC (The lower right corner is the cancerous tissue area, the upper left corner is the normal tissue area)

We performed HE staining on liver sections from HCC and adjacent paracancerous tissue to compare their histological features (Figure 1). In the upper left region of the section, adjacent paracancerous tissue exhibited relatively regular hepatocyte arrangement, uniform morphology, relatively consistent nuclear size, and intact tissue architecture. In contrast, HCC tissue in the lower right region exhibited disorganized cellular arrangement, with darker, unequally sized nuclear staining, and associated disruption of the hepatic plate architecture.

3.2 Ki-67 IF shows high proliferative activity in HCC

Next, we used DAPI and Ki-67 double immunofluorescence staining to observe sections of hepatocellular carcinoma (HCC) and adjacent tissues (Figure 2). The blue signal marks cell nuclei, while the green signal corresponds to Ki-67 protein immunofluorescence. Overlaying the two produces a yellow-green color. A diagonal dividing line is visible in the image. In the lower left corner of the image, cancerous tissue shows densely packed nuclei, with numerous and intensely fluorescent green dots within the nuclei. Overlapping with the blue DAPI signal results in a bright yellow-green color, indicating high proliferative activity. In the upper right corner of the image, normal tissue shows relatively loosely packed nuclei, with sparse Ki-67 positive signals within the nuclei. Notably, a certain degree of green fluorescence is also observed in adjacent normal tissue, but this signal is mostly diffuse or continuously patchy and localized in the cytoplasm. This is primarily due to nonspecific antibody binding (e.g., cross-reactivity with mitochondrial proteins or the cytoskeleton), tissue autofluorescence caused by endogenous hepatocyte pigments (e.g., lipofuscin and bile pigments), and background enhancement caused by fixation and permeabilization conditions, rather than true Ki-67 expression. When interpreting, signal localization and morphological characteristics should be considered. True Ki-67-positive signals should appear as discrete dots, clearly localized within the nucleus and colocalized with the DAPI blue signal. Background signals are often diffuse cytoplasmic staining with little overlap with the nucleus. By establishing a no-primary antibody control or an isotype control, as well as optimizing imaging parameters, background interference can be effectively eliminated.

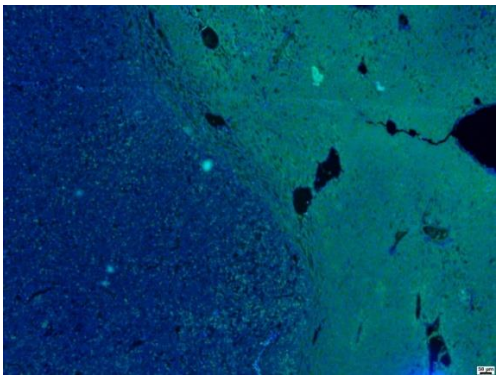

Figure 2 IF image of ki-67 (The lower left corner is the cancerous tissue area; the upper right corner is the normal tissue area)

4. Conclusion

This study aimed to systematically analyze the differences in structural characteristics and cell proliferation activity between HCC and adjacent tissues by combining morphology and immunolabeling. First, we used HE staining to comprehensively observe and record the histological structure of HCC and adjacent liver tissues, including changes in cell arrangement, nuclear morphology, cytoplasmic ratio, and sinusoidal structure, to provide morphological background

support for subsequent proliferation marker analysis. Subsequently, we used Ki-67 IF double staining to detect and compare the cell proliferation of the two tissues. Ki-67 signal-positive cells are mainly distributed in the cell nucleus, which can directly reflect the proportion of cells in the G1, S, G2, and M phases, thereby intuitively revealing the degree of cell proliferation activity in the tissue. The results of this study provide important evidence for understanding the proliferation characteristics of HCC and its differences from adjacent tissues. However, Ki-67 IF still has many limitations in practical application. First, there is no unified operating procedure and interpretation standard for this method. Different laboratories have differences in slice thickness, fixation time, antibody dilution ratio, imaging parameters, etc., which directly affect the sensitivity and specificity of the detection and reduce the reproducibility of the results [3]. In addition, the limitations of the method itself also lead to a certain degree of subjectivity in the evaluation, especially in the setting of positive thresholds and boundary judgments, different observers may come to different conclusions [4]. More importantly, Ki-67 can only reflect the proportion of proliferating cells and cannot identify tumor cells in a quiescent state. Therefore, for samples containing a large number of low-proliferation or dormant cancer cells, the true burden of the tumor may be underestimated, resulting in false negative results. This problem suggests that it is urgent to establish a unified and standardized detection system, such as measuring protein content or absorbance by spectrophotometry, and fixing the entire process of tissue processing, staining and imaging to reduce inter-experimental differences and human errors.

