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NF1 and KRAS mutations in pancreatic cancer secondary to alcoholic chronic pancreatitis

Takaaki Murase[1], Kazuko Sakai[2], Takao Satou[3], Kazuto Nishio[2], Yoshifumi Takeyama[1]

[1] *Department of Surgery, Kindai University Faculty of Medicine, Osaka, Japan*
[2] *Department of Genome Biology, Kindai University Faculty of Medicine, Osaka, Japan*
[3] *Division of Hospital Pathology, Kindai University Hospital, Osaka, Japan*

Abstract

The risk of developing pancreatic cancer is significantly higher in the patients with chronic pancreatitis than in those without chronic pancreatitis (CP). However, the genetic mechanisms for this increased risk remain unclear. We hypothesized that different genetic mechanisms may exist in the process of carcinogenesis secondary to CP. We included patients with pancreatic cancer who underwent pancreatectomy between 2012 and 2016 at Kindai University Hospital. Among them, 3 patients had alcoholic CP for more than 2 years. We examined 3 types of tissue samples from each patient: cancerous, CP, and normal tissues. We extracted DNA from each tissue type and used next-generation sequencing (NGS) to detect mutations. We found genomic comutation of *KRAS* and *NF1* in 1 patient. There were no mutations in normal tissues, but mutations occurred in CP tissues. The rate of dissection of pancreatic ductal adenocarcinoma (PDAC) from cancerous tissues was approximately 30%, and the variant frequency of *NF1* and *KRAS* was 34% and 32%, respectively. The rate of dissection of pancreatic ductal tissue from CP tissues was approximately 20%, and the variant frequency of *NF1* and *KRAS* was 19% and 21%, respectively. Comutation of *NF1* and *KRAS* may be a carcinogenic mechanism of pancreatic cancer in patients with alcoholic CP. *NF1* and *KRAS* mutations may be a therapeutic target in patients with pancreatic cancer secondary to CP.

Key words : Pancreatic cancer, Chronic pancreatitis, Gene mutation, NF1, KRAS, Next-generation sequencing

Introduction

Pancreatic cancer has one of the highest rates of mortality.[1] Approximately 80% of patients with pancreatic cancer have unresectable disease at the time of diagnosis, and the 5-year survival rate of patients who undergo resection is as low as approximately 20% in Japan.[2] Conventional chemotherapy achieves a median survival time of only 8-11 months.[3-5] On the other hand, molecular targeted therapy shows considerable efficacy for specific types of cancers, such as lung cancer. [6-9]

Genetic mutations in *KRAS, CDKN2A, TP53,* or *SMAD4* are commonly detected in pancreatic ductal adenocarcinoma (PDAC).[10-12] However, targeted therapy for these mutations has not been established in clinical practice. It is essential to discover other candidate genes for targeted therapy.

The risk of developing pancreatic cancer is approximately 6 to 28 times higher in patients with chronic pancreatitis (CP) than in those without CP.[13,14] Furthermore, early image diagnosis of pancreatic cancer among CP patients is very difficult due to atrophy and calcification of the pancreas[15]. Kudo et al showed that 9 of 218 patients with CP developed pancreatic cancer, and 7 patients among them were diagnosed with pancreatic

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cancer more than two years after the diagnosis of CP. The 7 patients were all unresectable due to disease progression when diagnosed with pancreatic cancer[16]. However, the genetic mechanisms for this increased risk remain unclear. It is necessary to elucidate the genetic mechanism of progression to pancreatic cancer in CP. We hypothesized that different genetic mechanisms may exist in the process of carcinogenesis secondary to CP compared with those involved in general PDAC.

In this study, we analyzed surgical PDAC specimens obtained from three patients with CP. We compared the genetic differences in three types of tissue specimens: cancerous, CP, and normal tissues.

Materials and Methods

We included patients with pancreatic cancer who underwent pancreatectomy between January 2012 and December 2016 at Kindai University Hospital. Among them, 3 patients had CP for more than 2 years. This study was approved by the Ethics Committee of Kindai University Faculty of Medicine (27-029).

