Utility of Serum DNA as a Marker for KRAS Mutations in Pancreatic Cancer Tissue

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Keywords

KRAS, droplet digital polymerase chain reaction, liquid biopsy, pancreatic cancer

Abstract

Background/Objectives: The detection of cancer-specific DNA in peripheral blood, known as a liquid biopsy, has been reported recently. Most such studies have used plasma as a sample; however, whether or not serum can be used as effectively is unclear. We attempted to clarify suitable samples for detecting KRAS mutations in circulating DNA in the blood of pancreatic cancer patients using droplet digital polymerase chain reaction (PCR).

Methods: DNA was extracted from the tissue, plasma, and serum of 40 pancreatic cancer patients. The presence of KRAS mutations G12D, G12V, and G12R was analyzed by droplet digital PCR.

Results: The amount of DNA isolated from the serum was much higher than that from plasma (1.0- to 42.0-fold). At least 1 KRAS mutation was observed in 93% of cancer tissues, whereas we detected the mutations in only 48% of the serum and plasma DNA samples. The G12D mutation was the most prevalent of the three mutations, followed by the G12V mutation. The presence of the G12D KRAS mutation in the plasma, serum, or tissue did not correlate to the overall survival; however, the prognosis of the patients with a KRAS mutation at G12V in the plasma or serum was significantly poorer than that of the patients without the mutation ($P < 0.01$).

Conclusions: Serum and plasma were found to be good materials for detecting cancer-specific DNA in the peripheral blood and the presence of KRAS mutations in blood-derived DNA may be used as a prognostic biomarker for patients with pancreatic cancer.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is associated with particularly poor outcomes. It is the eighth leading cause of cancer death in males and ninth among females worldwide [1]. During the progression from normal ductal epithelium to PDAC, multiple genetic alterations occur sequentially, such as mutations in KRAS, p16, p53, DPC4, and BRCA2 [2]. The mutations in KRAS occur at the pancreatic intraepithelial neoplasm (PanIN) stage, which is the most common precursor to invasive pancreatic adenocarcinoma [2, 3]. The frequency of mutations in KRAS is very high in pancreatic cancer (75%-90%) [3-6], so KRAS mutations are expected to be useful biomarkers for early detection and/or as prognostic factors.

Until recently, resected or biopsied tissue samples were needed to analyze the presence of mutations in KRAS. However, there are some disadvantages in the examination of tissue samples. First, it is invasive to obtain such samples from patients, and retrieving samples multiple times during treatment is difficult, as the repetition of biopsies simply increases the risk of complications. Second, we might miss crucial mutations occurring in only a small portion of the tissue, as there might be molecular heterogeneities in the tumor. To overcome these issues, a noninvasive method of assessing the total tumor characteristics—liquid biopsy—is under development. Cell-free circulating tumor DNA (ctDNA) theoretically comes from cancer cells, at rapidly growing sites in the tumor where cancer cells divide and collapse frequently; they are therefore representative of the genome at the poorest differentiation site in the tumor. Recently, the development of highly sensitive techniques, such as the digital polymerase chain reaction (PCR), beads,

emulsions, amplification, magnetics (BEAMing) or amplification refractory mutation systems (ARMs), have enabled the detection of cancer-specific DNA in the blood [7-10]. Generally, plasma has been used as the material for detecting ctDNA; very few techniques have used serum as a source because the serum contains genomic DNA released from white blood cells during the clotting process, which can interfere with the detection of ctDNA [11-14]. We recently examined the ctDNA in serum samples using droplet digital PCR and found that KRAS mutations could be detected at a relatively high rate (62.5%) in the serum of the patients with advanced pancreatic cancer [15]. However, whether plasma is superior or inferior to serum for the detection of ctDNA in this highly sensitive system is unclear.

In this study, we compared the detection rates of KRAS mutations in ctDNA samples extracted from the serum and plasma of patients with pancreatic cancer and tried to determine the best samples for a liquid biopsy with digital droplet PCR.

Methods

Characteristics of the Patients

We selected patients with histologically diagnosed pancreatic cancer who were admitted to our institute between September 2013 and February 2016. Written informed consent for this study was obtained from all patients prior to their enrollment. Forty patients were enrolled in this study. Ten healthy individuals with no prior history of any cancers were recruited as controls. This study was approved by the institutional review board of Okayama University Hospital and was conducted in accordance with the Declaration of Helsinki.

DNA extraction

Tissue samples were collected from all pancreatic cancer patients by surgical resection or endoscopic ultrasound fine needle aspiration (EUS-FNA). DNA was extracted from 16 surgically resected formalin-fixed, paraffin-embedded (FFPE) specimens using a QIAamp DNA FFPE Tissue Kit (Qiagen, Valencia, CA, USA), and from 24 samples obtained by EUS-FNA using a QIAamp DNA Mini Kit (Qiagen). Paired plasma and serum samples were also collected from all patients, with isolation performed within 3 h after blood sampling. Fifty microliters of circulating cell-free DNA was extracted from 1 mL each of paired plasma and serum using a QIAamp Circulating Nucleic Acid Kit (Qiagen) in accordance with the manufacturer's instructions. DNA eluent was frozen at -30 °C until analyzed with droplet digital PCR. DNA

from paired plasma and serum samples of healthy individuals was also extracted by the same way. All obtained DNAs were quantified using a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA [please describe the company location]).

