The value of using cocktail double staining for improving accuracy of Ki-67 labeling index

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Objective: Investigate using LCA/Ki-67 cocktail double staining to improve accuracy of the counting of Ki-67 labeling index (LI) in meningeoma. Methods: A total of 29 cases diagnosed with benign meningeoma in the pathology department of the first affiliated hospital of Zhengzhou university from January 2014 to September 2014 were collected. Meningeoma tissues of all the cases were stained with the antibody LCA and Ki-67 by cocktail double immunostains, and then we use the immunohistochemical (IHC) staining for Ki-67’s single chromosome and LCA’s single chromosome. Ki-67 LI was counted under microscope and the data were analyzed statistically. Results: Expression of Ki-67 in IHC group was significantly higher than that in cocktail double immunostains group and the difference was considered statistically significant. Conclusion: The cocktail double staining can improve accuracy of the counting of Ki-67 LI; therefore, it has a certain clinical value.

1. Introduction

Meningeoma is a common benign tumor arising from meningeal tissue of the brain[1]. As one of the most commonly used index for clinical detection of cellular proliferation, Ki-67 is widely used in the diagnosis of meningeoma[2–4]. Accurate counting of Ki-67 is greatly significant in the pathological diagnosis of meningeoma. However, in practice, we often find inflammatory cells, vascular endothelial cells, fibroblasts and other cells in meningeoma tissues. As a result, usually a large number of detected Ki-67-positive cells are inflammatory cells in the proliferative phase, making it difficult to obtain an accurate counting of Ki-67 labeling index[5]. LCA refers to leukocyte common antigen and can be used to label T lymphocytes, B lymphocytes, polymorphonuclear leukocytes, monocytes and inflammatory cells. In this study, Ki-67/LCA cocktail double staining[6,7] was used on meningeoma tissue sections. Cells with both Ki-67 and LCA expressed were inflammatory cells, and cells with only Ki-67 expressed were tumor cells in the proliferative phase. The results were also compared with the counting of Ki-67-positive cells in single staining to improve accuracy of Ki-67 counting.

2. Material and methods

2.1. Clinical data

A total of 29 cases diagnosed with benign meningeoma (WHO I) in the pathology department of the first affiliated hospital of Zhengzhou university from January 2014 to September 2014, with no vascular invasion or tissue invasion, were collected. All specimens were subjected to 100% formalin fixation, paraffin embedding, and sectioning with the thickness of 3 μm.

2.2. Methods

Cocktail double staining was used on meningeoma tissue sections to detect the expression of Ki-67 and LCA. Reagent kits for immunohistochemical cocktail double staining were bought from Fuzhou Maixin Biotech. Co., Ltd. Specific steps: (1) Deparaffinizing, hydrating and rinsing three times with PBS for 3 min; adding citric acid solution (pH 6.0) for antigen retrieval at 220 W for 2 min; after the sections reaching room temperature,
rinsing three times with PBS for 3 min. (2) Incubating in peroxidase blocking solution at room temperature for 10 min before rinsing three times with PBS for 3 min. (3) Incubating in the serum of normal non-immunized animals at room temperature for 10 min. (4) Removing the serum; overnight incubation with the primary antibody (the concentration of mixed Ki-67 and LCA being 1:100) in the refrigerator at 4 °C; the second day, taking out the incubation box and putting it aside for 1 h at room temperature before rinsing three times with PBS for 3 min. (5) Removing PBS; incubating with newly labeled secondary antibody at room temperature for 25 min and then rinsing three times with PBS for 3 min before washing twice with distilled water (the secondary antibody was the mixture of equal rabbit and mouse secondary antibody newly prepared according to the number of stained sections). (6) Removing distilled water; adding dropwise adequate amount of newly prepared AP-Red solution to each section for 10-25 min color development, with shiny red meaning positive reaction; observing and controlling the degree of color development by microscope; washing three times with distilled water. (7) Removing distilled water; adding dropwise adequate amount of newly prepared DAB to each section, with dark brown meaning positive reaction; observing and controlling the degree of color development by microscope; washing three times with distilled water. (9) Removing distilled water; staining again with hematoxylin for 10-25 s; bluing in water; washing with water. (10) While sections were still wet, mounting sections with water based mounting medium; drying 30 min at 30°C. When the mounting medium was dry, allowing it cool naturally, mounting sections with neutral balsam, and then observing.

Meanwhile, immunohistochemical Elivision™ two-step method was used for the respective labeling and color development of Ki-67 and LCA so that the results could be compared with that of cocktail double staining.

2.3. Criterion for results

Single staining group: nuclei of cells labeled with Ki-67 were brownish yellow, see Figure 1. Cocktail double staining group: nuclei of cells labeled with both Ki-67 and LCA were brownish yellow; cell membranes were shiny red, see Figure 2.

