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Comparative genomic hybridization analysis for pancreatic cancer specimens obtained by endoscopic ultrasonography-guided fine-needle aspiration

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Background. Comparative genomic hybridization (CGH) analysis of pancreatic cancer has been done exclusively for surgical and autopsy specimens, because of the difficulty of tissue sampling without surgery. To overcome this difficulty, we applied CGH technology to cells obtained by endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA). ***Methods.*** In the present study, we performed EUS-FNA for 17 patients with pancreatic cancer before surgery. Tumor cells were selected by microdissection. DNA was extracted from the cells and amplified by degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR). Then CGH was carried out. ***Results.*** In the 15 patients with tubular adenocarcinoma, the most common loci of gains (including amplification) were 5p, 8q, and 20q (60% of the patients); and 1q, 7p, and 12p (27%). The most frequent losses were 17p (73%); 9p, 18q, and 19p (47%); and 8p (33%). These findings were similar to our previously reported data. Both of the patients with acinar cell carcinoma showed gains of 2q and 5p, and losses of 1p, 9p, 9q, 11p, 11q, 14q, 17p, 17q, and 18q. ***Conclusions.*** The results of this study suggest that comprehensive genetic analysis is possible for EUS-FNA biopsy specimens, with a combination of microdissection and DOP-PCR. This analytical strategy will enable us to evaluate the biological characteristics of pancreatic cancer before treatment.

Key words: comparative genomic hybridization, EUS-FNA, DOP-PCR, pancreatic cancer

Introduction

Pancreatic cancer (PC) is the fifth leading cause of death in Japan. Despite recent diagnostic and therapeutic progress, the prognosis of patients with PC still remains poor. This is because most of the patients already have lymph node metastasis, liver metastasis, and peritoneal dissemination at the time of their diagnosis.[1–3] In general, the biological characteristics of a tumor are primarily affected by genetic alterations of the tumor. Therefore, comprehensive analysis of genetic alterations is crucial to elucidate the oncogenetic mechanisms in PC.

Comparative genomic hybridization (CGH) is a powerful tool for analyzing cytogenetic aberrations of the entire genome in a single experiment.[4,5] To date, there are five reports of CGH analysis of PC.[6–10] However, all the reports deal exclusively with surgical samples as clinical materials. Because CGH requires a relatively large amount of DNA, it has been impossible to obtain tissue specimens sufficient for CGH analysis of PCs before surgery. In addition, due to the anatomical location of the pancreas, conventional biopsy is also difficult in PC. Some analyses other than CGH can be performed for cells contained in pancreatic juice, but they are too small in number and degraded to be suitable for CGH analysis.

Endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) biopsy was recently developed in the field of gastroendoscopy.[11–20] This method can be applied to the pathological diagnosis of a tumor. In PC, it has another advantage, in that it enables us to obtain tumor cells directly, with a low-invasive procedure, before beginning treatment of patients.

We have reported that the combined technique of CGH and degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR) analysis is useful and reliable for detecting genetic alterations in PC. This means that DOP-PCR CGH analysis can be

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applied to quite a small number of cells obtained by EUS-FNA biopsy. This is the first report on the detection of comprehensive genetic alterations in PC before treatment.

Patients, materials, and methods

Patients

Seventeen patients with tumors of the pancreas underwent EUS-FNA at the Yamaguchi University School of Medicine Hospital, from April 2002 to March 2004. All patients underwent abdominal US and computed tomography (CT) scanning before EUS-FNA, and were diagnosed as having PC. The patients' average age was 67 years (range, 33 to 82 years) and the male-to-female ratio was 11 : 6. The tumor location was the head of the pancreas in 10 patients, the body in 6, and tail in 1. Tumors were staged according to the International Union Against Cancer TNM classification of malignant tumors. Five tumors were classified as stage IIB, 7 as stage III, and 5 as stage IV. Histological diagnoses of the EUS-FNA specimens were made by two pathologists, independently. The clinicopathological features of the subjects are summarized in Table 1.

The study protocol was approved by the Institutional Review Board for Human Use at the Yamaguchi University School of Medicine in May 1995, and informed consent for this study was obtained from all patients.