At the experimental technical level, when Ki-67 is used as a single detection indicator, it is easily affected by insufficient tissue fixation, insufficient antigen retrieval, and operational errors, which can cause the staining signal to weaken or the background noise to increase. The literature suggests that Ki-67 and other cell cycle-related markers can be detected simultaneously to improve the accuracy and stability of the analysis, or that AI image recognition technology can be introduced to automatically count, thereby reducing the deviation caused by human subjective factors. In addition, common technical problems in IF detection also include antibody cross-reaction and tissue autofluorescence. In terms of antibody cross-reactivity, when the primary antibody is not specific enough, it may bind to non-target antigens, resulting in non-specific signals in the cytoplasm or extracellular matrix; when the secondary antibody is at high concentration or insufficiently blocked, it may also bind to Fc receptors or other non-specific proteins in the tissue, thereby significantly increasing background fluorescence [7]. In terms of autofluorescence, tissues such as the liver are rich in substances such as lipofuscin, bile pigment, and hemoglobin. These substances have strong endogenous fluorescence in the visible light band, especially in the green light region, which can easily mask nuclear localization signals or cause cytoplasmic false positives. In addition, certain tissue structural features can also lead to local false positive signals. For example, the sticky substances on the surface of the small intestinal villi can adsorb antibodies, which appear as bright areas during imaging, thereby affecting the positive rate determination. To address these problems, a study proposed a variety of optimization solutions, including using spectral separation technology to reduce autofluorescence interference, using high-resolution image post-processing to improve the signal-to-noise ratio, or selecting near-infrared fluorescent dyes (such as Cy5) to avoid the peak of the visible light region, thereby significantly improving image quality. In addition, long-term exposure will cause fluorescence signal attenuation, affecting the accuracy of quantitative analysis; crosstalk may also occur between different fluorescence channels, especially in multiple staining, which will make the attribution and interpretation of signals more complicated. Whether there is a strict linear relationship between fluorescence intensity and Ki-67 protein expression level is still controversial. With the continuous advancement of detection technology, a variety of new strategies have been introduced to optimize Ki-67 IF. For example, nanoantibodies can reduce nonspecific binding while improving staining uniformity due to their small molecular weight and strong tissue permeability; near-infrared dyes (superior to Cy5 and Cy7) can significantly reduce the interference

of autofluorescence and improve the signal penetration of thick tissue imaging, which is suitable for three-dimensional tissue sections or in situ analysis. In addition, combined with super-resolution microscopy technology, the positioning accuracy of Ki-67 can be improved to 20 nm, thereby more accurately analyzing its fine spatial distribution pattern in chromosome binding and cell cycle regulation. Experimental experience also indicates that the formation of nonspecific cytoplasmic signals is often due to a combination of factors, including poor primary antibody quality (binding to non-target antigens), excessive antibody concentration (resulting in nonspecific binding of both primary and secondary antibodies), and tissue characteristics (such as antibody adsorption by sticky substances). These factors must be mitigated in experimental design.

In summary, Ki-67 IF not only complements HE staining, providing intuitive morphological and molecular evidence for cell proliferation assessment, but also enables more accurate differentiation between benign and malignant lesions in clinical diagnosis. In the future, with the help of quantum dot-based rapid IF technology, Ki-67 detection has the potential to enable rapid intraoperative imaging and be used to predict patient response to treatment, dynamically monitor the proliferation of circulating tumor cells, and even evaluate drug efficacy in ex vivo organ culture systems. These advances not only expand the application of Ki-67 in basic research but also provide stronger technical support for precision diagnosis and treatment in clinical practice.

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