Digital droplet PCR

We used a QX200 droplet digital PCR system (Bio-Rad Laboratories, Hercules, CA, USA) to analyze the presence of mutations and wild-type status in KRAS, as described previously [15]. We used three types of probes for KRAS to analyze the mutations G12D, G12V, and G12R (Bio-Rad Laboratories), as these are the three major KRAS mutations in pancreatic cancer [16, 17]. Five microliters of DNA from tissue, plasma, and serum was added to 10 μL of Droplet PCR Supermix (Bio-Rad Laboratories), 2 μL of primer/probe mixture and 5 μL of sterile DNase-and RNase-free water. A total 22 μL of mixture was added to 70 μL of Droplet generator oil (Bio-Rad Laboratories) in order to generate droplets. The emulsion was thermal cycled, starting with enzyme activation of 10 minutes at 95 °C, followed by 40 cycles of 30 seconds at 94 °C and 1 minute at 60 °C. The cycling then finished with 10 minutes at 98 °C for enzyme deactivation. When the cycling was complete, the fluorescence signal of each droplet was measured.

Data analysis

The data of the fluorescence signals were analyzed with the Quanta software program, version 1.4.0 (Bio-

Rad Laboratories), in accordance with the manufacturer's instructions, to determine the number of droplets that were positive for wild-type KRAS and/or KRAS mutations. In this study, we set the cut-off values at 2000 for the x-axis (mutation) and 2000 for the y-axis (wild-type). In addition to these cut-off values, we set the detection rate at >0.0222%. The detection rate was estimated with a Poisson distribution.

Statistical analyses

The baseline characteristics were summarized as medians and ranges. The overall survival was measured from the day of diagnosis to the date of death or the last follow-up date. The overall survival curves were calculated using the Kaplan-Meier method, and the log-rank test was used to compare the overall survival curves. All significance tests were two-sided, and differences with a p-value less than 0.05 were considered significant. All statistical analyses were performed using the JMP software program, ver. 11.0 (SAS Institute Japan Ltd., Tokyo, Japan).

Results

Characteristics of the Patients

The median age was 71 years (range 49-92 years), and 26 patients (65%) were male. Median size of primary tumor was 30 mm (range 14-69 mm). The number of patients in stage I, II, III, and IV was 2 (5%), 21 (52%), 6 (15%), and 11 (28%), respectively. The median follow-up period was 7.8 months (range 0.3-30.2 months).

DNA concentrations in serum and plasma

We analyzed the amount of DNA in the paired plasma and serum samples of healthy individuals and of pancreatic cancer patients (Figure 1). The median plasma DNA concentrations in pancreatic cancer patients and healthy individuals were 17.9 and 10.7 ng/mL, respectively ($P < 0.01$). The DNA concentration in the plasma of stage III/IV pancreatic cancer patient tended to be higher than in stage I/II patients (22.6 and 15 ng/mL, respectively), but the difference was not significant ($P = 0.27$). The median serum DNA

concentrations in pancreatic cancer patients and healthy individuals were 129 and 110 ng/mL (P = 0.73), respectively. The concentrations in the serum were much higher than those in plasma, by 7.2- and 10.3-fold, respectively. However, as with plasma, no significant differences in the concentrations were observed between the pancreatic cancer patients at stage I/II and those at stage III/IV.

Determination of cut-off level for digital droplet PCR

We analyzed the KRAS mutations in DNA samples extracted from 10 paired plasma and serum samples of healthy individuals. We detected the KRAS mutation G12D in 1 of the serum samples, as 1 mutation-type droplet in 3807 wild-type droplets. The frequency of the mutation droplet estimated with Poisson distribution was 0.0222%. We therefore judged this mutation-type droplet to be a false positive and defined a frequency over 0.0222% as the threshold of true positive.

KRAS mutations in pancreatic cancer samples

We compared the frequencies of KRAS mutations at G12D, G12V, and G12R in tissue, plasma, and serum samples (Table 2). The G12D mutation was the most prevalent mutation detected in pancreatic cancer tissue (73%), followed by G12V (43%) and G12R (23%). In total, 37 patients (93%) had at least 1 mutation. The order of the mutation rates in the plasma and serum was the same as that observed in the tissue samples; however, the detection rates in the blood samples (both plasma and serum) were lower than those in the

tissue samples.

The mutation rates detected in the serum and plasma DNA were quite similar. The rate of the G12D mutation in plasma DNA and that in serum DNA were 38% and 40%, and those of the G12V mutation were 13% and 10%, respectively. We did not detect the KRAS mutation at G12R in either plasma or serum DNA.

The detection rates of the KRAS mutations at any of G12D, G12V, and G12R were the same for both blood sources (48%). In addition, no marked difference in the detection rate was observed between stage I/II and stage III/IV patients (Table 3).

Overall survival rate and KRAS mutations

In the follow-up period, 12 patients (30%) died of pancreatic cancer. The overall survival curves classified by KRAS mutation in tissue, plasma, and serum are shown in Figures 2 and 3. None of the KRAS mutations in tissue were related to the overall survival. However, while the KRAS mutation at G12D in plasma and serum was not related to the overall survival, the patients with the G12V mutation detected in serum or plasma showed a significantly shorter survival than those without the mutation ($p < 0.01$ and $p < 0.01$, respectively). All patients with the G12V mutation in serum or plasma were Stage III/IV, which was the only difference of the characteristics of the patients between the groups (Table 4a and 4b). In addition, all four patients with the G12V mutation in serum overlapped with those with the same mutation in plasma.

Discussion

We analyzed the feasibility of plasma DNA and serum DNA as the samples of detecting KRAS mutations in patients with pancreatic cancer. The median amount of serum DNA was 7.2 times higher than that of plasma. The DNA concentration in the plasma of pancreatic cancer patients was significantly higher than that of healthy individuals, although we did not observe any marked difference in the serum DNA concentration. Among the three KRAS mutations, the frequency of the G12D mutation was the highest in tissue, plasma, and serum (73%, 38%, and 40%, respectively). Although the frequency was low in blood samples, no marked difference was observed between the plasma and serum. In terms of a prognostic factor, the mutation of KRAS at G12V both in the serum and plasma samples was strongly correlated to a poor overall survival, whereas that in tissue did not affect the overall survival. Because we did not observe any marked difference between the plasma and serum DNA regarding the detection rates of KRAS mutations, either can be used as a sample for a liquid biopsy.

