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Analysis of Numerical Chromosome Aberration of Gastric Cancer: Application of Fluorescent in situ Hybridization using Chromosome Specific DNA Probes.

Hiroyuki Yamaguchi

First Department of Surgery, Nagasaki University School of Medicine

Abstract: An analysis in the numerical chromosome aberration of human gastric cancer was made by applying fluorescent in situ hybridization (FISH) with chromosome specific DNA probes. Thirteen primary tumors and four metastatic lymph nodes were surgically resected from thirteen gastric cancer patients and analyzed. FISH using α satellite DNA probes of chromosomes 1, 3, 7, 11, 17 and X was applied to interphase cells of each sample, then the signal spot number in each nucleus was counted. DNA ploidy from the flow cytometric analysis of nuclear DNA content was compared with chromosomal aberration from FISH analysis. Numerical chromosome aberration, especially a gain in chromosomes, occurred more frequently in DNA aneuploid cancers than in DNA diploid cancers. Numerical gain of chromosomes 7 and 17 were significantly more frequent in DNA aneuploid cancers. Since gastric cancer with a gain in chromosome 7 and/or 17 is often accompanied with a high level of metastasis in lymph nodes and/or distant metastasis, it is suggested that numerical gain of those chromosomes must be related to the ability of metastasis or growth in metastatic regions. Since the FISH study could detect numerical chromosome aberration in DNA diploid cancer, we will be able to further study the DNA diploidy of clinical materials by using this technique. Because FISH has made it possible to detect the chromosome number rapidly in the interphase cells, it is hopeful that human solid cancer chromosome analysis will rise as these materials for analysis increase and that we will take long strides in cytogenetic research of cancer in the near future.

Introduction

Although the close relationship between cancer and its chromosome aberrations has been well documented, we can not yet conclude that there is a cause/effect relationship in human solid cancer. One important reason is that it is very difficult to try and apply the conventional banding methods to solid cancer, as it is arduous to obtain metaphase cells suitable for chromosome analysis. Recently it became possible to detect the targeted chromosomes of interphase cells by utilizing fluorescent in situ hybridization (FISH) using chromosome specific DNA probes, which were developed in molecular biology. Application of this method has made it possible to analyze the chromosome number of human solid cancer cells as seen in the interphase.

It has not been long since this technique was developed, so that FISH is not prevalent as a mature method because it still holds some problems. It is more advantageous to use the FISH method and therefore analyze chromosomes from interphase cells, than to use conventional analysis methods. This is due to the fact that even though we can obtain good metaphase spreads, chromosomes may be affected by the process of modification and selection. FISH also has the merit of being able to compare DNA ploidy analyzed by flow cytometry and chromosomal ploidy of the same materials in the same condition. Therefore I tried to detect chromosome aberration of gastric cancer by applying this method to fresh surgical specimens, comparing the nuclear DNA content with the chromosome number and then evaluating the utility of FISH.

Materials and Methods

Between December 1989 and September 1990 at the First Department of Surgery of Nagasaki University School of Medicine, thirteen primary tumors, four metastatic lymph nodes and three normal gastric mucosa were surgically resected from thirteen patients who had been diagnosed with gastric cancer. The identification of gastric cancer was in accordance with the stipulations listed in the General Rules for the Gastric Cancer Study in Surgery and Pathology: the eleventh edition (Japanese Research Society for Gastric Cancer). Patients were eight males and five females, 46–80 years of age (mean 60 ± 10.8). The histological depths of cancer were one sm, two pm, one ss β, two ss γ and seven cases se or sei. The histological stages were two stage I, one stage II, seven stage III and three cases stage IV, most of them being advanced cases. The metastatic lymph nodes were resected from Cases 5, 7, 10 and 13 at the same time by gastrectomy, and were diagnosed as metastasis from a primary tumor using pathological procedures.

