Amplification of mutant *KRAS*^{G12D} in a patient with advanced metastatic pancreatic adenocarcinoma detected by liquid biopsy: A case report

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Abstract. Pancreatic ductal adenocarcinoma (PDAC) remains one of the deadliest cancer types. Activating oncogenic *KRAS* mutations are commonly observed in PDAC; however, oncogenic *KRAS* amplification is rarely observed, and its significance in prognosis and resistance to therapy remains poorly characterized. The present report describes the case of a 52-year-old male patient diagnosed with advanced PDAC with liver metastasis. The patient received modified FOLFIRINOX (mFFX) therapy to which the patient became intolerant with a strong inflammatory response. Subsequent treatment with gemcitabine plus nab-paclitaxel failed to control the disease. Targeted genetic analysis revealed *KRAS*^{G12D} and *TP53*^{R248Q} mutations in the primary tumor and liver metastases. Analysis of circulating tumor DNA (ctDNA) before the first line of treatment confirmed these genetic findings and revealed a >4-fold amplification of the mutant *KRAS*^{G12D} not detected in the primary tumor. Additionally, subsequent analysis confirmed a 5-fold amplification of the *KRAS*^{G12D} allele in liver metastasis. Consecutive monitoring of ctDNA revealed an initial decrease in the tumor burden 2 weeks after the first cycle of mFFX. However, coinciding with treatment intolerance, a sharp increase in tumor mutational levels and *KRAS*^{G12D} amplification was observed 1 month later. The patient died 70 days after treatment initiation. Overall, amplification of oncogenic *KRAS*^{G12D} was not only associated with an aggressive phenotype, but also supported cancer resistance to chemotherapy. Importantly, this case suggests that plasma detection of *KRAS*^{G12D} amplification is feasible in the clinical routine and constitutes a powerful tool for assessing tumor aggressiveness.

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive epithelial tumors with a 5-year survival rate of <10% (1), which dismal prognosis is greatly related to a diagnosis at late stages and few effective treatment options. The prognosis of patients with PDAC has barely changed over the past two decades, as there are no reliable biomarkers for early detection (2). Although modest advances have been made in treatment options with combination therapies (3-5), recurrence rates remain high (~80%), with patients relapsing within 2 years (6). Hence, implementation of new diagnostic methods, such as liquid biopsy, may help enhance detection accuracy and monitoring tumor progression in real time.

PDAC occurs due to the accumulation of multiple genetic alterations, including activation of oncogenes or loss of tumor-suppressors, as well as aberrant function of signaling pathways (7). Acquisition of mutations in *KRAS* (KRAS proto-oncogene, GTPase) is regarded as a driver event in PDAC. However, several *in vivo* studies showed that mutated *KRAS* alone is insufficient to trigger metastatic transformation (8). Combination with other frequently found inactivating mutations in genes such as *CDKN2A* (cyclin-dependent kinase inhibitor 2A), *SMAD4* (SMAD family member 4), and *TP53* (tumor protein p53), or epigenetic changes in key genes, are recognized to further enhance tumorigenesis and metastasis. In fact, 70-90% of PDAC cases harbor co-occurring *KRAS* and *TP53* mutations (9), constituting the most common genetic alterations in PDAC. Although overexpression of wild-type and mutated *KRAS* is well recognized in colorectal and non-small cell lung cancers (10,11), it remains poorly known in PDAC.

Here, we report the case of a patient with advanced PDAC with multiple liver metastases found to bear marked amplification of the oncogenic *KRAS*^{G12D} allele as detected by liquid biopsy. Mutant *KRAS* amplification may have important clinical implications, including increased risk for resistance to treatment.

Table I. Mutations detected in tissue samples.

	Primary tissue			Liver metastasis			White blood cells		
Mutation	Mol depth, x	Counts, n	VAF, %	Mol depth, x	Counts, n	VAF, %	Mol depth, x	Counts, n	VAF, %
KRAS-G12D	3,831	1,766	46.10	18,844[a]	17,245	91.51	3,529	0	0.00
TP53-R248Q	2,194	1,129	51.46	1,975	1,209	61.22	9,544	0	0.00
GNAS-R201H	4,073	0	0.00	4,949	0	0.00	6,812	6	0.10

[a]Mol depth surpassed maximum expected value for unamplified samples. GNAS, GNAS complex locus; Mol, molecular; VAF, variant allele frequency.

