Novel and simple method of double-detection using fluorescence *in situ* hybridization and fluorescence immunostaining of formalin-fixed paraffin-embedded tissue sections

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Received September 19, 2016; Accepted September 28, 2017

DOI: 10.3892/ol.2017.7413

Abstract. The importance of fluorescence *in situ* hybridization (FISH) for pathological diagnosis has been increasing. However, the procedures utilized for a conventional FISH method with formalin-fixed paraffin-embedded tissue sections are complicated and it is difficult to perform as a routine laboratory test. In addition, there are difficulties with differentiation of targeted cells in observations with a fluorescence microscope. The present study reported a novel method that utilizes FISH in combination with fluorescence immunostaining as a simple double-detection technique that addresses these problems. Using this novel method, various genetic aberrations, as well as protein overexpression were easily visualized in isologous sections. In particular, FISH signals with our method clearly identify target cells in samples with poor differentiation between tumor cells coexisting with normal cells. It is proposed that this simple technique is widely applicable as a routine laboratory test and future developments are expected.

Introduction

The requirement for genetic testing for tumor pathological diagnosis is increasing due to the association between tumor genesis and genetic abnormalities, thus objective diagnosis may be achieved by investigating such aberrations. Furthermore, genetic diagnosis methods, including fluorescence *in situ* hybridization (FISH) are important for determining the target drug for individual patients. For example, in cases of breast cancer, it is important to investigate the expression of human epidermal growth factor receptor 2 (HER2) protein or determine HER2 genetic amplification when considering administration of the molecular targeting drug trastuzumab (1). As a result, institutions are increasing adopting FISH for routine pathological examinations. In addition, FISH is frequently used to diagnose hematologic malignancy as important gene abnormalities have been observed in affected patients (2). For example, the important chimaera gene for oncogenesis, which has been observed in soft portions of tumors, such as synovial sarcoma (2). It is considered that the importance of FISH for examining solid tumors may increase in the same manner.

When using FISH, various problems can be encountered including those associated with reproducibility. The protocol for detection is complicated and considerable difficulties with obtaining stable results are faced when performing examinations for HER2 administration. Furthermore, a formalin-fixed paraffin-embedded (FFPE) technique is typically used for pathological examinations. However, signal strength and rate of detection with FISH have been reported to be affected by formalin fixation time (3). Additionally, it is difficult to distinguish target cells during observations with a fluorescence microscope.

To address these problems, the present study aimed to simplify the FISH protocol and develop a double-detection method that includes fluorescence immunostaining of FFPE tissue sections. In the present study, experiments were performed to validate this novel method.

Materials and methods

Cases. FFPE sections from 32 cases (20 mammary gland and 12 stomach) that underwent an examination of HER2 at Tsuchiura Kyodo General Hospital (Tsuchiura, Japan) between May and November in 2015 were used. All samples were biopsied. The samples that were fixed for >48 h were excluded from the subject. The mean age of the patients was 46 years (range, 32-68 years). All the mammary gland samples were collected from female patients, while stomach samples were collected from 7 males and 5 females. In addition, FFPE sections of lymph nodes from 1 patient with Hodgkin's disease were examined. Written informed consent was obtained from all of the patients who provided specimens. Approval for the present study was obtained from Tsuchiura Kyodo General Hospital Ethical Review Board. All diagnoses of HER2 were obtained based on the HER2 guidelines of the Japanese Society of Pathology (4).

