Preparation of Single Cell Suspensions from Paraffin-Embedded Tissue for FISH and its Clinical Application for Gastric Cancer Cells

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In the application of paraffin-embedded sections for fluorescence in situ hybridization (FISH), the effects of cell dispersion were compared among the three methods, Schutte Hedley and heiden methods, respectively. The cell shapes were best preserved by Schutte method. The satisfactory results of FISH were obtained by Schutte method.

On the other hand, cell collection was excellent in Heiden method. In applying for paraffin-embedded section, there is a great advantage toward extension of the use by means of trimming procedure on the section.

In the 10 paraffin embedded sections of gastric cancer, numerical aberrations of chromosome 3, 7, 11, 17 X and Y were evaluated by using chromosome 3, 7, 11, 17 X and Y specific probes. The numerical aberrations of chromosome 3, 7, 11 and 17 were frequent and common regardless of patient's ages and disease stages.

The present study offers insight into the cytogenetic research for solid tumor by using paraffin-embedded sections which provides essential benefit from expansion of clinical application.

Introduction

Fluorescence in situ hybridization makes it possible to detect aberration of chromosomal number in interphase nuclei with ease and simplicity. Application of FISH has been extended in the field of malignant disorders originated in hematopoetic tissues as well as solid malignant tumors.

There are few reports concerning cytogenetic analysis by using paraffin-embedded sections instead of fresh material.

Meanwhile, there are certain problems with analysis of interphase cytogenetics by using fresh material. In this method, cell loss is abundant in the process of sample treatment, requiring a large amount of samples. Therefore, it is not suitable for carcinomas in early stage which include relatively limited number of cancer cells. The consuming time from taking sample to treating is limited.

On the other hand, the use of paraffin-embedded sections is of great help to promote a retrospective study. A retrospective study may be promoted by development of cytogenetic analytical method using paraffin-embedded sections in which the prognosis are already known. Detailed evaluation has become feasible by timing of paraffin-embedded section even in minute carcinomas.

The wide availability of FISH by paraffin-embedded section is expected in the progression of cytogenetic study. In this study, establishment of cell dispersion method from paraffin-embedded sections is investigated from cell suspension to develop FISH by paraffin-embedded sections. Application of FISH by paraffin-embedded sections is not yet established. For clinical application of FISH by paraffin-embedded section, cell dispersion methods are compared among Schutte's method with the use of trypsin, Hedley's method with the use of pepsin and Heiden's method with the use of protease. Furthermore, chromosomal aberration was also evaluated in gastric cancer cells.

Materials and Methods

The test specimens were male's spleen tissues routinely embedded in paraffin, and peripheral blood lymphocytes (46, XY) were used as controls. The biotin-labeled chromosome-specific repetitive DNA probes, D3Z1 (chromosome 3), D7Z1 (chromosome 7), D11Z1 (chromosome 11), D17Z1 (chromosome 17), DXZ1 (chromosome X) and DYZ1 (chromosome Y) were used.

Sections of 50μm were excised from paraffin embedded tissues, followed by deparaffinization and rehydration in xylene twice for 60 minutes, in 100, 95, 70 and 50% ethanol each for 30 minutes and in distilled water for 20 minutes. Then three different digestions were performed as below.

1. Schutte's method

Samples were incubated in 0.25% Trypsin (DIFCO, 1:250) in citrate buffer (3mM trisodiumcitrate, 0.1% Nonidet P40, 1.5mM sperminetetracloride, 0.5mM Tris, pH 7.6) overnight at 37°C. After vortexing and filtration over a nylon mesh (50μm), the cells suspension were centrifuged and resuspended in distilled water.
2. Hedley's method

Samples were treated with 0.5% Pepsin (Sigma, P-7012) in 0.9% NaCl, pH 1.5 for 30 minutes at 37 °C, and centrifuged. The pellet was washed with distilled water and resuspended in distilled water.

3. Heiden's method

Samples were treated with 0.1% Protease XXIV (Sigma) in 0.1M Tris, 0.07M NaCl, pH 7.2 for 30 min at 37 °C. After centrifuged and washed the cells were resuspended in distilled water.