Case report

A 52-years-old Japanese man with no relevant medical history visited our hospital in early July 2019 with chief complaints of persistent upper abdominal pain for 2 months. The patient had a 36-year history of smoking and daily alcohol consumption. No family history of cancer was reported. Physical examination showed high fever (>38.0˚C) and tenderness in the upper abdomen. Laboratory data revealed mild liver dysfunction and normal levels of carbohydrate antigen 19-9 (CA19-9; 31.9 U/ml), with duke pancreatic monoclonal antigen type 2 (DUPAN-2) >1,600 U/ml. Abdominal contrast-enhanced computed tomography (CT) revealed a hypovascular tumor mass of 25 mm in the head of the pancreas. Multiple liver metastases with different masses were detected and no metastases at other sites were evident on CT (Fig. 1A). The patient was immediately admitted, and endoscopic ultrasound-guided fine needle aspiration was performed on the primary tumor and metastases. Histological analysis confirmed that it was an adenocarcinoma, classified as cT3 cN0 cM1(Hep) and cStage IV according to the Union for International Cancer Control criteria and Tumor-Node-Metastasis classification (12). The patient started FOLFIRINOX (mFFX; folinic acid, fluorouracil, irinotecan, and oxaliplatin combo) therapy 2 weeks after the diagnosis in July 2019. On day 3 of treatment, the patient experienced liver dysfunction with increased levels of uric acid and creatinine. On day 6, a strong myelosuppressive effect [white blood cell (WBC): 600/μl, neutrophils: 256/μl, and platelets: 40,000/μl] was observed along with disseminated intravascular coagulation and acute renal failure. On day 8, the patient experienced encephalopathy and a marked increase in the levels of procalcitonin (PCT; 56.9 ng/ml) and C-reactive protein (CRP; 21.62 mg/dl) were observed, most likely as a result of tumor tissue damage. On day 19, a CT assessment revealed a reduction in the primary lesion; thus, the mFFX treatment was initially considered to be effective. However, soon after, a strong fever recurred with increased CRP levels. Therefore, mFFX re-administration was considered severely adverse and intolerable. With the patient informed consent, the regimen was changed and treatment continued with an intravenously administrated second-line therapy, named GnP, comprising gemcitabine (1,000 mg/m^2) and nab-paclitaxel (125 mg/m^2) (13). GnP did not induce adverse reactions as potent as mFFX, but still resulted in an inflammatory response and elevated levels of procalcitonin (Fig. 2). Despite a slight recovery after chemotherapy, the patient general condition continued to deteriorated and a myriad of new metastatic liver tumors emerged with uncontrollable growth patterns. The patient died of gastrointestinal bleeding associated with disseminated intravascular coagulation 70 days after treatment initiation.

Genetic analysis was performed in both tumor tissue and liver biopsies by amplicon-based next-generation sequencing (NGS) with the Ion AmpliSeq Comprehensive Cancer Panel (Thermo Fisher Scientific, Inc.) of 509 genes. Activating *KRAS*^{G12D} and *TP53*^{R248Q} mutations, along with increased copy number variations of the proto-oncogenes *MYC* (MYC proto-oncogene, bHLH transcription factor) and *MAF* (MAF bZIP transcription factor) were detected in both primary tumor and metastasis samples (Tables I and II). Plasma samples were collected just before the first-line chemotherapy and in weeks 2 and 4 after treatment initiation, and circulating tumor DNA (ctDNA) was analyzed (Tables III and IV). Ultradeep targeted NGS with the Oncomine pan-cancer cell-free assay (Thermo Fisher Scientific) was used to investigate genetic alterations in 52 genes. ctDNA from before the first line of treatment confirmed the genetic alterations found in the primary tumor and revealed an amplification in the mutant *KRAS*^{G12D} allele [variant allele frequency (VAF) = 87.2%). Mutant allele amplification was detected based on the capped molecular depth (19,999x] obtained in the ctDNA sequence, which surpassed by at least 4-fold the maximum expected molecular depth in unamplified regions (4,980x) based on the sample input (16.6 ng). A 5-fold amplification of *KRAS*^{G12D} in the metastasis samples (VAF=91.5%) was subsequently confirmed by digital polymerase chain reaction and NGS sequencing. A *GNAS*^{R201H} mutation (VAF=0.05%) was also detected in the plasma liquid biopsy. Since this mutation was not present in the primary tumor tissue, genomic DNA from the WBCs was also analyzed. The *GNAS*^{R201H} mutation was confirmed in the WBCs (VAF=0.1%), indicating its association with clonal hematopoiesis rather than with the pancreatic tumor (Table I). Analysis of ctDNA 2 weeks after the first mFFX cycle showed an initial decrease in *KRAS*^{G12D} and *TP53*^{R248Q} frequency (VAF = 25.3 and 3.9%, respectively). However, ctDNA analysis at week 4 of mFFX indicated an upregulation of the mutant VAF levels and in *KRAS*^{G12D} amplification, close to the levels prior to treatment (Fig. 1B; Table III).