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Key words: fluorescence *in situ* hybridization, fluorescence immunohistochemistry, double-detection, human epidermal growth factor receptor 2

Simplified FISH method and FISH combined with fluorescence immunostaining. FISH was performed with a Path-vision HER2 DNA kit (Abbott Pharmaceutical Co., Ltd., Lake Bluff, IL, USA). For the FISH method protocol, 4-μm dewaxed sections were washed with xylene, rehydrated in a descending alcohol series and incubated in antigen activation fluid (pH 9.0; cat no. 415211; Nichirei Biosciences, Inc., Tokyo, Japan) for 30 min at 99˚C, with the incubation time extended to 45 min for surgical samples that underwent extensive fixation, followed by cooling for 20 min. Following drying, the FISH probe (from the kit) was added and the section was incubated for 6 h in a moisture chamber at 42˚C after denaturing treatment for 5 min at 94˚C. The sections were then washed with Tris buffer (cat. no. 102189; LSI Medience Corp., Tokyo, Japan), including 0.3% Nonidet P-40 (cat. no. 25223-04; Nacalai Tesque, Inc., Kyoto, Japan) at 42˚C for 15 min, prior to being air dried and cover-slipped in DAPI.

A novel double-detection method was developed using a combination of FISH and fluorescence immunostaining. All processes were performed in a chamber at 42˚C. First, the section was treated with antigen activation solution (pH 9.0; Nichirei Bioscience) for 30 min and cooled for 20 min. The section was then reacted with the primary HER2 antibody (clone CB11; dilution, 1:300; cat. no. NCL-L-CB11; Leica Microsystems GmbH, Wetzlar, Germany) for 60 min, followed by incubation with a biotinylated secondary antibody (cat. no. 426072; undiluted; Nichirei Biosciences, Inc.) for 30 min and Alexa Fluor 488-labelled streptavidin (cat. no. S11223; Thermo Fisher Scientific Inc., Waltham, MA, USA; dilution, 1:30) for a further 30 min. Subsequently, re-fixation in 4% formalin was performed for 5 min at room temperature; then, after 15 min washing with Tris buffer (LSI Medience Corp.), including 0.3% Nonidet P-40 (Nacalai Tesque, Inc.) and drying, FISH was performed using the aforementioned simplified protocol.

Correlation between HER2 protein and gene amplification. The association between HER2 protein and gene amplification was investigated using 12 stomach and 20 mammary biopsy specimens. The routine laboratory tests were scored, as described previously (4). A FISH examination was also performed in cases with a score of 2. In addition, double-detection was performed using FISH and fluorescence immunostaining for HER2 in all 32 cases to examine HER2 protein overexpression, then the HER2/CEP17 ratio was calculated, and compared the results with those of routine laboratory testing.

Cytokeratin and HER2 genetic double-detection. In a conventional FISH examination, it is difficult to distinguish target cells under a fluorescence microscope. Our double-detection method was performed with cytokeratin, a representative marker of epithelial cells, to examine 3 biopsy samples obtained from subjects with poorly differentiated gastric cancer, with AE1/AE3 utilized as the cytokeratin antibody (cat no. M3515; dilution, 1:300; Dako; Agilent Technologies, Inc. Santa Clara, CA, USA).

Cluster of differentiation (CD)30+ IgH chimaera gene double-detection in Hodgkin's disease. In Hodgkin's disease, Hodgkin's cells coexist with non-tumor cells. Using our double-detection method, FISH was performed along with identification of tumor cells. One FFPE section of a Hodgkin's disease specimen was immunostained with an anti-CD30 antibody (clone JCM182; dilution, 1:150; cat. no. NCL-L-CD30-591; Leica Microsystems GmbH) to identify Hodgkin's cells, after which it was reacted with an IgH break-apart probe (cat. no. KBI 10729; Kreatech Biotechnology B.V., Amsterdam, Netherland) and the condition of the IgH gene in those cells was observed using fluorescence microscope at x400 magnification.

Statistical analysis. In order to investigate the correlation between HER2 protein and gene amplification, the Spearman's rank correlation test was performed using SPSS Statistical software 24.0 (IBM Corp., Armonk, NY, USA).