Then, according to the method of Arnoldus et al., the resultant cells were fixed in ethanol-acetic acid (3:1), developed on a slide glass coated with poly-L-lysine, followed by treatments with 0.5% pepsin/0.2N HCl, 70% acetic acid and 1% hydroxyammonium chloride/PBS. The chromosome denature was made in 70% formamide, 2 x SSC solution at 70 °C for 2 minutes. Similarly, a hybridization mixture (50% formamide+10% dextran sulfate + 500μg/ml each probe) denatured at 70 °C for 10 minutes was dropped on the cells on the slide glass, and in the moist chamber under the cover glass, hybridization was carried on at 37 °C overnight. The washing after the hybridization was made with the following solutions in the order of 2 x SSC for 10 minutes x 2, 60% formamide, 2 x SSC 10 minutes, 2 x SSC 10 minutes, and 0.05% Tween 20/PBS for 5 minutes. Thereafter, 1% bovine serum albumine was dropped and after warming under the cover glass at 37 °C for 30 minutes, after washing with Tween 20, 5μg/ml of FITC-labeled avidin was reacted under the cover glass at 37 °C for 20 minutes. Further, after washing with Tween 20, again reacted with the FITC-labeled avidin DCS for 20 minutes, and finally, counterstained with 1 μg/ml of propidium iodide solution.

Results

1. Cell morphology

Prior to hybridization in PI-staining, the shapes of nuclei had kept normal in Schutte' method and intranuclear structures were clearly observed. In contrast, the nuclei in Hedley' method had become smaller and picnotic and intranuclear structures were nuclear with uniformity. On the other hand, in Heiden' method, the nuclei were smaller in size, the shapes of nuclei were kept almost normal, revealing a definite intranuclear structure as shown in Fig. 1.

2. DNA histogram

Nuclear DNA content was evaluated by using PI staining on the hystogram. FL2 gain was similarly set in the three methods. A sharp G,M peak was seen in Shutte' method and a few debris contained in both of Shutte and Hedley' method. In contrast, Heiden' method contaminated a large number of debris. Shift to the left on the histogram was seen in 52 channels by Hedley' method and in 22 channels by Heiden' method in contrast with 60 channels in Shutte. The increasing order of shift to the left Heiden, Hedley and Schutte' method, respectively as shown in Fig. 2.

The results in FISH were as follows

![Fig. 1. PI staining of the cells deparaffinized form paraffin embedded tissue (spleen)](image)

lt.: Schutte', mid.: Hedley', rt.: Heiden'

![Fig. 2. DNA histograms of the deparaffinized splenic cells.](image)
3. Non-specific fluorescence

In this study, numerical chromosome 17 and Y aberration were compared among the three methods. Non-specific fluorescence was intensified by Hedley method as shown in Fig. 3.

4. Successful rates in each probes

Comparisons of the result by FISH were made by using chromosome 3, 7, 11, 17, x and Y specific probes. Schutte method succeed in chromosome 3, 7 and 11 specific probes. On the other hand, no signal spot was seen by Hedley and Hedley and Heiden methods.

In chromosome 17, X and Y specific probes, significant spots were gain in the three methods but the results by Schutte' method were excellent as shown in Table 1.

The rates of cell collection from the same sample were evaluated in comparison with the three methods. Cell dispersion by the three methods was made in the four samples and cell counts in cell suspension were compared. The mean cell counts were \(2.67 \times 10^9/cm^3\) by Heiden, \(3.17 \times 10^9/cm^3\) by Hedley and \(1.59 \times 10^9/cm^3\) by Schutte' method. Heiden method was maximum. In contrast, collected cells in fresh material were lessened and showed one tenth of the lowest values obtained by Schutte' method from paraffin-embedded sections.

<table>
<thead>
<tr>
<th></th>
<th>17</th>
<th>X</th>
<th>Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Schutte's method</td>
<td>3/3 (100%)</td>
<td>3/3 (100%)</td>
<td>3/3 (100%)</td>
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<tr>
<td>Hedley's method</td>
<td>3/3 (100%)</td>
<td>2/3 (67%)</td>
<td>2/3 (67%)</td>
</tr>
<tr>
<td>Heiden's method</td>
<td>2/3 (67%)</td>
<td>2/3 (67%)</td>
<td>3/3 (100%)</td>
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Table 2 revealed a result of numerical 3, 7, 11, 17, X and Y chromosome aberration in paraffin-embedded sections obtained from 10 patients with gastric cancer. Aberration of chromosome 7 in gastric cancer cells was not correlated with patient's ages. And also there was no relationship to the disease stage. Furthermore, the most numerical chromosome aberrations were 7, 11 and 17 chromosomes.

5. Application for minute carcinomas of gastric cancer by trimming

Samples obtained from minute gastric carcinomas were trimmed on the slices. The sites of carcinomas were selectively taken. The procedure of cell disparrison by Schutte' method was made and FISH was applied.