We examined 3 types of tissue samples from each patient: cancerous, CP, and normal tissues. Surgical specimens from cancerous and CP regions of the pancreas were available as formalin-fixed, paraffin-embedded (FFPE) or frozen tissue samples. Whole blood or other organ tissue was used as a normal sample for analysis.

The collected specimens were subjected to histological review by a pathologist. We collected tissue to extract DNA. DNA was extracted using an Allprep DNA/RNA FFPE kit for FFPE tissues, an Allprep DNA/RNA Mini kit for frozen tissues, and a QIAamp DNA Mini Kit for whole blood (all from Qiagen, Valencia, CA). DNA was then subjected to next-generation sequencing (NGS) panels to detect mutations. The quality and quantity of the DNA were verified using a NanoDrop 2000 (Thermo Scientific Wilmington, DE) and PicoGreen DNA assay kit (Life Technologies, Carlsbad, CA). The extracted DNA was stored at -80℃ until analysis.

For DNA sequencing, 10 ng of DNA was subjected to multiplex PCR amplification with an Ion AmpliSeq Library Kit 2.0 and Ion AmpliSeq Comprehensive Cancer Panel (Life Technologies, Carlsbad, CA), which cover 409 cancer related genes.[17] After multiplex PCR, Ion Xpress Barcode Adapters (Life Technologies) were ligated to the PCR products, which were then purified with the use of Agencourt AMPure XP Reagent (Beckman Coulter, Brea, CA). The purified libraries were pooled and then sequenced with the use of an Ion Proton instrument, Ion PI Sequencing 200 Kit, and Ion PI v3 Chip Kit (all from Life Technologies, Carlsbad, CA).

DNA sequencing data were accessed through the Torrent Suite v.4.2 program (Life Technologies, Carlsbad, CA). Reads were aligned against the Human Genome version 19,[18] and variants were called with the use of Variant Call Format ver 4.2. Raw variants were filtered with the following annotations: synonymous variants, quality score<100, and sequencing error, which was manually checked with the Integrative Genomics Viewer.[19] Germline mutations were excluded with matched normal regions and the Human Genetic Variation Database.[20]

Results

The patient characteristics are shown in Table 1. All the patients had CP for longer than 2 years (4-15 years) before the diagnosis of pancreatic cancer. The etiology of CP was alcoholic CP in all patients.

The specimen characteristics are shown in Table 2. For normal tissues, we collected whole blood in cases 1 and 2 and used tissue from an FFPE block of the stomach in case 3. Because case 1 and case 2 were prospectively included, and case 3 was retrospectively included, we obtained whole blood in cases 1 and 2, and because there was almost no normal tissue remaining in the pancreas in case 3, normal tissue was collected from the stomach, which was resected at the time of surgery. In the CP tissue specimens from the 3 cases, we used frozen tissue from case 1 and FFPE block from case 2 and 3. The frozen tissue sample from case 2 was not pathologically adequate because of a lack of target cells; thus, we used tissue from an FFPE block in case 2.

For DNA extraction, we collected tissue by manual dissection. The dissected area contained a relatively large number of target cells that stained with hematoxylin and eosin (HE), as confirmed by a pathologist. Target cells indicate PDAC in cancerous tissues. In addition, target cells represent the pancreatic duct, which includes pancreatic intraepithelial neoplasia (PanIN) 1-2 in CP tissues. The content rate of target cells in the sequenced specimens is shown in Table 2.

We analyzed the 409 cancer related genes

Table 1. Characteristics of 3 patients with pancreatic cancer secondary to chronic pancreatitis

Characteristics	Case 1	Case 2	Case 3
Sex	male	male	male
Age	75	72	55
Smoking history	yes	yes	yes
Alcohol consumption	yes	yes	yes
Diabetes	yes	yes	yes
Pancreatic stone	yes	yes	yes
Etiology of CP	alcohol	alcohol	alcohol
Duration of CP until surgery, (year)	7	4	15
Surgical procedure	TP	SSPPD	TP
Pathological diagnosis	PDAC with CP	PDAC with CP	PDAC with CP

CP, chronic pancreatitis; TP, total pancreatectomy; SSPPD, subtotal stomach-preserving pancreaticoduodenectomy; PDAC, pancreatic ductal adenocarcinoma

Table 2. Specimen characteristics from 3 tissue types: cancerous, chronic pancreatitis, and normal tissue.