Sample Preparation

About 1 cm³ of both primary tumor and normal gastric
Hybridization was performed overnight in a moist chamber slides, then sealed with rubber cement under a coverslip. and then 20 ml of the hybridization mix was added to the Denaturation of the probes was done at 70 °C for 10 min, treatment, the slides were incubated in 0.25% acetic anhy-

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\text{mide/2xSSC at 70 °C for 2 min to denature target DNA, and then rinsed twice in}
\]

70% formaldehyde/0.1 M Tris-HCl pH = 8.0, and then rinsed twice in 2xSSC (0.3 M NaCl, 30 mM sodium citrate, pH = 7.0).

**DNA probe**

The α-satellite DNA probes specific for chromosomes 1, 3, 7, 11, 17 and X (biotinylated D1Z5, D3Z1, D7Z1, D11Z1, D17Z1, DXZ1) were obtained commercially (ONCOR CO.) DNA probes were used in the hybridization mix; 50% formamide, 10% dextran sulfate, 500 μg/ml salmon sperm DNA, 0.5 μg/ml DNA probe, 2xSSC (0.3 M NaCl, 50 mM sodium citrate, pH = 7.0).

**Hybridization**

FISH with D17Z1 and DXZ1 were first applied to the samples from normal gastric mucosa, and then respectively applied to all samples from cancerous tissue with probes. Cell suspension fixed in ethanol/acetic acid was dropped onto a poly-L-lysine coated slide and air dried. After rinsing twice in PBS, pretreatment was performed in 0.01% pepsin (SIGMA CO.) 0.2N HCl at 37 °C for 15 min and then postfixed in 4% paraformaldehyde/0.1 M PBS at 4 °C for 10 min. After dehydation in a series of cold ethanol treatment, the slides were incubated in 0.25% acetic anhy-

drate/0.1 M Tris-HCl pH = 8.0, and then rinsed twice in 2xSSC. Nuclei on the slide were incubated in 70% formal-

\[
\text{mide/2xSSC at 70 °C for 2 min to denature target DNA, and then dehydrated in a series of cold ethanol treatment.}
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Denaturation of the probes was done at 70 °C for 10 min, and then 20 ml of the hybridization mix was added to the slides, then sealed with rubber cement under a coverslip. Hybridization was performed overnight in a moist chamber at 37 °C.

**Cytochemical Study**

After hybridization, the slides were washed in 60% formamide/2xSSC (two times at 42 °C for 10 min) followed by washes in 2xSSC (two times at 37 °C for 5 min) and in 0.05% Tween-20/PBS at 37 °C for 5 min. Thereafter the slides were incubated with 5% nonfat dry milk/0.05% Tween-20 in 4xSSC (MST buffer) for 5 min at room temperature. Detection of biotinylated probes was achieved by incubation with 5 μg/ml FITC conjugated avidin DCS (Vector Labo. Inc.)/MST buffer for 20 min at 37 °C. All washes were carried out in 0.05% Tween-20/PBS. The probe linked fluorescence was amplified by incubation with 5 μg/ml biotinylated anti-avidin antibody (Vector Labo. Inc.)/MST buffer for 20 min at 37 °C and again incubation for 20 min with 5 μg/ml FITC conjugated avidin DCS. All preparations were counterstained with 1 μg/ml propidium iodide (SIGMA) and then visualized on a fluorescence microscope (OLYMPUS BH-2). Fluorescence signal spots per nucleus were counted on about 400 nuclei. The Student’s t test was applied in all statistical comparisons.

**Result**

**Normal Gastric Mucosa**

The result of FISH applied to the normal gastric mucosa from three male patients using chromosome 17 and X specific probes (D17Z1, DXZ1) is displayed in Table 1. The average appearance rate of nuclei with no signal spot in chromosome X was 11.9%, and nuclei with one signal spot was 81.4%. The average appearance rate of nuclei with two, three and four signal spots were 5.7%, 0.9% ans 0.1% respectively. With regard to chromosome 17, nuclei with no and one signal spot were 1.1% and 14.5% respectively. Nuclei with two signal spots were 82.7% and those with three and four signal spots were 1.1%, and 0.5% respectively. Although normal male cells containing chro-
mosome X should be monosomic and chromosome 17 disomic, nuclei with one or two signal spots from the FISH study were a little over 80%. Nuclei with less than two spots in chromosome 17 and with less than one spot in chromosome X were both over 10%, and nuclei with a spot number greater than that of normal chromosomes ranged from under 1% to 7%. At this time, in order to analyze the FISH study applied to solid cancer consisting of hetero-
geneous cell populations, evaluation of the results were performed as follows for the sake of convenience.