EUS-FNA

Standard EUS was performed first, using a radial scanning echoendoscope (JF-UM200-7.5; Olympus, Tokyo, Japan), to identify and evaluate the tumor lesions. Pancreatic tumors could be observed through the wall of the stomach and duodenum on the ultrasound screen of the echoendoscope. EUS-FNA employs an ultrasound apparatus (EU-C2000; Olympus) and a linear scanning instrument (GF-UC2000P-OL5; Olympus). Then FNA was performed, using an automatic aspiration device (NA-11 J-KB; Olympus) and a 22-gauge needle. Color doppler examination was used to verify that there was no blood flow in the lesion or at the aspiration site. Tumor tissues were obtained by moving the needle forward and backward, in the lesion 8–12 times, while applying negative pressure (Fig. 1b).

Histopathological examination

For histological diagnosis, after fixation of the specimen in formalin, serial sections were cut, and stained with hematoxylin and eosin (Fig. 2b). For CGH analysis, smear preparations were made from the specimens and fixed in 99.5% ethanol immediately after EUS-FNA, and then stained with hematoxylin (Fig. 2c). Of the 17 patients, 15 had tumors that were classified histologically as tubular adenocarcinoma and 2 had acinar-cell carcinoma (Table 1).

Table 1. Summary of patients' clinicopathological features

Patient no.	Age (years)	Sex	Location	Histology	T	N	M	Stage	Gain of 1q	Gain of 5q	Gain of 7p	Gain of 8q	Gain of 20q
1	82	M	Ph	Tub	3	1	0	IIB	−	+	+	+	−
2	67	M	Ph	Tub	3	1	0	IIB	+	+	+	+	−
3	71	F	Ph	Tub	3	1	0	IIB	−	−	−	++	+
4	58	M	Ph	Tub	3	1	0	IIB	−	−	−	−	−
5	75	F	Ph	Tub	3	1	0	IIB	−	−	+	−	−
6	59	M	Pb	Tub	4	1	0	III	+	−	−	+	+
7	68	M	Ph	Tub	4	1	0	III	−	+	+	+	+
8	75	M	Ph	Tub	4	1	0	III	−	−	−	−	−
9	73	M	Pb	Tub	4	1	0	III	−	+	−	+	+
10	70	F	Ph	Tub	4	1	0	III	−	+	−	−	−
11	77	M	Pb	Tub	4	1	0	III	−	−	−	+	+
12	71	F	Pb	Tub	3	1	1	IV	−	+	−	−	+
13	63	F	Pb	Tub	4	1	1	IV	+	+	−	−	++
14	69	M	Ph	Tub	4	1	1	IV	−	+	−	+	++
15	82	F	Ph	Tub	4	1	1	IV	+	+	−	+	++
16	33	M	Pb	Acinar	4	1	0	III	−	+	−	−	+
17	62	M	Pt	Acinar	4	1	1	IV	++	++	++	++	−

Pb, body of the pancreas; Ph, head of the pancreas; Pt, tail of the pancreas; Tub, tubular adenocarcinoma; acinar, acinar cell carcinoma; +, gain; ++, amplification; −, no change