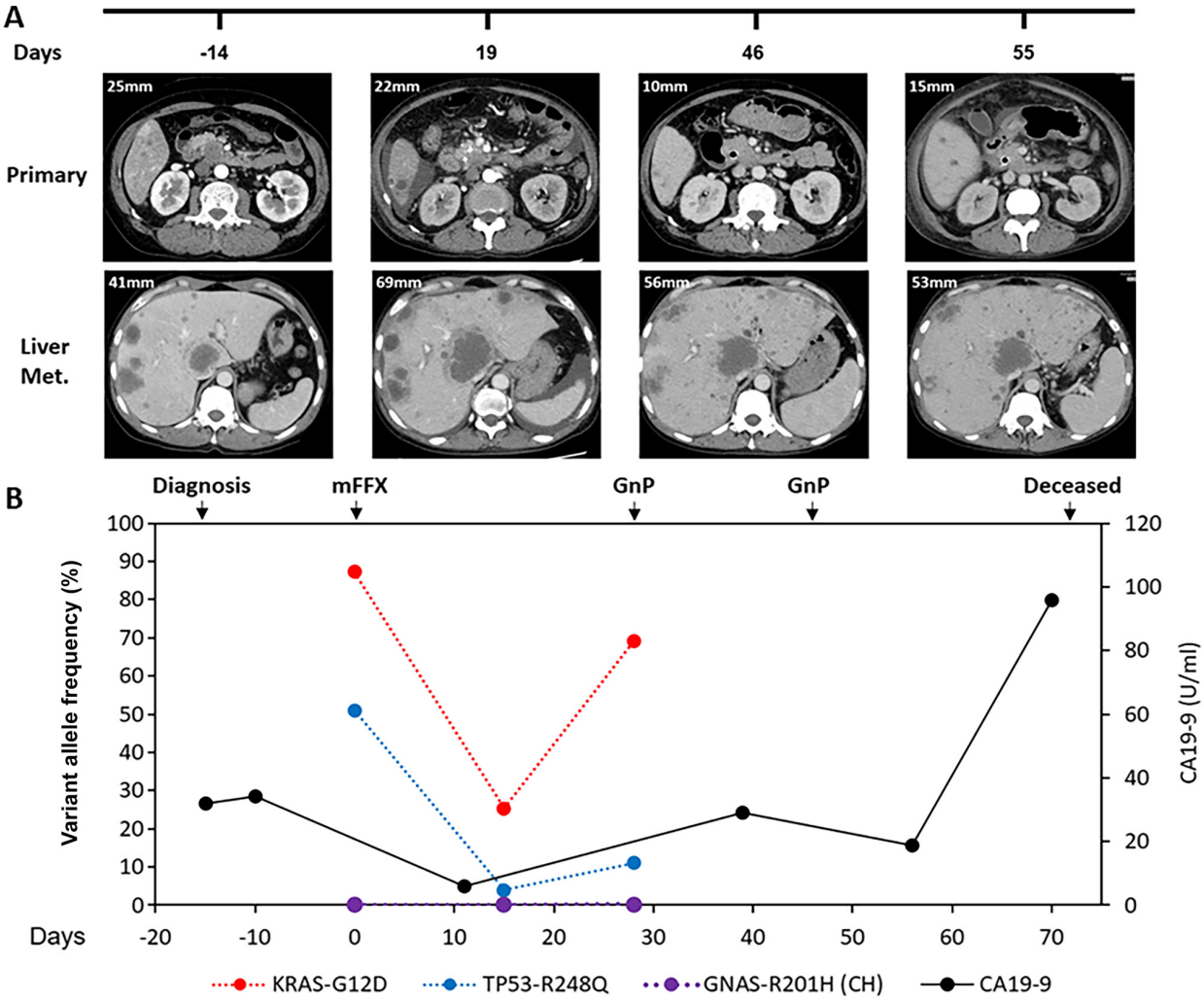

Figure 1. Dynamics of ctDNA during the course of treatment. (A) CT scan depicting the evolution of the primary tumor and liver metastasis from diagnosis (day 14) to after treatment initiation (day 55). The diameter of the primary tumor lesion and the main liver metastatic tumor is shown at the top of each image. (B) ctDNA dynamics of the detected mutations in association with treatment. The black line represents the dynamics of the tumor marker CA19-9. mFFX and GnP treatment cycles are indicated with arrows. CH, clonal hematopoiesis; GnP, gemcitabine plus nab-paclitaxel; mFFX, modified FOLFIRINOX (folinic acid, fluorouracil, irinotecan, and oxaliplatin combo); ctDNA, circulating tumor DNA; Met., metastasis; GNAS, GNAS complex locus.