When nuclei with less than two signal spots in chro-
nome 1, 3, 7, 11, 17 or less than one signal spot in chromosome X were present over 20%, it should be ruled that a population (stem line) that loses a chromosome must exist and that this shall be designated “L”. When nuclei with signal spots greater that two in chromosome I, 3, 7,
The result of FISH using chromosomes X and 17 (DXZ1, Z17Z1) applied to normal gastric mucosa. Signal spot number per nucleus was counted using 400 nuclei, which were well morphologically reserved and clear counterstained. The percentages of cells with each respective number of signal spots is indicated. The upper figure is a histogram of the results.

**Table 1.**
The nuclear DNA content of thirteen primary tumors and four metastatic lymph nodes was analyzed with flow cytometry, showing eight lesions to be DNA diploid and nine lesions to be DNA aneuploid. The result of the FISH study from all seventeen lesions (primary tumor and metastatic lymph nodes) was evaluated according to the criteria prescribed, and compared with pathological studies and DNA ploidy (Table 2). Because of sample shortages, FISH with D1Z5, D3Z1 and DXZ1 could not be applied to the Case 4 primary tumor, and likewise with D3Z1 to the Case 8 lymph node. For example in Case 3, a flow cytometric study revealed DNA aneuploid (Di = 1.20) and the FISH study indicated that the population lost one chromosome 3, gained one more chromosome 7, and a gain in one and two chromosome 11 were present (Fig. 1a, 1b, 2).

In Case 13, flow cytometric measurement of nuclear DNA content showed DNA aneuploid (Di = 1.37, 1.87) in the primary tumor and DNA diploid in the metastatic lymph node. FISH analysis of the chromosome number 11, 17 or greater than one signal spot in chromosome X were present over 10%, it should be ruled that a population (stem line) that gains a chromosome must exist, and that this shall be designated “G”. For example, when nuclei with three or four signal spots were present over 10% respectively using the chromosome 17 specific probe (D17Z1), it was ruled that the population which had a gain by one or two more of chromosome 17 must exist, and furthermore be designated “GG2”.

**Primary Tumor and Metastatic Lymph Node**

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**Table 2.**
FISH study applied to thirteen primary tumors and four metastatic lymph nodes using D1Z5, D3Z1, D7Z1, D11Z1, D17Z1, and X was evaluated, and the result of evaluation is displayed above. T: primary tumor, LN: metastatic lymph node, DD: DNA diploid, DA: DNA aneuploid; *: absence of population with abnormal number of that chromosome, G: presence of population with one gain of that chromosome, G2: presence of population with two gain of that chromosome, GG2: combination of both G and G2 L: presence of population with one loss of that chromosome, NI: non informative because of unsatisfactory hybridization, /: not applied due to sample shortage, nO: means n (~) |
Fig. 1a. FISH using the chromosome 17 specific probe (D17Z1) as applied to the primary gastric tumor from case 3. Most of the nuclei contain two signal spots.

Fig. 1b. FISH using the chromosome 11 specific probe (D11Z1) as applied to the primary gastric tumor from case 3. Two, three, four or five signal spots are seen in each nucleus.

Fig. 2. The primary tumor from case 3. Analysis of nuclear DNA content by FCM revealed DNA aneuploid (DI=1.20). Analysis of the chromosome number by FISH revealed a loss of chromosome 3 and gain in chromosomes 7, 11 and a 2 signal spot gain in chromosome 11.