Results

Correlation between HER2 protein and gene amplification. The results are summarized in Table I. HER2 protein and genes were distinctly observed with the use of the double staining method (Fig. 1). In the routine laboratory tests, 8 cases were given an HER2 protein score of 2+. Those were subjected to FISH, which identified 4 cases as positive. Furthermore, 11 cases had an HER2 score of 3+, thus the total number of positive cases was 15. Protein overexpression and gene amplification were recognized in all of these positive cases with our double-detection method. The mean HER2/CEP17 ratio for the 17 negative cases was 1.27, while that of the 15 positive cases was 5.98. Thus, these results demonstrated that double-detection provided results equal to those obtained with routine laboratory testing. A FISH examination was also performed in 8 cases that underwent routine laboratory testing and the correlation with the HER2/CEP17 ratio results obtained from the double-detection method was investigated, which indicated a significant and positive correlation (Fig. 2). Thus, a similar FISH ratio using double-detection was obtained.

Cytokeratin and HER2 genetic double-detection. The results of the investigation in 3 cases of poorly differentiation gastric cancer demonstrated distinct validation of the presence of epithelial cells and observation of the HER2 gene in all cases. Since the HER2 protein was negative in all of these cases, FISH was not performed. Furthermore, gene amplification was not identified even with the double-detection method (Fig. 3). Thus, the method may precisely observe objective cellular genetic conditions.

CD30+IgH chimaera gene double-detection in Hodgkin's disease. Using the double-detection method, Hodgkin's cells were identified using the CD30 antibody and the genetic condition was confirmed with an IgH probe (Fig. 4A). However, it was difficult to distinguish Hodgkin's cells with a simple FISH examination (Fig. 4B).

Discussion

In the present study, a novel double-detection method was developed to detect proteins and the genetic condition of isologous FFPE sections used for routine pathological examinations. The protocol is simple and easy, and similar to that used for

Table I. Results of routine testing and double detection method.

	Routine		Double-detection				
Case no.	Immunostaining	Fish ratio	Immunostaining	Gene amplification	Fish ratio	Specimen type	Organ
1	0	ND	-	-	1.09	Biopsy	Mammary gland
2	0	ND	-	-	1.24	Biopsy	Mammary gland
3	0	ND	+	-	1.09	Biopsy	Stomach
4	0	ND	+	-	1.1	Biopsy	Stomach
5	0	ND	+	-	1.21	Biopsy	Mammary gland
6	0	ND	+	-	1.38	Biopsy	Mammary gland
7	0	ND	+	-	1.44	Biopsy	Stomach
8	1+	ND	+	-	1.02	Biopsy	Mammary gland
9	1+	ND	+	-	1.06	Biopsy	Mammary gland
10	1+	ND	+	-	1.3	Biopsy	Mammary gland
11	1+	ND	+	-	1.5	Biopsy	Mammary gland
12	1+	ND	+	-	1.53	Biopsy	Mammary gland
13	1+	ND	+	-	1.55	Biopsy	Mammary gland
14	2+	1.12	++	-	1.05	Surgical	Mammary gland
15	2+	1.08	++	-	1.17	Biopsy	Stomach
16	2+	1.29	++	-	1.25	Biopsy	Mammary gland
17	2+	1.63	++	-	1.55	Surgical	Mammary gland
18	2+	2.96	++	+	2.66	Biopsy	Mammary gland
19	2+	5.82	++	+	5.18	Biopsy	Stomach
20	2+	9.43	+++	+	9.13	Biopsy	Stomach
21	2+	8.67	++	+	9.75	Biopsy	Mammary gland
22	3+	ND	++	+	2.67	Biopsy	Stomach
23	3+	ND	+++	+	2.45	Biopsy	Mammary gland
24	3+	ND	+++	+	2.54	Surgical	Mammary gland
25	3+	ND	++	+	3.25	Biopsy	Stomach
26	3+	ND	++	+	4.41	Biopsy	Stomach
27	3+	ND	+++	+	6.57	Biopsy	Stomach
28	3+	ND	++	+	7.36	Surgical	Mammary gland
29	3+	ND	++	+	7.56	Biopsy	Stomach
30	3+	ND	+++	+	8.2	Surgical	Mammary gland
31	3+	ND	+++	+	8.41	Biopsy	Stomach
32	3+	ND	+++	+	9.57	Biopsy	Mammary gland

FISH, fluorescence *in situ* hybridization; ND, not tested.