Several studies have been reported that the DNA in serum was 4- to 24-fold that in plasma [12-14, 18]. In our study, the amount of DNA in serum was 7.2 times higher than that in the paired plasma samples, which concurred well with previous findings. This is mainly the result of the clotting process while isolating serum. When blood clots, the cells in the blood collapse, and their DNA flows out into the serum. Conventional methods like RT-PCR or an RFLP analysis are affected by the amount of non-mutated normal DNA derived from blood cells, which subsequently reduce their sensitivity for the detection of cancer-

specific mutations. In this study, we clearly showed that droplet digital PCR was able to overcome this interference and demonstrated the usefulness of serum for detecting ctDNA.

We previously reported that KRAS mutations in ctDNA are a better biomarker than those in tissue in terms of predicting the overall survival [15]. We were able to reproduce this advantage when using ctDNA in the present study. The KRAS mutation G12V in serum or plasma was a good marker for a poor prognosis. However, the major mutation detected in ctDNA, mutation G12D, did not have any obvious correlation with the prognosis. This mutation may instead be useful as a biomarker reflecting the therapeutic effect if monitored during therapy, as about 40% of patients with pancreatic cancer are positive for this mutation in ctDNA.

In our first report, we focused on the detection rate of KRAS mutations in serum by examining patients with relatively advanced pancreatic cancer. In contrast, half of the patients were suffering from less-advanced cancer (stage I/II) in the present study; we were therefore able to evaluate the differences in the DNA concentrations and mutation detection rates across clinical stages. Although, there were no significant differences in the serum and plasma DNA concentrations between stages I/II and III/IV, the plasma DNA concentration in the advanced-stage patients was slightly higher than in less-advanced-stage patients, which concurred with the previous findings [19]. In contrast, the detection rates of the KRAS mutations were almost the same between the stage I/II and stage III/IV patients. These results indicated that we could detect genetic alterations by examining blood samples, even in patients with early-stage pancreatic

cancer. The sensitivity for detecting a rare mutation was approximately 0.01%, which was sufficient to detect a KRAS mutation in blood samples with innumerable normal DNA [7]. No marked differences in the detection rate were observed between serum and plasma samples, meaning that both are appropriate sample sources for this KRAS mutation analysis, as far as we determined.

Several limitations associated with the present study warrant mention. First, the sensitivity of a liquid biopsy is lower than that using tissue and is not sufficient for screening pancreatic cancer in a clinical setting. This is likely due to the low amount of ctDNA in the blood compared to the tumor-derived DNA in tissue samples. Increasing the sample volume might help improve the sensitivity. Second, this is a retrospective study, and the number of patients was small. Although we were able to reproduce the relationship between the serum KRAS G12V mutation and the overall survival with a new cohort in this study, a prospective study is required to verify the relationship conclusively.

In conclusion, the detection rates of KRAS mutations in serum and plasma DNA were almost equal, regardless of the differences in the amount of extracted DNA, and both serum and plasma DNA can be used as prognostic biomarkers for pancreatic cancer.

Acknowledgements:

The authors declare no conflicts of interest in association with this study. We would like to thank Takehiro Matsubara (Okayama University Hospital Biobank, Okayama University Hospital) for allowing us access to the droplet digital PCR machine. We also thank Hiroyuki Tanaka (Department of Diagnostic Pathology, Okayama University Hospital) for his helpful participation in this work.

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Table1. Patient Characteristics

		N = 40
Age (years)		71 (49-92)
Sex (male, %)		26 (65%)
Diabetes mellitus		16 (41%)
Size (mm)		30 (14-69)
Stage of disease	I	2 (5%)
(UICC)	II	22 (55%)
	III	7 (18%)
	IV	9 (22%)
CEA (ng/mL)		4.4 (0.2-1379)
CA19-9 (IU/mL)		112 (0.6-91973)
SPAN-1 (IU/mL)		77 (10-62000)
DUPAN2 (IU/mL)		292 (25-16000)
Initial therapy	BSC	3 (8%)
	Chemo therapy	15 (37%)
	Surgery	22 (55%)
		Median (range)

CEA: carcinoembryonic antigen, CA19-9: carbohydrate antigen 19-9, SPAN-1: s-pancreas-1 antigen, DUPAN-2: duke pancreatic monoclonal antigen type 2, BSC: best supportive care

Table2. Frequency of KRAS mutations in tissue, serum, and plasma

	Tissue	Plasma	Serum	P-value
G12D	29 (73%)	15 (38%)	16 (40%)	0.82
G12V	17 (43%)	5 (13%)	4 (10%)	0.72
G12R	9 (23%)	0 (0%)	0 (0%)	-
KRAS (G12D or G12V or G12R)	37 (93%)	19 (48%)	19 (48%)	1.00

The P-value indicates the proportion between plasma and serum.