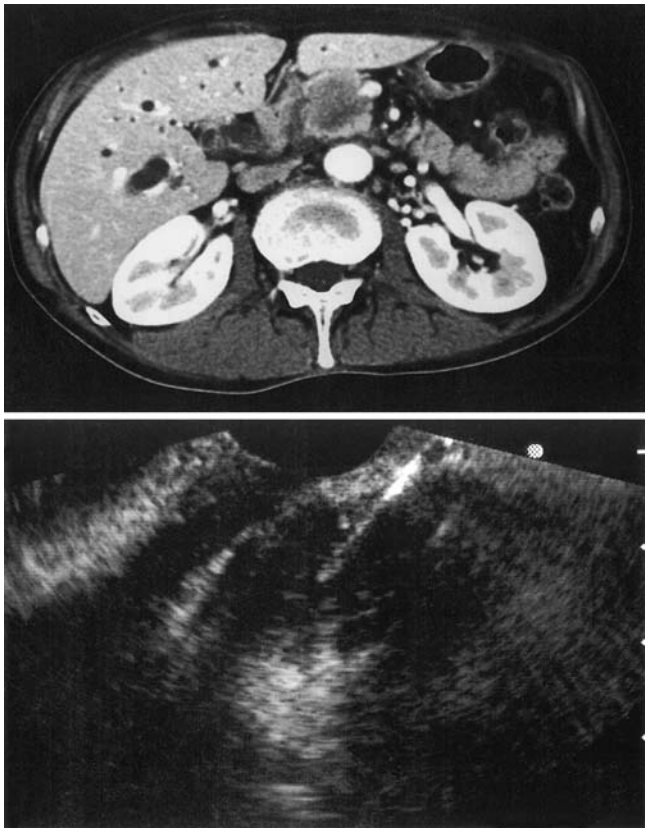

Fig. 1. a Abdominal computed tomography (CT); **b** endoscopic ultrasound-guided-fine-needle aspiration (EUS-FNA). **a** A tumor, 3 cm in diameter, is seen in the head of the pancreas. **b** The same pancreatic tumor as that shown in **a**, seen with the linear scanning echo endoscope used for EUS-FNA. The needle is clearly visible inside the lesion

Microdissection and extraction of DNA

Tumor cells were selectively collected from stained tissues by using a laser capture microdissection (LCM) apparatus (LCM200; Arcturus, USA; Fig. 2d). Approximately 50–100 tumor cells were microdissected and incubated in 400 μg/ml proteinase K solution for 24 h at 55°C to extract DNA. In addition, reference DNA from peripheral blood lymphocytes was also extracted, using a DNA extraction kit (SepaGene, Sankoujunyaku, Japan).

DOP-PCR

DOP-PCR was performed for both tumor DNA and reference DNA, using a universal primer, 6-MW (5′-CCGACTCGAGNNNNNNATGTGG-3′), as previously described.[6,21–23]

CGH and digital image analyses

CGH and digital image analyses were carried out as previously described.[6,7,21–27] Briefly, DNA extracted from tumors and lymphocytes was labeled by nick-translation with SpectrumGreen (Vysis, Downers Grove, IL, USA) and SpectrumRed (Vysis), respectively. Each labeled DNA sample (200 ng) and 10 μg of Cot-1 DNA (Gibco BRL, Gaithersbury, MD, USA) were mixed in 10 μl of hybridization buffer and co-hybridized onto normal denatured metaphase chromosomes for 48 h at 37°C. The slides were mounted in anti-fade solution containing 0.15 mg/ml 4,6-diamino-2-phenylindole (DAPI) as a counterstain. Images were captured with an Olympus BX50 fluorescence microscope (Olympus, Tokyo, Japan), equipped with a 100 × UplanApo objective lens and a cooled charge-coupled device (CCD) camera (SenSys 1400; Photometrics, Tucson, AZ, USA). At least 10, and in most cases approximately 20, representative images were analyzed (Fig. 3). Increases and decreases in DNA sequence copy numbers were defined by green-to-red ratios of 1:2 and 0:8, respectively, as previously described.[6,21,22] High-level copy number increases in subregions (amplifications), in contrast to whole-arm gains, were defined when the tumor/control ratio was 1:4.

Results

Cellularity of the specimens obtained by EUS-FNA

We could obtain tumor tissues from all the study patients with PC, using EUS-FNA. More than 100 tumor cells were harvested in one procedure. On average, 30% of the cells within the specimens were tumor cells. Contamination by normal cells such as fibroblasts and leukocytes affected the cellularity of tumor cells (Fig. 2a). These nontumor cells were removed completely using LCM.

CGH analysis

CGH analysis revealed at least four abnormal regions in all patients. Recurrent alterations are summarized in Fig. 4.