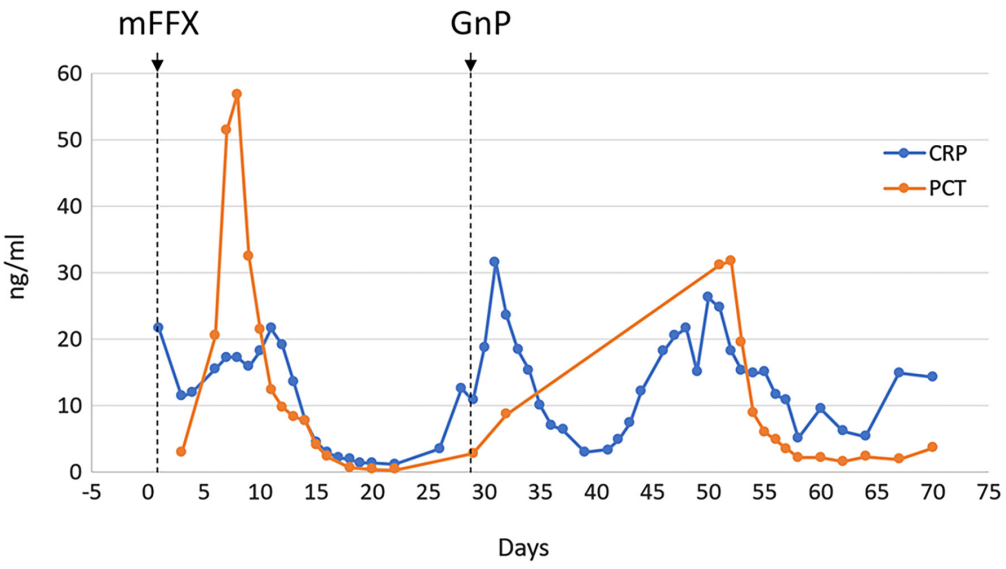

Figure 2. Levels of PCT and CRP in response to treatment. GnP, gemcitabine plus nab-paclitaxel; mFFX, modified FOLFIRINOX (folinic acid, fluorouracil, irinotecan, and oxaliplatin combo); PCT, procalcitonin; CRP, C-reactive protein.

Table II. CNVs detected in tissue samples.

	Primary tissue		Liver metastasis		White blood cells	
Gene <CNV>	Copy no.	CNV ratio	Copy no.	CNV ratio	Copy no.	CNV ratio
KRAS	4	2.0	10	5.0	0	0
MYC	6	3.1	6	3.0	0	0
MAF	7	3.5	8	4.0	0	0

CNV, copy number variation; GNAS, GNAS complex locus; MAF, MAF bZIP transcription factor.

Table III. Mutations detected in ctDNA samples.