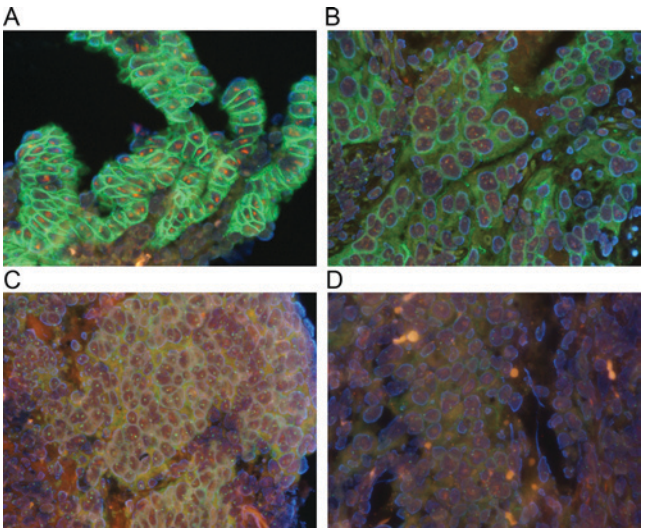

Figure 1. Detection of human epidermal growth factor receptor 2 protein and gene amplification using double-detection (magnification, x400). (A) Score of 3 and positive amplification. (B) Score of 2 and borderline amplification. (C) Score of 1 and negative amplification. (D) Score of 0 and negative amplification.

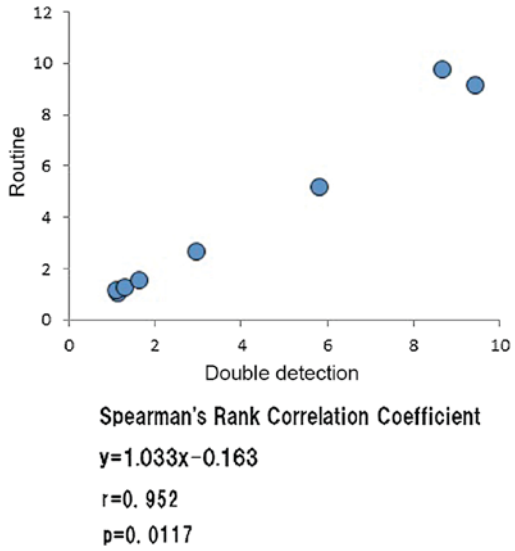

Figure 2. Correlation between routine testing and double-detection method for human epidermal growth factor receptor 2/CEP17 ratio.

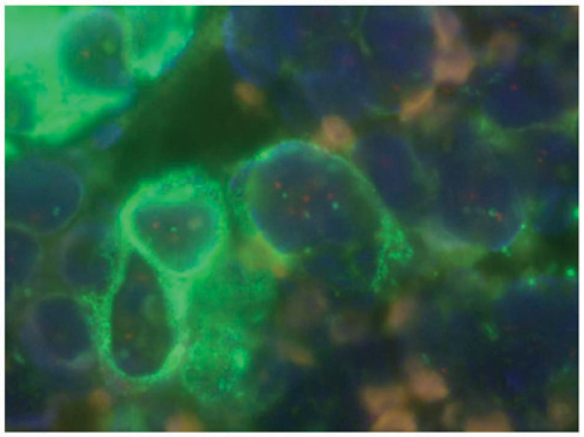

Figure 3. Detection of epithelial cells using cytokeratin immunostaining and human epidermal growth factor receptor 2 gene expression (magnification, x1,000).