Table3. Comparison of the DNA concentrations and mutation frequency in stage I/II or III/IV

		Stage I/II (n = 24)	Stage III/IV (n = 16)	P-value
Mutation rate	Tissue	21 (88%)	16 (100%)	0.26
	Plasma	9 (38%)	10 (63%)	0.20
	Serum	9 (38%)	10 (63%)	0.20

Table 4a. Characteristics of the patients with or without G12V mutation in serum

	With G12V mutation in serum (n = 4)	Without G12V mutation in serum (n = 36)	P-value
Age (years)	74.5 (59-81)	70.5 (49-92)	0.73
Sex (male, %)	2 (50%)	24 (67%)	0.60
Diabetes mellitus	2 (50%)	14 (39%)	1.00
Size (mm)	33 (20-40)	30 (14-69)	0.91
Stage (III/IV, %)	4 (100%)	12 (33%)	0.02
CEA (ng/mL)	64.0 (1.36-115.8)	4.3 (0.2-1379)	0.11
CA19-9 (IU/mL)	15.6 (0.6-117)	157.2 (0.6-91973)	0.09
SPAN-1 (IU/mL)	69 (18.4-181.8)	77.4 (10-62000)	0.79
DUPAN2 (IU/mL)	2227 (25-16000)	292 (25-16000)	0.66

Median (range)

Abbreviations were the same as indicated in Table 1.

Table 4b. Characteristics of the patients with or without G12V mutation in plasma

—	With G12V mutation in plasma (n =5)	Without G12V mutation in plasma (n = 35)	P-value
Age (years)	81 (59-91)	70 (49-92)	0.29
Sex (male, %)	2 (40%)	24 (69%)	0.32
Diabetes mellitus	2 (40%)	14 (40%)	1.00
Size (mm)	28 (20-40)	30 (14-69)	0.77
Stage (III/IV, %)	4 (100%)	12 (33%)	0.02
CEA (ng/mL)	41.7 (1.36-115.8)	4.2 (0.2-1379)	0.10
CA19-9 (IU/mL)	30.2 (0.6-336.1)	155.6 (0.6-91973)	0.19
SPAN-1 (IU/mL)	78.6 (18.4-181.8)	10 (10-62000)	0.82
DUPAN2 (IU/mL)	338 (25-16000)	270.5 (25-16000)	0.63

Median (range)

Abbreviations were the same as indicated in Table 1.

Figure legends

Figure 1. Comparison of the DNA concentrations between plasma and serum samples

The median serum DNA concentrations in pancreatic cancer patients and healthy individuals were much higher than those in plasma, by 7.2- and 10.3-fold, respectively. The concentration of plasma DNA in pancreatic cancer patients was higher than that in healthy volunteers, whereas no marked difference was observed in serum samples. The horizontal bars in the boxes and numbers beside them indicate the median values.

Figure 2. The survival of the patients with pancreatic cancer with different KRAS mutations in cancer tissue

None of the three KRAS mutations in tissue (G12D, G12V, G12R) were related to the overall survival. G12D in tissue (a), G12V in tissue (b) and G12R in tissue (c).

Figure 3. The survival of the patients with pancreatic cancer with or without KRAS mutations in serum or plasma DNA

The patients with the G12V mutation in serum or plasma showed a significantly shorter survival than the patients without this mutation ($p < 0.01$ and $p < 0.01$, respectively). G12D in plasma (a), G12V in plasma (b), G12D in serum (c) and G12V in serum (d).