Ki-67 counting was conducted in areas with more positive cells. The number of positive cells among at least 1 000 tumor cells was counted under high power field[s], excluding positive vascular endothelial cells. The percentage of positive cells was represented by labeling index (LI): LI=number of positive cells/1 000 tumor cells × 100%.

Single staining group: (1) The percentage of brownish yellow cells among 1 000 cells in a region with higher density was Ki-67 LI. Cocktail double staining group: (2) The percentage of brownish yellow cells and cells with two colors (with brownish yellow nuclei and shiny red membranes) among 1 000 cells in a region with higher density was total Ki-67 LI. (3) The percentage of cells just brownish yellow among 1000 cells in the same region was Ki-67 LI of real tumor cells.

2.4. Data collection and analysis

SPSS 17.0 software was used for statistical analysis. The results were also analyzed by Wilcoxon matched-pairs signed-ranks test and all the data were represented by (Mean±SD). When \( P<0.05 \), the difference was considered statistically significant.

3. Results

29 cases were all benign WHO I meningeoma, including 17 males and 12 females. Results showed that: both in single staining and cocktail double staining, the percentages of Ki-67-positive cells were relatively low (10%). Single staining group: (1)The average Ki-67LI was (4.66±2.70)% Cocktail double staining group: (2) The average of total Ki-67 LI (covering both cells only labeled by Ki-67 and cells labeled by both Ki-67 and LCA) was (4.59±2.56)%; (3) The average Ki-67 LI of real tumor cells (only brownish yellow Ki-67 positive cells) was (3.07±2.40)%. (1) and (2) were analyzed by Wilcoxon matched-pairs signed-ranks test and results (Z=0.272, \( P=0.785 \)) showed that the difference was considered statistically non-significant, indicating that total Ki-67-positive cells in single staining and cocktail double staining had (including tumor cells and LCA positive inflammatory cells) no difference and that cocktail double staining was stable and feasible. (1) and (3) were analyzed by Wilcoxon matched-pairs signed-ranks test and results (Z=4.482,
P<0.001) showed that the difference was considered statistically significant, indicating that after excluding inflammatory cells (both Ki-67-positive and LCA-positive cells) in cocktail double staining, the rest Ki-67-positive cells were real tumor cells.

4. Discussions

Ki-67 is a nuclear antigen that can be exclusively detected within the nucleus of proliferative cells. Its monoclonal antibody can be present in nuclei during all active phases of the cell cycle (G1, S, G2, and mitosis), but absent from resting cells (G0 and G1[2]). Ki-67 is therefore considered to be an index that can fully reflect the proliferative activity of a given cell population. Ki-67-positive cells are often measured by Ki-67 labeling index, which has become one of the most frequently used immunohistochemical markers in routine pathological diagnosis. Clinical data show that the level of Ki-67 proliferation index is closely associated with the differentiation, invasion, metastasis and prognosis of many tumors and it is also one of the important reference indexes for the prognosis of some malignant tumors[9-11]. Ki-67 can be used as an important indicator for predicting the recurrence of meningioma and differentiating benign and malignant meningioma[1]. Ki-67 can also be used as a basis for grading certain tumors, such as gastrointestinal neuroendocrine tumors and meningiomas. Therefore, accurate counting of Ki-67 is very important in pathological diagnosis of meningioma. However in practice, Ki-67 in common immunohistochemical staining is not only present in tumor cells during the proliferative phase, but also in inflammatory cells, fibroblasts, vascular endothelial cells and other cells during the proliferative phase, which makes it very difficult to accurately count proliferative tumor cells[12].

Immunohistochemical double-staining make it possible to obtain more accurate Ki-67 labeling index of proliferative meningioma cells. Traditional immunohistochemical double staining works by catalyzing two different chromogenic substrates (BCIP/NBT and AEC) in two different enzyme systems, namely horseradish peroxidase (HRP) system and alkaline phosphatase (AP) system, to present two colors (blue-black and dark red) before double staining and AEC) in two different enzyme systems, namely horseradish peroxidase (HRP) system and alkaline phosphatase (AP) system, to present two colors (blue-black and dark red) before double staining and AEC. As a result, with shiny red from AP-Red and brownish yellow from DAB totally different from blue, it's easy to distinguish them. Ki-67 is a nuclear antigen present in nucleus while LCA is a cell membrane antigen present in cell membrane. As they are present in different regions, it's possible that they are present in the same cell of the same tissue[15].

In conclusion, in the diagnosis of meningioma, LCA/Ki-67 cocktail double staining can be used to improve the accuracy of Ki-67LI, which will provide more reliable information for its clinical diagnosis and pathological grading.

References