Tubular adenocarcinoma

The common loci of gains (including amplification) were, in order of frequency, 5p, 8q, and 20q (60% of the patients); and 1q, 7p, and 12p (27%); with minimal overlapping regions at 5p15, 8q24, 20q11–q13.2, 1q23–q31, 7p13–p14, and 12p12, respectively. The most frequent losses were 17p (73%); 9p, 18q, and 19p (47%); and 8p (33%); minimal overlapping regions of 17p, 9p, 18q, and 8p were located at 17p13, 9p24, 18q21–q22, and 8p23, respectively, whereas 19p showed losses of the whole chromosome arm (Table 2). In all patients who showed liver metastasis on imaging at the time of EUS-FNA, gain of 20q was noted. Gain of 20q was not

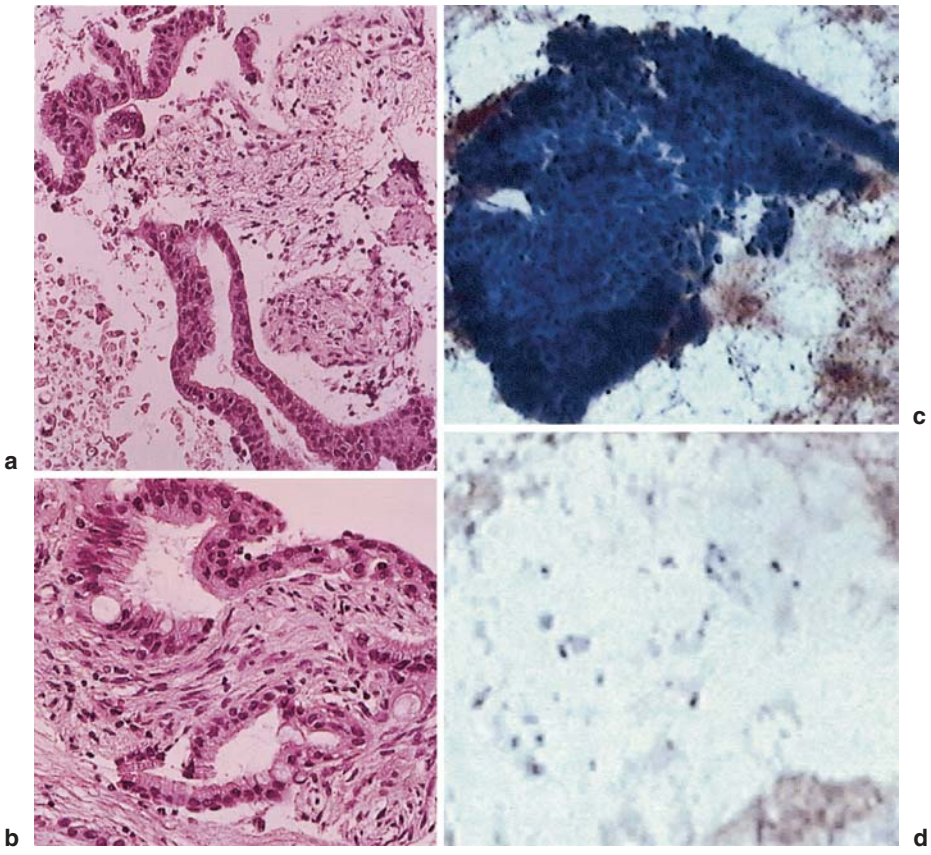

Fig. 2. **a** Tissues collected by diagnostic EUS-FNA (patient 13 in Table 1). The tumor cells are surrounded by normal cells such as fibroblasts and leukocytes. **b** Tissues collected by diagnostic EUS-FNA (patient 2). Papillo-tubular tissues and columnar proliferation of cancer cells can be seen **c** Tissues collected by EUS-FNA for comparative genomic hybridization (CGH; patient 9). **d** Tumor Cells collected by laser capture microdissection (LCM200) (patient 9). **a** and **b** H&E, ×200; **c** hematoxylin, ×200