Figure 1

Comparison of the DNA concentrations between plasma and serum samples

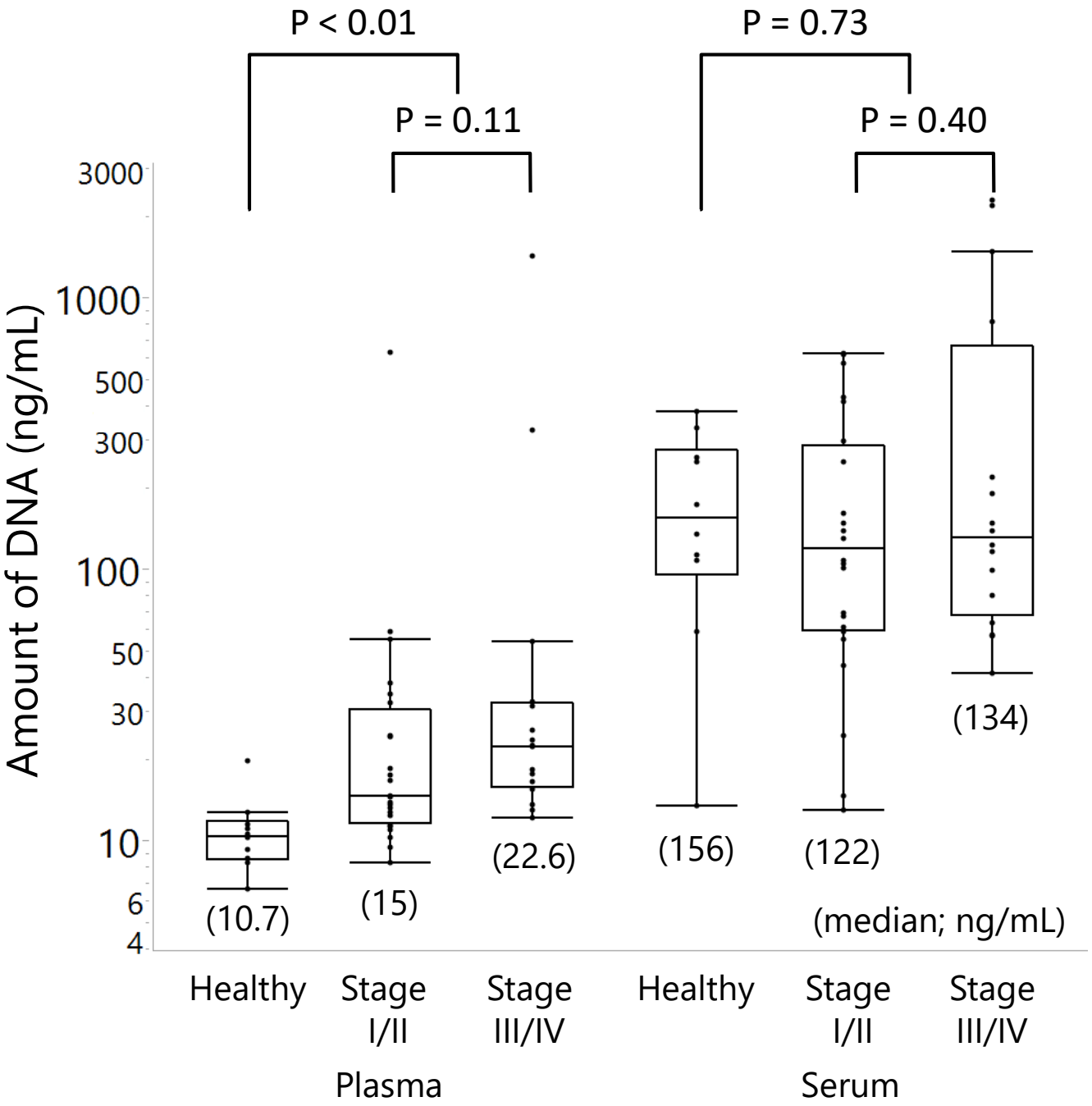

P < 0.01
P = 0.11
P = 0.73
P = 0.40
Amount of DNA (ng/mL)
3000
1000
500
300
100
50
30
10
6
4
(10.7)
(15)
(22.6)
(156)
(122)
(134)
(median; ng/mL)
Healthy
Stage I/II
Stage III/IV
Healthy
Stage I/II
Stage III/IV
Plasma
Serum

Figure 2

The survival of the patients with pancreatic cancer with different KRAS mutations in cancer tissue

Figure 2a

G12D in tissue

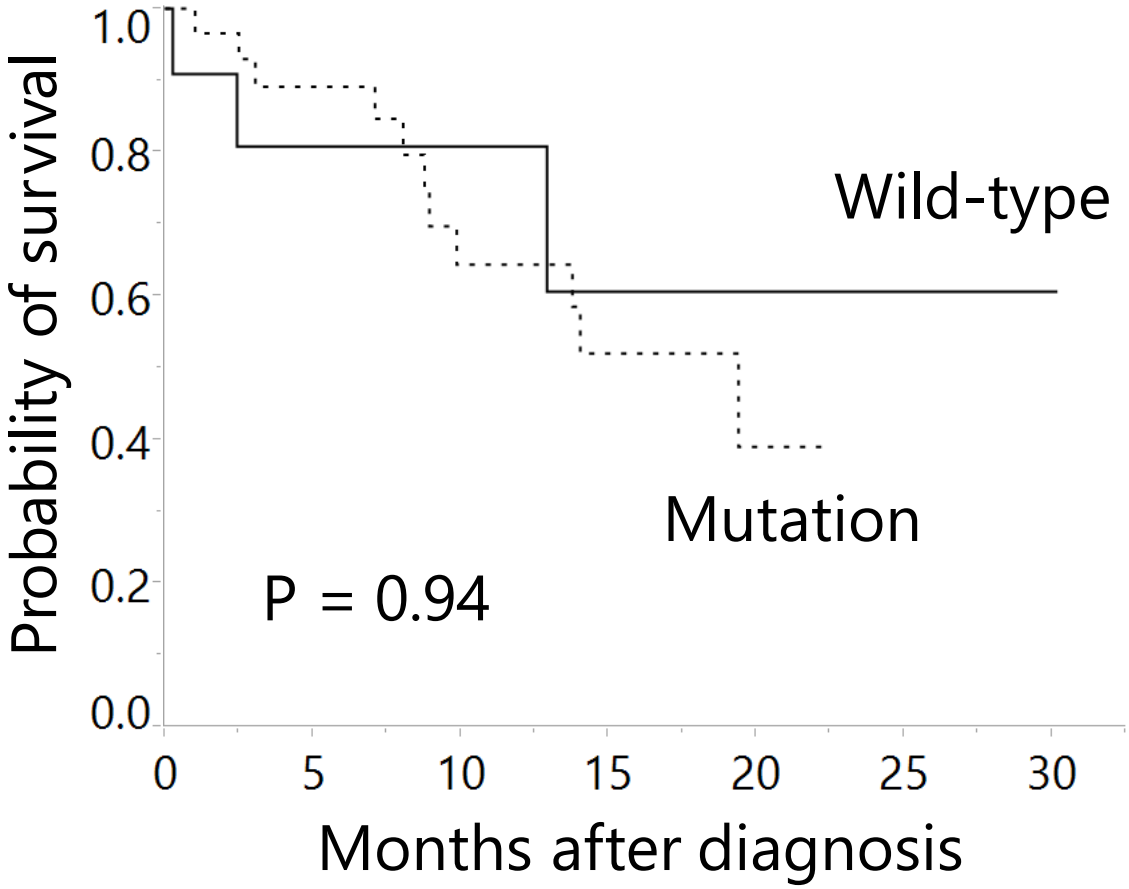

Probability of survival
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
30
Months after diagnosis
Wild-type
Mutation
P = 0.94

