Detection of numerical aberration in chromosome 17 and c-erbB2 gene amplification in epithelial ovarian cancer using recently established dual color FISH

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Summary

This paper presents a new dual color FISH technique that will allow the use of cell specimens isolated from formaldehyde-fixed and paraffin-embedded (F-P) tissues. The new FISH method was used to examine numerical aberration in chromosome 17 and c-erbB2 gene amplification in 26 F-P epithelial ovarian cancer tissues. Numerical aberration of chromosome 17 appears frequently in clear cell adenocarcinoma. The frequency of amplification of the c-erbB2 gene in stage III and IV cases was significantly higher than in stage I cases. The FISH technique as established here may serve as a molecular tool for examination of clinico-pathological significance in F-P tissues.

Key words: Dual color FISH; Formaldehyde-fixed and paraffin-embedded tissue; Numerical aberration of chromosome 17; Amplification of c-erbB2 gene; Epithelial ovarian cancer.

Introduction

Many cancers have been reported to display abnormalities in chromosomes, oncogenic amplification, and loss of tumor suppressor genes [1-5]. Loss of p53 and amplification of c-erbB2 located on chromosome 17 in epithelial ovarian cancer has been investigated using polymerase chain reaction (PCR) and in situ hybridization (ISH) techniques [6-8]. The ISH method has been established in morphological studies and provides a great deal of information about fresh cell or tissue specimens. Recently, though, examination of numerical aberration in chromosome 17 in uterine cervical and endometrial neoplasia using fluoresence in situ hybridization (FISH) has proved useful as an additional method for the differential diagnosis of those lesions [9].

FISH is useful as a molecular technique to detect chromosomes or genes in the nuclear morphology of cell or tissue specimens. Only fresh cells or tissue have been recommended for use with FISH, which makes it difficult to perform FISH with specimens of tissues that were fixed in formaldehyde and embedded in paraffin.

Thus, the authors have determined the appropriate conditions for the dual color FISH technique in order to detect chromosomes and oncogenes in formaldehyde-fixed and paraffin-embedded (F-P) tissue specimens. The authors also examined the relationship between the clinical significance and the numerical aberration of chromosome 17 as well as c-erbB2 amplification in epithelial ovarian cancer by means of the new dual color FISH.

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Materials and Methods

Tissues

F-P cancer tissues were obtained from 26 patients with epithelial ovarian cancer who were surgically treated at this hospital between 1989 and 1996. Histological types were classified into nine cases with serous adenocarcinoma, nine with mucinous adenocarcinoma, five with endometrioid adenocarcinoma, and three with clear cell adenocarcinoma. The breakdown for stages of epithelial ovarian cancer consisted of 13 patients with stage I, two with stage II, six with stage III, and five with stage IV.

Dual color FISH protocol in F-P cancer tissue

After paraffin was removed, the tissue specimens were treated with 0.5% pepsin for 30 min and subsequently with 200 μg/ml proteinase K (PK) for 15 min. Then, these isolated cells were smeared on silane-coated glass (Matsunami, Japan), fixed in 75% methanol and 25% acetic acid, and air dried. Cells were denatured in denaturing solution (70% formamide/2x SSC, pH 7.0) at 70°C for 15 minutes, dehydrated in an ethanol series, and air dried. They were then treated with 10μg/ml PK for 7 minutes. All of the cells lost the cytoplasm through this procedure.

A probe mixture solution was prepared as follows. One μl of chromosome-specific α-satellite DNA probe (a digoxigenin-labeled) chromosome 17 specific probe, Oncor, USA), 1 μl of human placental DNA (Sigma, USA), and 1 μl c-erbB2 DNA probe (Locus 17q11.2-q12, biotin labeled, Oncor, USA) were mixed in 7 μl of hybridization mixture solution (55% formamide/10% dextran sulfate/2xSSC, pH 7.0). The probe mixture solution was denatured in a water bath at 70°C for 5 minutes and kept at 37°C until hybridization. The cells were incubated with 10 μl of the probe mixture solution, covered with slips, and sealed with paper bond. Hybridization was carried out for 18 hours at 37°C in a moist chamber. Then, after the cover slips were removed, the cells on the slides were washed three times in washing solution (51% formamide/2xSSC, pH 7.0) and twice in 2xSSC, pH 7.0 at 45°C for 10 mintues. c-erbB2 on the cells was stained with fluorescein isothiocyanate (FITC)-conjugated

avidin after incubation with 1% BSA. The cells were incubated with PNM (PN buffer/5% non fat dry milk, 0.02% Sodium Azide) for 5 minutes to block non-specific binding. They were then incubated with rhodamine-conjugated antidigoxigenin antibody (3:47/PNM, Boehringer Mannheim, Germany) and biotin-labeled anti-avidin antibody (Oncor, USA) at 37°C for 60 minutes. The signals of c-erbB2 were again augmented with FITC-conjugated avidin. Finally, they were stained with DAPI (4,6-diamidino-2-phenylindole). The signals for chromosome 17 appeared as a red spot and those for c-erbB2 as a green spot when observed under a fluorescence microscope (Figure 1).