	Before treatment			2 weeks after treatment			4 weeks after treatment		
Mutation	Mol depth, x	Counts, n	VAF, %	Mol depth, x	Counts, n	VAF, %	Mol depth, x	Counts, n	VAF, %
KRAS-G12D	19,999[a]	17,449	87.20	7,111	1,798	25.30	13,736[a]	9,493	69.10
TP53-R248Q	3,144	1,602	51.00	4,207	165	3.92	3,794	418	11.00
GNAS-R201H	3,787	4	0.05	6,175	5	0.08	6,606	8	0.12

[a]Mol depth surpassed maximum expected value for unamplified samples. Mol, molecular; VAF, variant allele frequency; GNAS, GNAS complex locus.

Table IV. CNVs detected in ctDNA samples.

	Before treatment		2 weeks after treatment		4 weeks after treatment	
Gene <CNV>	Copy no.	CNV ratio	Copy no.	CNV ratio	Copy no.	CNV ratio
MYC	2.8	1.4	2.1	1.0	2.4	1.2

CNV, copy number variation.

Discussion

KRAS activating mutations and *TP53*, *CDKN2A*, and *SMAD4* loss-of-function alterations are the most common genetic alterations found in PDAC. Nevertheless, a large number of infrequent mutations and copy number variations in multiple genes are also detected, resulting in significant interindividual heterogeneity (7,14). In addition, the oncogenic effect of MYC is well established as a critical effector of activated RAS in several cancer types, including PDAC (15).

Allelic imbalance caused by amplification of mutant *KRAS* is more frequently reported in high-grade tumors of NSCLC and can affect its response to therapy (10,11). Amplification of *KRAS*^{mut} in PDAC, although less documented, confers an increased metastatic potential by inducing robust epithelial-mesenchymal transition signatures, being associated to worse prognosis (16). However, one of the challenges in accurately detecting gene amplification in PDAC is the presence of high stromal cell content within the tumor tissue (17), with non-neoplastic stroma confounding precise gene dosage and comprehensive interpretation of copy number alterations. Although tissue biopsies are the gold standard for diagnosis and molecular characterization of tumors, the analysis of ctDNA from liquid biopsies can avoid the interference of non-neoplastic stromal cells and capture the intrinsic influence of tumor heterogeneity during the course of the disease. In this case, we detected *KRAS*^{G12D} amplification in the ctDNA but not in the primary tumor. Amplification was later confirmed in the metastatic tissue, reflecting the heterogeneous evolution of the tumor. *KRAS*^{G12D} amplification was associated with rapid tumor growth, suggesting that it may play an important role in promoting the metastatic spread of PDAC cells. In addition, the poor response of the patient to both lines of treatment suggests that the presence of amplified *KRAS*^{G12D} may also impair the tumor sensitivity to chemotherapy. Recognition of this information in advance may help predict treatment-related deterioration of the patient general condition. This is of particular importance for selecting treatment approaches that consider the rate of tumor collapse to help control poor prognosis PDAC cases. Hence, albeit underrated in the clinical setting, amplification of oncogenic *KRAS*^{G12D}, which constitutes a key driving force that adds to an aggressive PDAC phenotype, can be detected in ctDNA through routine liquid biopsy.

This case highlights the importance of accurate detection of gene-dosage gains in oncogenic *KRAS* mutations in PDAC. $KRAS^{G12D}$ amplification in combination with *TP53* mutation and deregulated MYC expression may be associated an aggressive PDAC phenotype. Hence, in light of the heterogeneous characteristics of aggressive pancreatic cancers, monitoring tumor evolution through liquid biopsies can help identify such cases at earlier stages. Importantly, the amplification of oncogenic $KRAS^{G12D}$ can be successfully detected through liquid biopsy and is feasible for implementation in the clinical setting.

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Availability of data and materials

All data generated or analysed during this study are included in this published article.

Authors' contributions

MM, YN, SKL, YK and FPS were involved in the conception, design and execution of the study and manuscript writing. MM, SKL and FPS collected and analyzed the data. MM and YK provided study material. MM and FPS confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The patient provided informed consent in writing for participation in the study approved by the Japanese Foundation for Cancer Research Review Board (IRB 2018-1016).

Patient consent for publication

The patient provided informed consent in writing regarding the publication of the study approved by the Japanese Foundation for Cancer Research Review Board (IRB 2018-1016).

Competing interests

The authors declare that they have no competing interests.

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