Fig. 3. The primary tumor and metastatic lymph node from case 13. Analysis of nuclear DNA content by FCM revealed DNA aneuploid (DI = 1.37 and DI = 1.87). Analysis of chromosome number by FISH revealed a gain in chromosomes 3, 7, 11, 17 and X.
lower than in the relatively early group (stages I and II). However, the abnormality rates of the four chromosomes were higher in the former than in the latter. This especially true concerning chromosome X, where aberration did not occur in any case in stage I or II, but did occur with 8 cases out of 10 in stage III and IV. Of the 8 cases, 7 case showed a gain in chromosome X and one case showed a loss in chromosome X. The abnormality rates were therefore significantly higher (p < 0.01) in stages III, IV than in stages I, II (Table 4).

The incidence rate of populations with an abnormal chromosome number was evaluated between pathological stages. Numerical aberration, especially a gain chromosome showed significant frequency (p < 0.01) in the advanced group (stages III and IV) compared with the relatively early group (stages I and II). The incidence of numerical loss showed no significant differences in each chromosome between the two groups.

All seventeen cancer lesions (primary and metastatic) were then divided into DNA diploid and DNA aneuploid groups and numerical chromosome aberration was evaluated. Populations with abnormal chromosome number were also detected in DNA diploid cancer lesions. The abnormality rates were 43.6% in the DNA diploid and 69.8% in the DNA aneuploid groups. From Table 5, we see that the rates of chromosome gain were 28.2% in the DNA diploid group and 53.5% in DNA aneuploid group, and therefore were significantly higher (p < 0.05) in the latter versus the former group.

The abnormality rates in each chromosome in the DNA diploid and DNA aneuploid group were evaluated as shown in Table 6. Five cases whose primary lesions had populations with a gain of chromosome 7 or 17 were reevaluated, and found to all be DNA aneuploid lesions. Case 1, 5 and 13 which had a gain in both chromosome 7 and 17, were all found to have a high level of lymph nodes metastasis, although case 1 was “sm” with peritoneal dissemination.
Discussion

Although chromosome aberration of human solid cancer has been researched for many years, the reports are still insufficient and the results show a great number of different directions. The same is true with most of the human gastric cancer researches, which end with the consensus, as I do that investigation in this area has been and is difficult, although my investigation has shed more light in this area. Genetic research of cancer has been improved by the development of a new molecular biological technique called "in situ hybridization" which is able to be applied to interphase cells. The word "interphase cytogenetics" has been proposed. It is one of them to detect the targeted chromosome number of a cell, as it is in interphase, using DNA probes specific to the repetitive sequence on the centromeric region of each chromosome. The study of human solid cancer utilizing this method is already beginning to be reported. At this time I tried to analyze chromosome aberration of human gastric cancer using fluorescent in situ hybridization (FISH) in which the hybridized probes are cytochemically fluorescence-labeled. Since this method has some problems which must be improved for future use, it is important that we recognize the present shortcomings during research. An example is demonstrated from the result of FISH applied to normal gastric mucosa. Although they are normal cells which should be disomic in each chromosome (and monosomic in the male sex chromosome), the number of signal spots in each cell is not always two (or one), but scattered. This occurs from two causes. First, the probes are limited because they cannot access and hybridize to all targeted sequences, therefore showing nuclei which have fewer signal spots than the actual chromosome number. Second, we overestimate the spot number which results in an amount that is greater than the actual chromosome number, because we cannot distinguish the spot due to cross-hybridization from the true signal spot.

There is therefore room for improvement with this technique, such as improving the accessibility of the probe to target DNA and making conditions more stringent consequently enhancing specificity of hybridization. There are further problems that might be recognized as numerical aberration, such as when a structural or functional abnormality involving the centromere takes place on the targeted chromosome. Since the success rate is still low in some probes, it can not yet be valued for all chromosomes. In this study the success rate was low: 37.5% for D1Z5 and 60% for D3Z1, and hence the analysis of chromosomes 1 and 3 was insufficient.