In the DNA ploidy analysis, diploidy pattern was delineated on the histogram as shown in Fig. 4. The results of FISH revealed an increase in chromosome 7 and 17 numbers to 7 or 8 as shown in Fig. 5.
Table 2. Numerical chromosome aberration in gastric cancer cells dispersed by Schutte method

<table>
<thead>
<tr>
<th>Case</th>
<th>stage</th>
<th>#3</th>
<th>#7</th>
<th>#11</th>
<th>#17</th>
<th>#X</th>
<th>#Y</th>
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<tbody>
<tr>
<td>M 64 y</td>
<td>I</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F 80</td>
<td>II</td>
<td></td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M 67</td>
<td>III</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>M 62</td>
<td>III</td>
<td>3</td>
<td>1</td>
<td>2</td>
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<tr>
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<td>III</td>
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<tr>
<td>M 82</td>
<td>III</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>2</td>
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<tr>
<td>M 79</td>
<td>III</td>
<td>3</td>
<td>3</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>F 76</td>
<td>IV</td>
<td>3</td>
<td>4</td>
<td>1</td>
<td>3</td>
<td></td>
<td></td>
</tr>
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<td></td>
<td></td>
<td>5/10</td>
<td>6/10</td>
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<td>6/10</td>
<td>2/10</td>
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Large nuclei with hyperdiploid spot number are present in chromosome 7, 11 and 17. Chromosome Y aberration was in one and it lacks Y chromosome.

In gastric cancer cells, numerical chromosome 3, 7, 11 and 17 aberration is common, reflecting representative of malignant potential which indicates biological behavior of tumor cells.

Discussion

Recently, molecular genetics have been developed in the analysis of structural aberration of chromosome for malignant tumors. Most of which used to be a sample of fresh material. Knowledge about numerical chromosome aberration using paraffin-embedded sections is scanty. Benefit from the use of paraffin-embedded sections is that a small amount of samples are enough to conduct, it is feasible as far as paraffin-embedded sections may be preserved, retrospective study can be promised, and also this method can make the most of trimming on the section.

This study aims to clarify the possibility as to whether or not paraffin-embedded sections are applicable for FISH. With respect to cellular structure, preservation of cellular structure was satisfactory by either Schutte or Heiden method.

Furthermore, Schutte method in this study indicated the best result concerning preservation of cellular structure as well as the successful rate of hybridization by using chromosome 3, 7, 11, 17, X and Y specific probes. It is assumed that the reasons for failure in getting a better result by Hedley are based on no aneuploidy peak in the analysis of DNA ploidy pattern. And also failure in application of Hedley's method may attribute to potent digestion of cells for action of pepsin by which it results in picnosis of nuclei and it leads to get unacceptable and unuseful results of FISH. Heiden's method developed by modification of Hedley's method is superior to other methods in the analysis of DNA ploidy pattern. Meanwhile, it is not necessarily satisfactory for investigation by FISH.

In the research of FISH, it is required that probe should be infiltrated into nuclei and form the reciprocal region. Schutte's method is the best for FISH research. It is confirmed that chromosomal aberration of minute gastric cancer is detectable for help of trimming procedure on the section by using paraffin-embedded section.

This study sheds light on cytogenetic study on chromosomal aberration of various malignant solid tumors with respect to carcinogenesis, tumor progression and determination of drug effect. Research of interphase in cytogenetics is of great value in studying human cancer. It is sure that fluorescence in situ hybridization provides a rapid and simple tools of testing chromosome aberration.

In this study, it is defined that FISH is feasible by Schutte method for using paraffin-embedded sections instead of frozen material. This is an enormous advantage over clinical application for cytogenetic research of solid tumors. The loss of the 7, 11, X and Y chromosomes were indentified in gastric cancer cells.

A missing Y chromosome in human male karyotype has been described for older patients. In this series, the loss of Y chromosome was not correlated with the age of the patients. It is assumed that loss of the Y chromosome may be a tumor specific abnormality.

Abnormality on chromosome arms such as deletion and translocations sometimes fail to be identified by FISH. Recently advances in centrometric probes makes it possible to be available for two third of human genome. And also chromosome-specific library probes are now available.

In gastric cancer cells, Creamer reported that numerical chromosome 7 and 17 aberration in aneuploid DNA cells are frequent rather than that in diploid. On the other hand, Numerical chromosome aberration of chromosome 8 and 9 as reported by Ochi have been defined in gastric cancers. It is well known that the epidermal growth factor receptor and c-erbB-2 genes are located on chromosome 7 and 17. In addition, numerical aberrations of those chromosomes are associated with amplification or overexpression of genes and result in a poor prognosis with a rapid progression of the tumor and its metastasis.

In this study, numerical chromosome 3, 7, 11, 17 and X aberrations were commonly seen in gastric cancer cells. These were not correlated with progression of the disease stage. Teysier reported complicated process of numerical and structural chromosome aberrations with progression of the tumor.

Recent research is the start toward clinical application of cytogenetic study for various solid tumors. Further study should be accumulated to elucidate carcinogenesis and progression of the tumors.

References