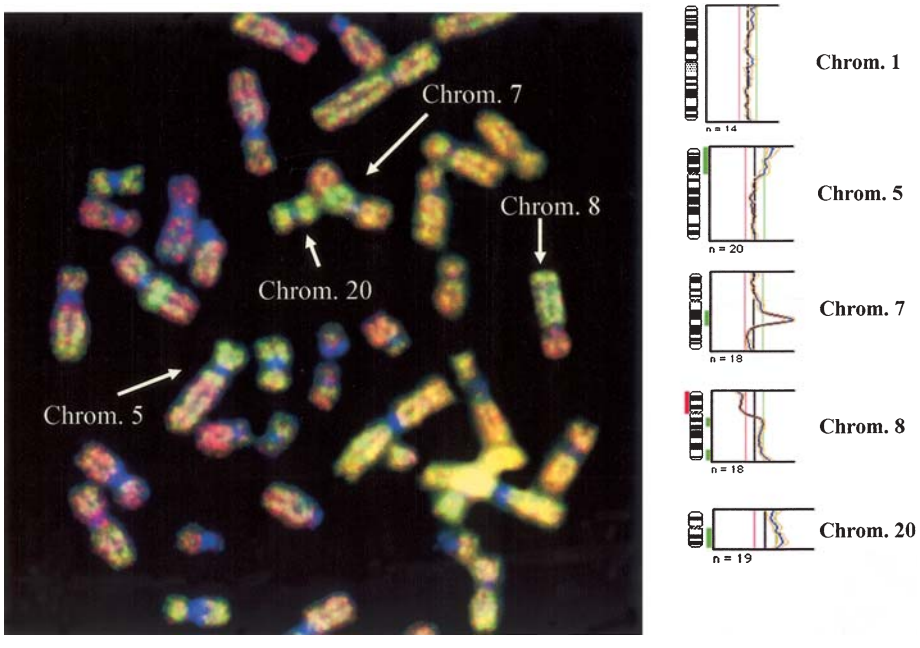

Fig. 3. Fluorescence image of CGH. Gains of 5p, 7q, 8q, and 20q are seen, as well as loss of 8p (case 9)

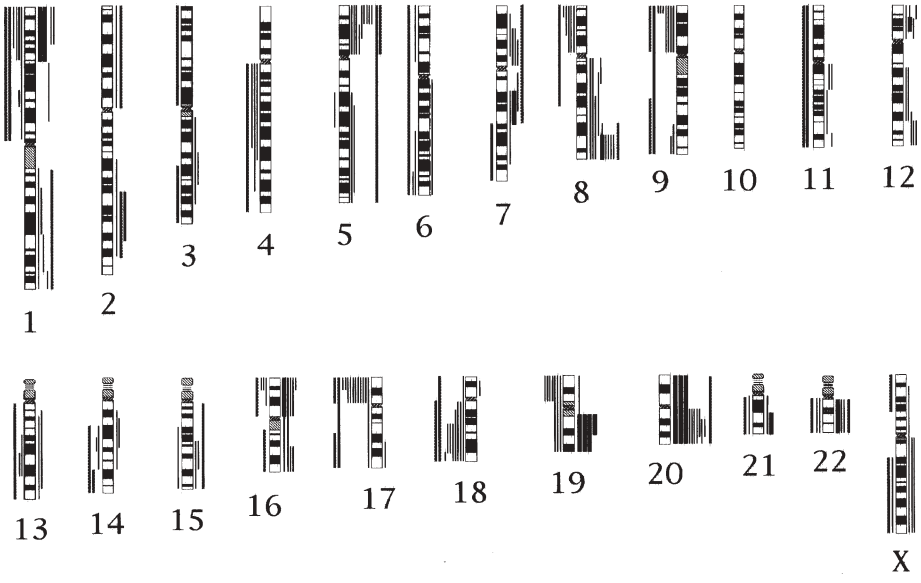

Fig. 4. CGH profiles of 17 patients with pancreatic cancer. Gains of chromosomes are shown on the *right* and losses are shown on the *left*. *Thick lines* denote amplification. Tubular adenocarcinomas are indicated with *straight lines*; acinar cell carcinomas, with *dotted lines*

Table 2. High-frequency DNA copy number alterations in tubular adenocarcinoma

Gain			Loss		
Locus	Frequency (%)	MOR	Locus	Frequency (%)	MOR
5p	60	5p15	17p	73	17p13
8q	60	8q24	9p	47	9p24
20q	60	20q11–q13.2	18q	47	18q21–q22
1q	27	1q23–q31	19p	47	Whole arm
7p	27	7p13–p14	8p	33	8p23
12p	27	12p12	4q	20	4q12–q27

MOR, minimal overlapping region

detected in 6 of the 11 patients without liver metastasis. There was no significant correlation between the genetic alterations and the clinicopathological features.