In the present situation, we can expect that if we apply FISH to solid cancer consisting of a heterogeneous population and try to detect numerical chromosome aberration, there would be a complication which would interfere with the evaluation. I proposed criteria on how to evaluate the results when FISH is applied to gastric cancer by referring to the result of normal gastric mucosa. When nuclei with more than two (one in chromosome X of male) signal spots are present in over 10%, it should be ruled that the population (stemline) which has that number of chromosomes must exist. When the nuclei with less than two (one in chromosome X of male) signal spots are present in over 20%, it should be ruled that the population (stemline) which has that number of the chromosomes must also exist. It is in this way that the problem of whether or not the population (stemline) with an abnormal chromosome number existing over some fixed amount was valued in this study. This criteria is certainly reasonable for research in both following situations: whether DNA diploidy from flow cytometry analysis reveals chromosomal diploidy or not, and what kind of chromosome aberration has occurred in a population detected as the DNA aneuploid peak by flow cytometric analysis.

The incidence of chromosome aberration in human gastric cancer was beyond our expectations. This result indicates the interesting fact that although the nuclear DNA content of the cancer is still DNA diploid, tumor cells with an abnormal chromosome number have already appeared in it. Although it cannot yet be referred to as chromosomal ploidy yet, the incidence of numerical chromosome aberration is recognized in DNA diploid cancer. I consider the intrinsic character of cancer that DNA diploid reveals, an interesting problem even though it is generally known that most of DNA diploid cancer has a better prognosis, compared to DNA aneuploid cancer. The result that the incidence of numerical chromosome aberration in DNA aneuploid cancer was significantly higher than in DNA diploid cancer indicates that an increase in nuclear DNA content may be one phenomena that relates to numerical gain in chromosomes.

When comparing the incidence of chromosome aberration according to stages, that of numerical gain in chromosome X was significantly higher in stages III and IV than in stages I and II. According to the DNA ploidy in the primary tumor, the incidence of numerical gain in chromosomes 7 and 17 was significantly higher in DNA aneuploid that in DNA diploid. Comparing these results with those of the other few studies of human gastric cancer reports, Ferti et al. reported that the numerical gain and structural abnormality of chromosomes 8 and 9 had been frequent in the study of five cases by conventional methods. Ochi et al. reported that deletion of chromosome Y and numerical
gain of chromosome 12 had been frequent in the study of five cases, and Van Dekken et al.\textsuperscript{10} reported a targeted cytogenetic study in which 10 gastric adenocarcinoma had been analyzed by FISH using chromosome specific DNA probes and therefore used the same method which was employed in this study. Although the methods used by Van Dekken et al. to estimate their results were different from the methods we used here, he described that hyperdiploid cases in DNA flow cytometric histograms were chromosomal hyperdiploid aneuploidy and that a significant clonal absence of the Y chromosome was found in males. Since we cannot find similar results between previous gastric cancer papers and this paper, it is still too difficult to conclude that the gain in chromosomes 7 and 17 reported in this paper is an essential phenomena associated with the progression of gastric cancer, and not an accidental one. The meaning of this phenomena will be clear in the future after similar studies have been accumulated. Teyssier\textsuperscript{11,12} however, from the result of chromosomal analysis of so many malignant and non-malignant solid tumors, described that since recurrent duplication of chromosome 7 is a common feature, acquisition of an increased number of copies of this chromosome may represent a selective genetic event which confers a proliferative advantage to cells. The Epidermal Growth Factor Receptor gene and c-erbB-2 gene are located on chromosome 7 and chromosome 17 respectively, and it was then confirmed in several reports that human solid cancer associated with amplification or overexpression of both these genes had a high level of metastasis or poor prognosis.\textsuperscript{13-15} This present result, that gastric cancer associated with a gain in chromosomes 7 and 17 had high level of lymph node metastasis or distant metastasis, suggests the possibility that chromosome aberration involving these genes may affect the ability of metastasis and growth in human gastric cancer, too.