Year of surgery		Cancer (content rate*1, %)		Chronic pancreatitis (content rate*2, %)		Normal
Case 1	2016	Frozen tissue	(30)	Frozen tissue	(20)	Whole blood
Case 2	2015	FFPE	(30)	FFPE	(20)	Whole blood
Case 3	2012	FFPE	(40)	FFPE	(20)	FFPE (stomach*3)

*1 the ratio of pancreatic ductal adenocarcinoma to all nucleated cells in the dissected cancerous tissue
*2 the ratio of pancreatic duct cells to all nucleated cells in the dissected chronic pancreatitis tissue
*3 stomach tissue included in the surgical specimen was used as a normal sample because a blood sample was not obtained prospectively.
FFPE indicates formalin-fixed paraffin-embedded tissue.

using NGS. The mean depth of cancerous, CP and normal tissues was 2004, 1999 and 1133, respectively, in case 1, 2070, 1773 and 1708, respectively, in case 2, and 3499, 330 and 4387, respectively, in case 3. The filtered variant call data are shown in Table 3. In case 1, *NF1* and *KRAS* were altered in both cancerous and CP tissues. Furthermore, the variant frequency was as high as 20-30%. The target cell content rates of cancerous and CP tissues were almost equal to the variant frequency of *NF1* and *KRAS* in cancerous and CP tissues. The Integrative Genomics Viewer shows the data of the 2 variants (Figure 1). There were no mutations in normal tissue in case 1, but there were mutations in CP tissues. In case 2, *PAX8*, *PIK3C2B* and *DST* were slightly changed relative to the content of target cells. The variant frequency of these genes were low; furthermore, there were few variants of the same genes in cancerous and CP tissues. In case 3, *TAF1*, *DCC*, *KRAS*, *TP53*, and *ERCC5* were slightly changed relative to the content of target cells. In particular, the variant frequency of *TAF1* was relatively high, but there were no changes in CP and normal tissues. The mean depth of CP in case 3 was as low as 330. Because this data was less than 1/10 of the mean depth of cancerous and normal tissues, this was not reliable data in CP tissues. There were no mutations of *NF1* in cases 2 and 3. On the other hand, no copy number variations were detected in all cases.

Table 3. Genetic variant frequency in 3 types of tissue samples: cancerous, chronic pancreatitis and normal tissue

Patient	Gene	Amino acid change	Variant frequency, % (variant read count/ total read count)					
			Cancer		Chronic pancreatitis		Normal	
Case 1	***NF1***	p. L1227V	**34**	(425/1262)	**19**	(213/1126)	**0**	(1/1480)
	KRAS	p. Q61H	**32**	(903/2828)	**21**	(661/3083)	**0**	(0/622)
Case 2	*PAX8*	p. W340C	6	(115/2087)	1	(13/2181)	0	(0/1040)
	PIK3C2B	p. R1499Q	6	(331/5606)	0	(10/5211)	0	(1/3132)
	DST	p. W4606G	5	(96/1813)	1	(4/688)	0	(0/1651)
	ERCC2	p. S541R	0	(6/2255)	5	(125/2367)	0	(4/1921)
Case 3	*TAF1*	p. T1537I	19	(32/166)	0	(0/8)	0	(0/15)
	DCC	p. R773H	7	(220/3143)	0	(0/1121)	0	(0/905)
	KRAS	p. G12D	7	(123/1794)	0	(0/168)	0	(33/7602)
	TP53	p. K132R	6	(146/2652)	0	(0/545)	0	(0/411)
	ERCC5	p. G1053E	6	(257/4544)	0	(0/11)	1	(5/477)
	ATM	p. R337C	5	(153/2940)	0	(0/82)	0	(0/376)