Figure 2b

G12V in tissue

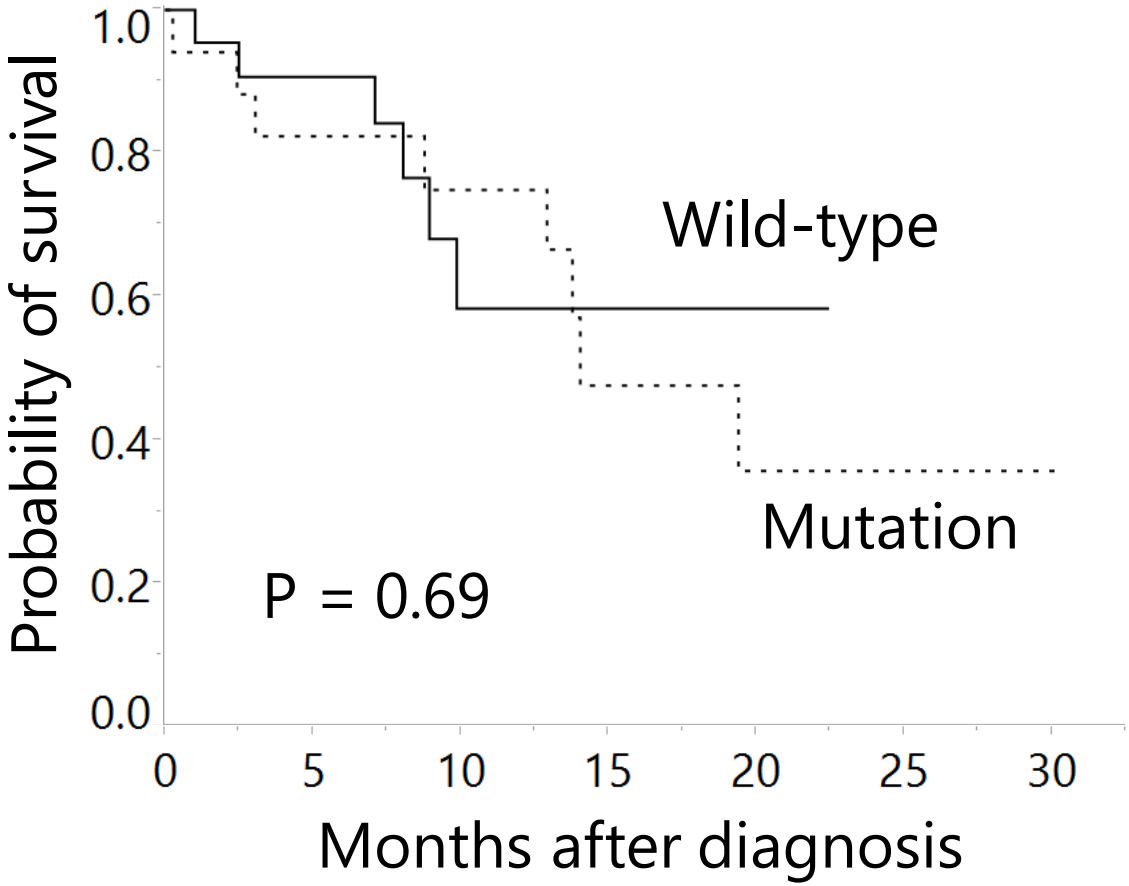
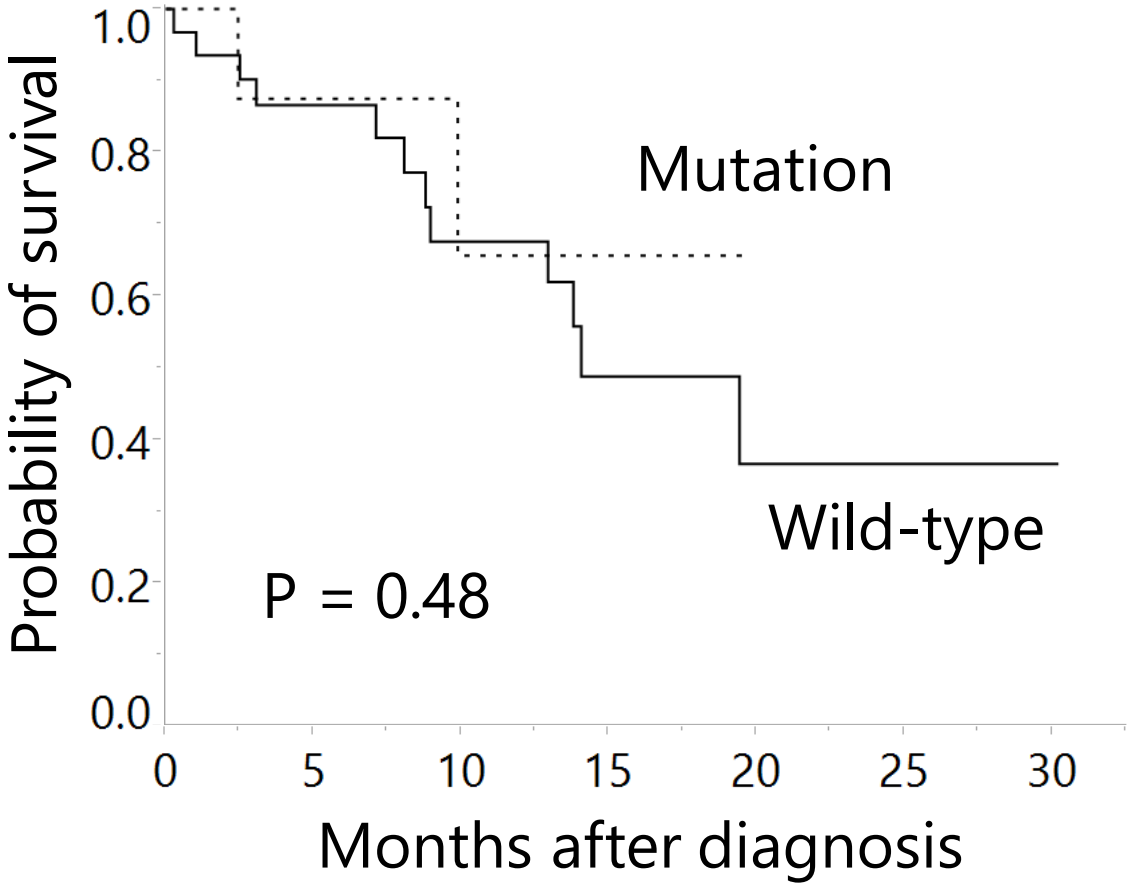