Investigation of cancer is now being made more clearly through genetic determination. However, Teyssier\textsuperscript{16} said it must be considered a complicated process which gradually affects the multiple structural and functional levels of the genome, and is incompatible with the simple activation or depression model of oncogenic genes. It must be true that numerical and structural chromosome changes play an important role in the process of tumor progression. Although many studies in chromosomal abnormalities of cancer have been reported, most of them concern those of the non-epithelial tumor and few are those of human solid cancer.\textsuperscript{16,20} It is said that even if the studies had concerned solid cancer, the subjects in these would mainly consist of metastatic tumor cells, derived from ascites or pleural effusion, and those from primary tumors are no more than 1% of the total.\textsuperscript{17,22} The most important reason for this, is that it is too difficult to obtain suitable metaphase cells from human solid cancer, although metaphase cells are indispensable for conventional cytogenetic study. Even if we could obtain suitable cells, they are not always representative of the targeted tumor. This is due to the modification and selection which occurs in the process when tumor cells are cultured and induced to metaphase with medium and drug.

In the present situation, this technique which is able to detect the targeted gene or chromosome as it is in interphase, may be a break through in this field. In this study, it was possible to apply FISH to cells easily isolated from fresh tissue samples of human gastric cancer without complicated processes involving cell culture, and to concurrently analyze the nuclear DNA content of the same sample by flow cytometry. The simple procedure and broad applicability of this method will be the driving force in cytogenetics in which research will continue to expand and discover new directions. I hope that the study in chromosomal abnormality of cancer will continue and that the data from these studies will accumulate, thereby demonstrating the relationship between oncogenesis or progression of cancer, and chromosome aberration.

**Conclusion**

I tried to investigate numerical chromosome aberration of human gastric cancer by application of Fluorescent in situ hybridization. Chromosome specific DNA probes were used with fresh tissue samples that were surgically resected from gastric cancer patients. Their nuclear DNA content was compared and these results yielded the following conclusions:

1) It was possible to detect numerical aberration in chromosomes 1, 3, 7, 11, 17 and X of human gastric cancer, as seen in interphase.

2) It was confirmed that the presence of numerical chromosome aberration is present in DNA diploid cancer.

3) The incidence of numerical chromosome aberration was significantly higher in DNA aneuploid versus DNA diploid cancer, and gain in chromosomes 7 and 17 was significantly higher in comparison, according to chromosome number.

4) It was suggested that the gain in chromosomes 7 and 17 may be associated with the ability of metastasis or growth and progression in the metastatic region.

My special thanks to Professor Masao Tomita for his helpful and instructive deliberations, and to Dr. Yutaka Tagawa for practical and editorial guidance and the other doctors belonging to the First Department of Surgery of NAGASAKI University School of Medicine for their kind support.

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Common Cytogenetic Findings in Gastric Cancer

Cytogenetic Studies in Primary Gastric Cancer

Targeted Cytogenetic Analysis of Gastric Tumors by In Situ Hybridization With a Set of Chromosome-specific DNA Probes

Nonrandom Chromosomal Changes in Human Solid Tumors: Application of an Improved Culture Method

Frequent Clonal Chromosomal Changes In Human Non-Malignant Tumors

MOLECULAR DIAGNOSIS ON GASTRIC CANCER

Amplification and overexpression of the EGF receptor and c-erbB-2 proto-oncogenes in human stomach cancer

erbB genes-c-erbB-1/EGF receptor and c-erbB-2

Amplification, Overexpression, and Rearrangement of the erbB-2 Protooncogene in Primary Human Stomach Carcinomas

The Chromosomal Analysis of Human Solid Tumors

Cytogenetics of Breast Cancer

Cytogenetics of Colorectal Adenocarcinomas

Chromosomal Rearrangements in a Primary Hepatocellular Carcinoma

Chromosome Abnormalities of Gastric Cancer Detected in Cancerous Effusions