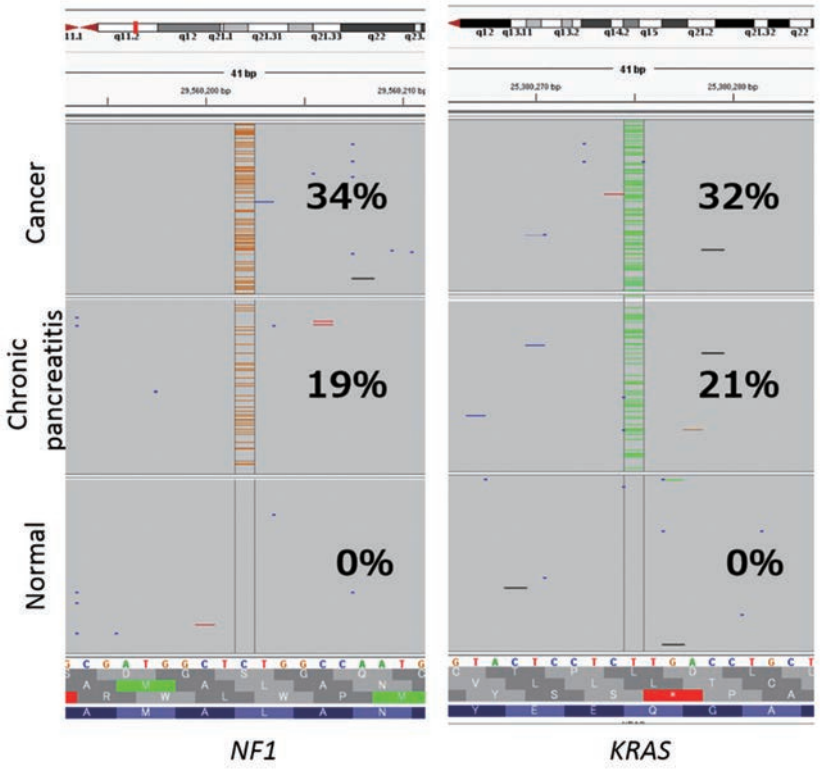

Figure 1. ***KRAS* and *NF1* mutation in Case 1.**
A screen shot from the Integrative Genomic Viewer. The center represents the DNA sequences, and the bottom is the reference genomic sequence. The sequencing data from case 1 indicate that *KRAS* and *NF1* were altered. The acquired L1227V C→G mutation (brown) in *NF1* and Q61H T→A mutation (green) in *KRAS* are shown. The variant frequency in *NF1* in cancerous, CP, and normal tissues was 34%, 19%, and 0%, respectively, and that in *KRAS* in cancerous, CP, and normal tissues was 32%, and 0% respectively.

Discussion

In this study, we analyzed genetic mutations of PDAC secondary to alcoholic CP. In case 1, we found considerable genomic comutation of *KRAS* and *NF1*. The content of PDAC in cancerous tissues was approximately 30%, and the variant frequency of *NF1* and *KRAS* was 34% and 32%, respectively. This suggests that PDAC has *NF1* and *KRAS* mutations, and these mutations occur during CP.