These signals were counted for at least 100 nuclei in respective specimens. The signals for chromosome 17 were classified into 1, 2 and 3 or more. When the signal number for c-erbB2 was greater than that of chromosome 17, the cell was considered to exhibit c-erbB2 gene amplification.

Statistical analyses were performed using the Kruskal-Wallis test or Mann-Whitney test. A result was deemed significant at $p < 0.05$.

Results

The signal number for chromosome 17 was not associated with the clinical stage (Table 1). The frequency of two signals of chromosome 17 in clear cell adenocarcinoma was significantly lower compared to that in the other three histological types (Table 1, $p < 0.05$, respectively). In other words, there is a significant difference in that there is an abnormal number of signals for chromosome 17 in clear cell adenocarcinoma.

The frequency of amplification of the c-erbB2 gene in stages III and IV was significantly higher than in stage I (Table 1, $p < 0.02$, respectively). Amplification of the c-erbB2 gene did not correlate with the histological type (Table 1).

Discussion

Although the sensitivity of FISH is slightly inferior to that of ISH using radioisotopes, FISH's specificity is very high and can obtain results in a shorter amount of time while not suffering from the drawback of radioactivity. Additionally, use of the FISH technique means that cells can be simultaneously incubated with more than one probe and consequently these signals can be observed concurrently. However, most of the samples used for FISH in the past were fresh tissue specimens. The attempt here was to establish an appropriate method of isolating and dispersing F-P tissues. The difficulty in establishing this new method was the way in which the probes would permeate the nuclear membrane. Because