Acinar-cell carcinoma

Both of the patients with acinar-cell carcinoma showed gains of 2q and 5p, and losses of 1p, 9p, 9q, 11p, 11q, 14q, 17p, 17q, and 18q.

Discussion

Although CGH requires a relatively large amount of DNA for analysis, it still has a great advantage for comprehensive genetic analysis of the tumor. Thus far, there have been five reports of CGH analysis of PC. However, all of them dealt exclusively with surgical or autopsy specimens.[6–10] This is due to the difficulty of tissue sampling without surgical resection. To overcome this difficulty, we applied the CGH technique to tumor cells obtained by EUS-FNA in patients with PC.

In the present study, we examined EUS-FNA specimens from 17 PCs, using DOP-PCR CGH. Tissue microdissection and DOP-PCR were essential for reliable CGH analysis, because the tumor tissues obtained by EUS-FNA contained elements of normal tissues, such as fibroblasts and leukocytes, and were too small to perform conventional CGH.

In this series, gains of 5p, 8q, 20q, 1q, 7p, and 12p, and losses of 17p, 18q, 8p, 9p, and 19p were frequent in the patients with tubular adenocarcinoma (Fig. 4). We have previously reported CGH data for 32 surgically removed PCs.[6] Our present data basically corresponded to our previous results. In the present study, it was difficult to collect surgical specimens for investigation from the patients because most of the patients were inoperable or refused surgery. Nor could we demonstrate direct accordance of the DNA alterations between specimens obtained by EUS-FNA and those obtained by surgical resection. However, it has been reported that the value of CGH profiles from FNA samples was similar to or better than that of the corre-

sponding operative samples in patients with breast cancer.[28] Based on this finding, we thought that similar results would be observed by comparison in patients with PCs. Our previous report revealed that gains of 1q, 5p, 7p, and 8q were linked to the occurrence of lymph node metastasis in PC, and that a gain of 20q was associated with liver metastasis.[6] In the present study, frequent gains of 1q, 5p, 7p, and 8q were also found in patients with lymph node metastasis. All the patients with liver metastasis of tubular adenocarcinoma showed a gain of 20q, whereas the gain of 20q was not detected in 6 of the 11 patients without liver metastasis. There was no significant correlation between the CGH profiles and clinicopathological findings in the present study, because our results were preliminary, due to the limited number of patients. However, our findings indicated that DOP-PCR CGH analysis of cells obtained by EUS-FNA provided reliable genetic information.

We have used conventional CGH to investigate patients with PC, and reported DNA alterations related to progression. In the present study, we used conventional CGH, because we thought it would be possible to investigate the biological characteristics of tumors, considering previous data related to progression before surgery, if CGH could be done using preoperative samples. This study demonstrated that CGH analysis was possible for EUS-FNA biopsy specimens from PC, with the combination of microdissection and DOP-PCR. As we have reported before, DNA alterations in PC specimens appeared to vary because of the heterogeneity of the specimens.[21] However, the differences were slight, and most of the alterations in the different specimens were similar. Although analysis needs to be conducted carefully, while taking heterogeneity into consideration in order to apply the results of the CGH analysis to clinical work, this analytical strategy enabled us to evaluate the biological characteristics of each tumor (including metastatic potential), and these characteristics are not usually predicted by imaging examinations before treatment. Moreover, this technique could potentially be applied to array CGH, which is now evolving. We think that array CGH could be used in future studies for more detailed investigation. By selecting the best therapy, considering the individual tumor's biological characteristics, the prognosis and quality of life of patients with PC may be improved.

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