Probability of survival
1.0
0.8
0.6
0.4
0.2
0.0
Wild-type
Mutation
P = 0.69
0
5
10
15
20
25
30
Months after diagnosis

Figure 2c

G12R in tissue


Probability of survival
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0.8
0.6
0.4
0.2
0.0
Mutation
Wild-type
P = 0.48
0
5
10
15
20
25
30
Months after diagnosis

Figure 3

The survival of the patients with pancreatic cancer with or without KRAS mutations in serum or plasma DNA

Figure 3a

G12D in plasma

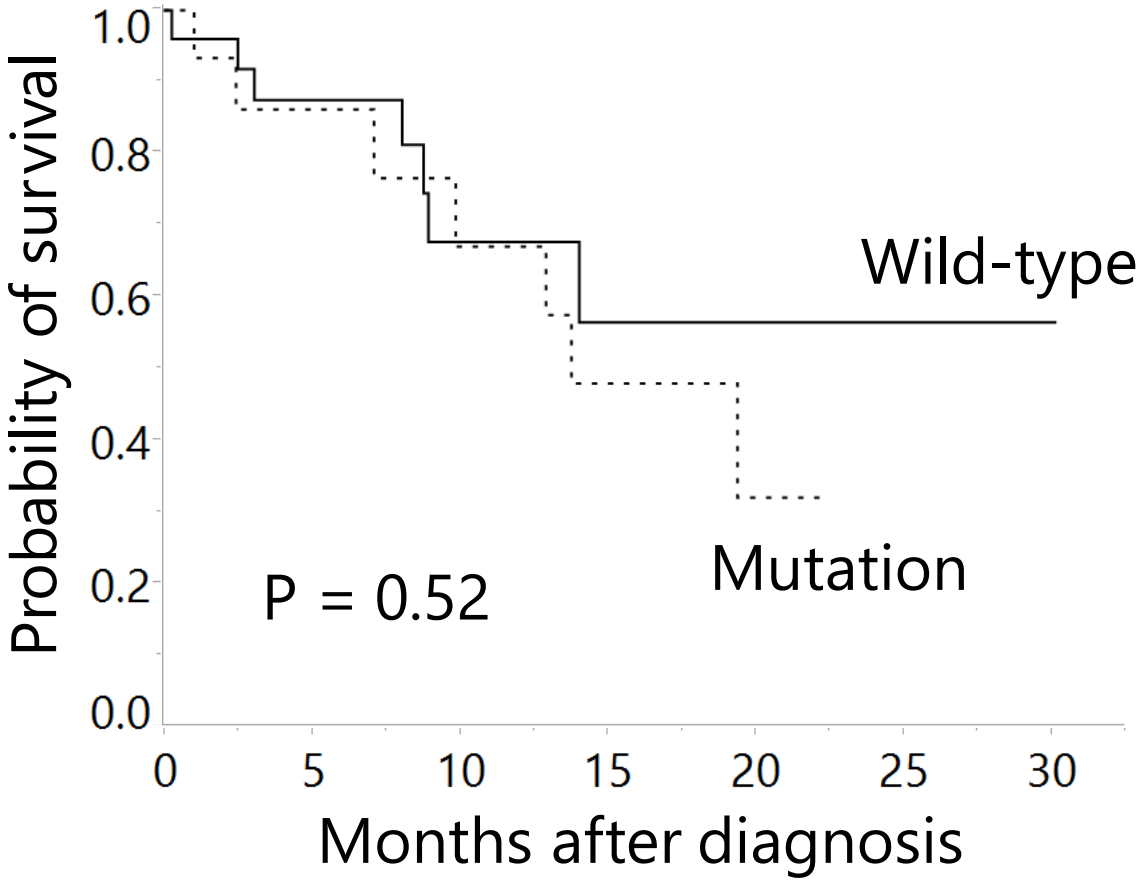

Probability of survival
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
30
Months after diagnosis
Wild-type
Mutation
P = 0.52