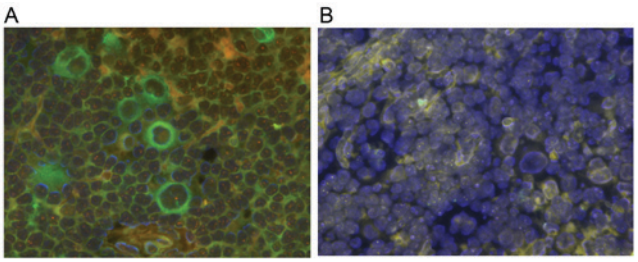

Figure 4. (A) Detection of Hodgkin's cells using cluster of differentiation 30 antibody immunostaining and IgH gene expression with double-detection (magnification, x400). (B) Detection of IgH in Hodgkin's disease section using conventional fluorescence *in situ* hybridization (magnification, x400).

double immunostaining. Thus, the present double-detection method may be applied as a part of routine laboratory testing.

In the present study with clinical samples, the results of our double-detection method were nearly the same as compared with routine laboratory tests, confirming its applicability. It is well known that the protein expression of HER2 is associated with gene amplification in gastric and breast cancer (5). When examining tissue sections, it is sometimes difficult to identify the target cells among various observed cells, though cytokeratin is useful to easily identify epithelial cells. Similarly, Hodgkin's cells were identified using CD30 in the present study. With our double-detection method, the genetic condition of targeted cells was observed.

Previous reports in other fields, including hematologic malignancy have presented double-detection methods (6). In 1992, Weber-Matthiesen *et al* (7,8) developed a method called Fluorescence Immuno-phenotyping and Interphase Cytogenetics as a Tool of the Investigation of Neoplasm. However, based on images presented in those reports, it was considered that improvements in the technique used for detection were required and it has yet to become a universal method. An examination using FFPE has also been reported (9). In that report, the images were low quality due to excessive proteolytic enzyme treatment and it is considered that improvements in the technique used for detection with that method are also necessary. With our method, heat treatment alone was used. Protease is usually used for pretreatment of ISH. The optimal treatment time of protease is associated with the formalin fixation time of a sample (10). Tissue samples in a routine laboratory test vary in terms of optimal protease treatment time, because the fixation time is not constant. This may be the reason why results of ISH vary. The methods used in the present study did not use protease, but used detergent instead. As a result, dispersion of signal intensity is suppressed. In the present study, observation was easy in comparison with previous reports.

Similar studies have used a visible light detection system. In 2005, Downs-Kelly *et al* (11) examined HER2 protein and gene double-detection, while Ni *et al* (12) in 2007 and Reisenbichler *et al* (13) in 2012 presented the same method. Those authors concluded that the techniques employed with

that method allowed for observation of gene and protein expressions, and examinations of both in detail. In 2012, Nitta *et al* (14) reported a method for detection of HER2 protein and gene expression using an automatic immunohistochemistry system, termed gene protein assay, which utilizes pigments, including DAB for visualization. Detection under visible light has numerous advantages. For example, there is no need for a fluorescence microscope or specially equipped darkroom and the preparations are permanently preserved. In contrast, our method using a fluorescence microscope has some merits. First, the choice of the target allows for the use of various probes, making it useful for a variety of applications. Second, an expensive detection system is not necessary. Therefore, our method is useful for pathological diagnosis using FFPE sections.

Another merit of our method is its simple protocol, though high quality FFPE sections are important to obtain good results. As for preparing routine FFPE sections, the methods and formalin fixation times are not uniform. It is well known that FFPE with an inferior condition results in incorrect immunostaining or FISH results. In the present study, good results were obtained with mammary gland needle biopsy specimens and gastric endoscopic biopsy specimens, for which a long period was not needed for fixation of the specimens.

In conclusion, the present study reported a novel and simple method of double-detection with FISH, and fluorescence immunostaining for use with FFPE sections. With this method, various genetic aberrations and protein overexpression were observed in isologous sections. Since the protocol is similar to that of double immunostaining, the method may be easily applied in a clinical laboratory setting.

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