NF1 encodes neurofibromin and appears to function as a negative regulator of the Ras signal transduction pathway. Neurofibromin is a GTPase-activating protein for Ras. Mutations in this gene have been linked to neurofibromatosis type 1, juvenile myelomonocytic leukemia (JMML) and other diseases.[21,22] *NF1* mutations in pancreatic cancer are rare. Among 176 patients with pancreatic cancer in The Cancer Genome Atlas (TCGA),[23] there are 4 patients carrying *NF1* variants. Interestingly, 3 of the 4 patients also had *KRAS* mutations. *NF1* deficiency is considered functionally equivalent to an oncogenic *KRAS* because *NF1* is a Ras-GTPase–activating protein. However, Cutts et al demonstrated a strong relationship between *NF1* deficiency and an oncogenic *KRAS*. In mice, expression of an oncogenic *KRAS* or inactivation of *NF1* in hematopoietic cells results in myeloproliferative disorders. They showed that the simultaneous inactivation of *NF1* and expression of *KRAS* G12D in mouse hematopoietic cells resulted in an earlier occurrence of acute myeloid leukemia (AML) and a lower survival rate than inactivation of *NF1* and mutation of *KRAS*. This suggests that comutation of *NF1* and *KRAS* led to earlier onset of myeloid malignancy and increased severity of disease compared with single mutations of *NF1* or *KRAS*.[24] The reason is thought to be because *NF1* might be involved in other signaling pathways. The domain from 1198 to 1551 in *NF1* is proposed to be the Ras-GTPase-activating domain of neurofibromin[25]. In case 1, the variant of *NF1* (L1227V) may have associated with carcinogenesis. The mutation frequencies of *KRAS* and *NF1* were almost the same in the same tissue. Therefore, the comutation of *KRAS* and *NF1* might have occurred in the same cell. Furthermore like in cancer tissue, each mutation was considered to be already homozygous in CP because the content rate of target cells and variant frequency were similar in CP and cancer tissue. In addition, there was no change in CNV, no appearance of any other somatic mutations in *KRAS* or *NF1* gene respectively, and no loss of heterozygosity.

Therefore this comutation is unlikely to be the direct cause of carcinogenesis. Additional other gene mutations not present in this panel and changes in gene expression levels might have promoted carcinogenesis in case 1. Current targeting therapy of *KRAS* and *NF1* mutations is ineffective. However, Hayashi et al reported that in non-small cell lung cancer cell lines with comutation of *RASA1* and *NF1* were more sensitive to MEK inhibitors than cell lines with single mutations of either *RASA1* or *NF1*.[26] *RASA1* and *NF1* suppress the Ras pathway upstream and behave similarly to oncogenic *KRAS*. Therefore, comutation in same pathway, such as in case 1, requires further study for its potential as a therapeutic target.

On the other hand, CP is considered to be a significant etiological factor for the development of pancreatic cancer. Inflammatory responses play a significant role in carcinogenesis. Activated inflammatory cells induce DNA damage and genomic instability.[27] Muligan et al have reported *KRAS* mutations in CP.[28] There are few genetic reports on carcinogenesis from alcoholic CP, and it is unclear whether mutations in genes other than *KRAS* occur in alcoholic CP. In this case, comutation of *KRAS* and *NF1* may have occurred due to chronic inflammation, and this comutation may have associated with carcinogenesis. Screening CP patients for pancreatic cancer is controversial even in high-risk groups of patients with hereditary or tropical forms of pancreatitis[14]. However, Mu et al reported *KRAS* mutations in samples obtained by fine-needle aspiration, (FNA) and endoscopic retrograde pancreatography (ERP) cytology may be useful for differentiating pancreatic cancer from CP.[29] By subgrouping mutations in *KRAS* and other gene mutations, it might be possible to anticipate the risk of cancer development in pancreatitis patients and to individually adjust subsequent follow-up in the future.

Limitation of this study is that it does not directly indicate that *NF1* and *KRAS* simultaneously mutated to promote the initiation of pancreatic cancer. In addition, in this report, the total number of subjects was small. However, it is difficult to obtain surgical specimens from patients who develop pancreatic cancer secondary to CP because of the low diagnostic rate. Further experiments are required.

Conclusion

We found comutation of *NF1* and *KRAS* in patients with pancreatic cancer secondary to alcoholic CP. This may be a carcinogenic mechanism of CP. This finding deepens our understanding of pancreatic cancer. *NF1*-based therapeutic approaches are currently limited; however, *NF1* mutation or comutation of *KRAS* and *NF1* may be therapeutic target in patients with pancreatic cancer secondary to CP in the future.

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