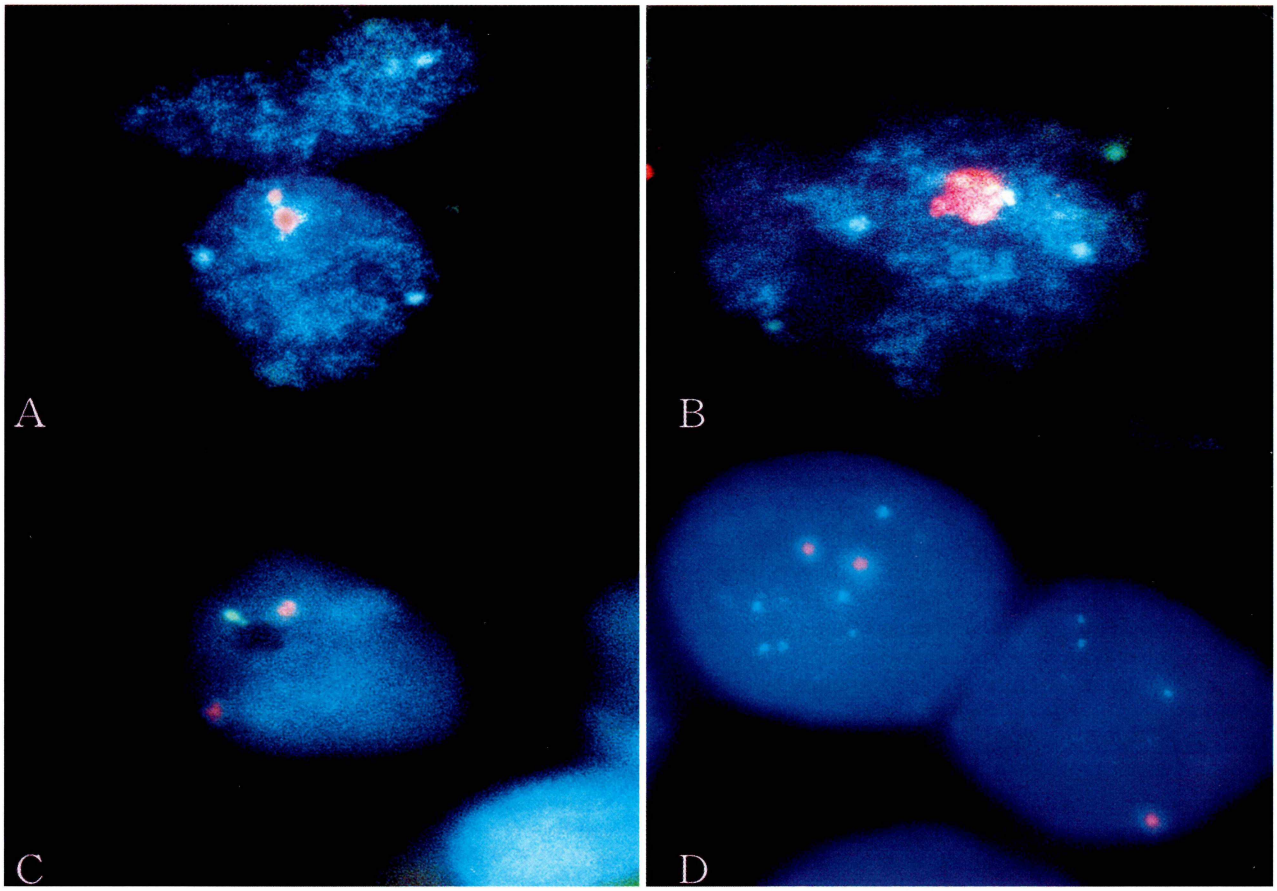

Figure 1. — Photographs of signals of chromosome 17 and the c-erbB2 gene in identical stage IV mucinous adenocarcinoma of the ovary provided by dual color FISH. Signal of chromosome 17 were noted as a red spot and those of the c-erbB2 gene as a green spot. A displays the cells without amplification of the c-erbB2 gene in a formaldehyde-fixed and paraffin-embedded (F-P) specimen. B shows the cells with amplification of the c-erbB2 gene in a F-P specimen. C shows the cells with amplification of the c-erbB2 gene in a fresh specimen. D shows the cells with amplification of the c-erbB2 gene in a fresh specimen.

Table 1. – *The frequency of numerial aberration of chromosome 17 and c-erbB2 gene amplification in epithelial ovarian cancers.*

Factor	No. of patients	Expression of chromosome 17 signal (%) 1	2	3 or more	Frequency (%) of c-erbB2 amplification
Stage					
I	13	17.3±7.8	27.0± 7.9	56.0± 8.8	21.8± 9.0**
II	2	15.5±6.3	33.0±15.5	51.5±21.2	30.5±14.8
III	6	20.1±8.3	26.1± 6.7	53.6± 6.3	33.2± 8.1
IV	5	24.2±6.4	22.4± 7.8	53.4±10.3	34.2± 9.6
Histological type					
serous	9	17.0±7.6	26.4± 6.8	56.5± 6.8	31.0±10.1
mucinous	9	16.7±8.6	27.4± 6.4	56.5±10.0	32.4± 9.1
endometrioid	5	18.2±6.1	32.4± 8.1	49.4± 7.7	34.0± 9.6
clear cell	3	20.3±8.7	17.6± 4.9*	62.0±11.3	23.0±10.0

* $p<0.05$, clear cell vs. the other 3 histological types; ** $p<0.02$, stage I vs. stage III and IV.

DNA must be hybridized with a probe that permeates the nucleus as part of the FISH method, it seemed unavoidable that chromosomal protein be removed. Thus, a method to isolate the cells from F-P tissue specimens using both pepsin and PK was devised and the nuclear membrane was dissolved by adding PK again. With this method, pepsin may first dissolve the cytoplasmic membrane and protein in the cytoplasm and then PK may denature the nuclear membrane. After the PK is added again to completely remove the nuclear membrane, the PK can then finally dissolve chromosomal protein.

FISH was performed using a chromosome 17 specific probe and c-erbB2 DNA probe with fresh specimens and F-P specimens obtained from identical cancer tissues. As a result, there was no difference in the frequency and intensity of signal expression for the two probes with both specimens (Figure 1). Thus, the present FISH method can serve as a molecular tool for examination of clinico-pathological significance in F-P tissues.

In the present study, numerical aberration of chromosome 17 occurred in 73% of epithelial ovarian cancer cells, and c-erbB2 gene amplification occurred in 29%. Numerical aberration of chromosome 17 significantly increased in clear cell adenocarcinoma, though it was not related to clinical stage. In contrast, amplification of the c-erbB2 gene was not associated with the histological type, though it was significant in advanced stages. Although no examination concerning the relationship between the numerical aberration of chromosome 17 and c-erbB2 gene amplification in epithelial ovarian cancer has been reported, the present results were similar to those for gastric cancer, breast cancer, and hepatocellular cancer [2, 3, 5]. Amplification of the c-erbB2 gene in breast cancer has been correlated with poor prognosis and lymph node metastasis [5]. Although the present study did not indicate the relationship between amplification of the c-erbB2 gene and prognosis nor of retroperitoneal lymph node metastasis in epithelial ovarian cancer (data not shown), the result concerning c-erbB2 gene amplification might suggest that c-erbB2 is associated with ovarian cancer progression.

Clear cell adenocarcinoma of the ovary has been reported to have chemoresistance, lead to poor prognosis, and is believed to possess aggressiveness [10]. The present result, where the numerical aberration of chromosome 17 was significantly higher in clear cell adenocarcinoma but c-erbB2 gene amplification did not change, suggests the association of other genes located on chromosome 17 for aggressiveness of clear cell adenocarcinoma. Biochemical techniques might be necessary in addition to FISH for further examination.

In any case, the new FISH method presented may prove helpful in retrospective studies with F-P tissue specimens as part of the accumulation of enormous amounts of clinical data.

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