Figure 3b

G12V in plasma

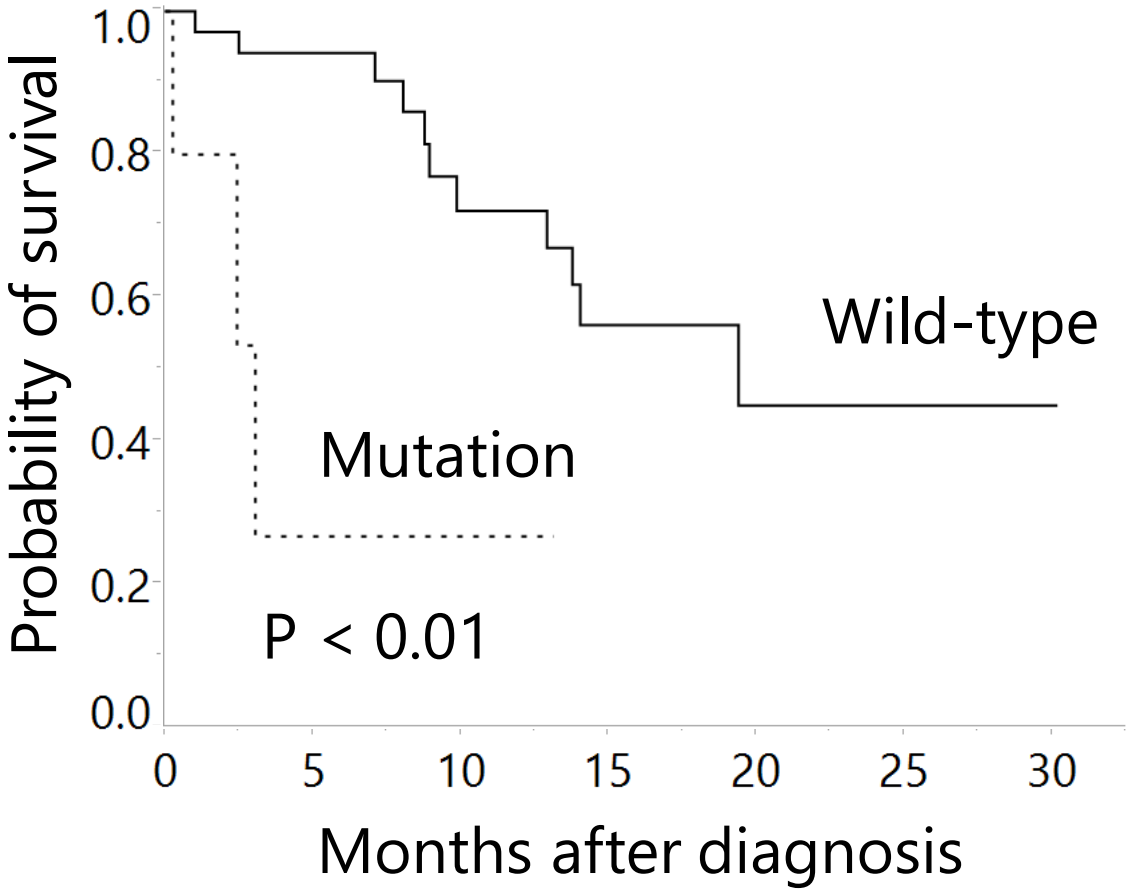

Probability of survival
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
30
Months after diagnosis
Wild-type
Mutation
P < 0.01

Figure 3c

G12D in serum

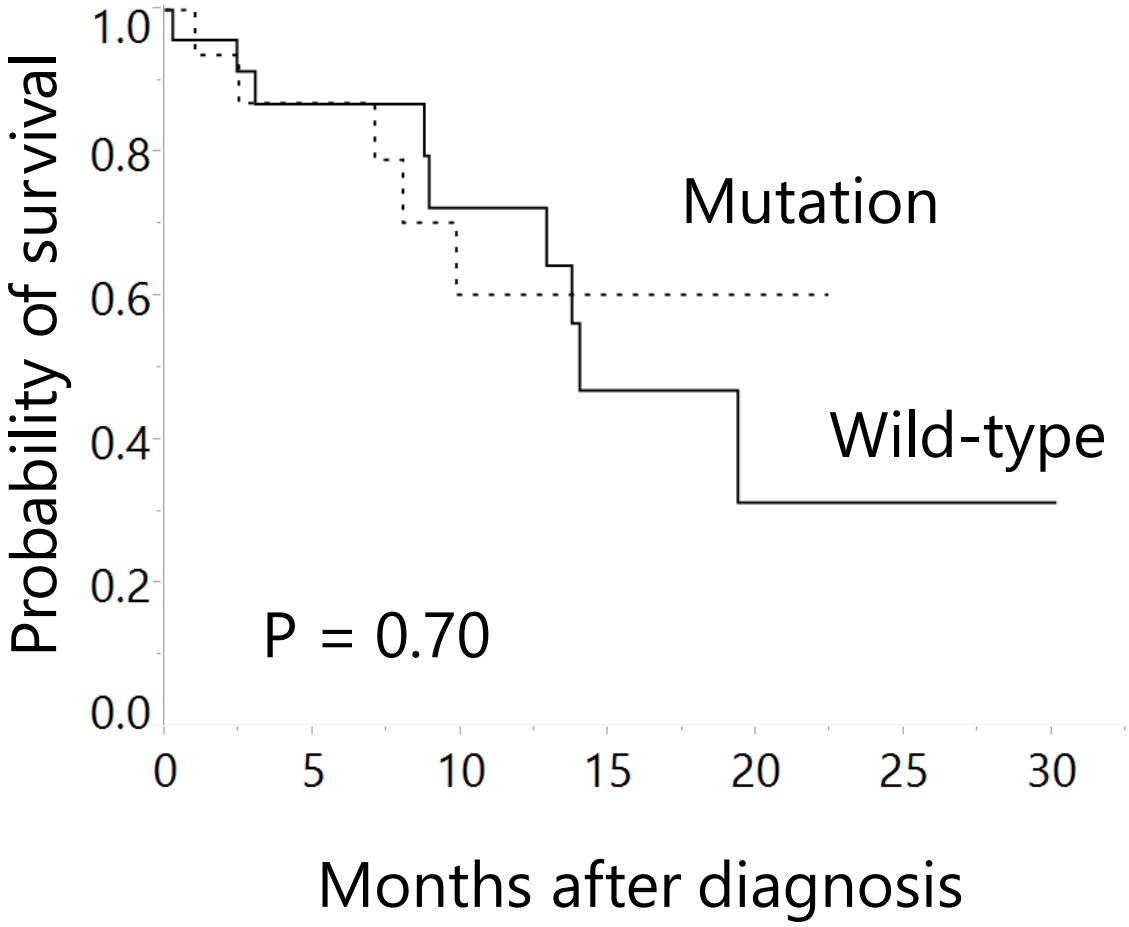
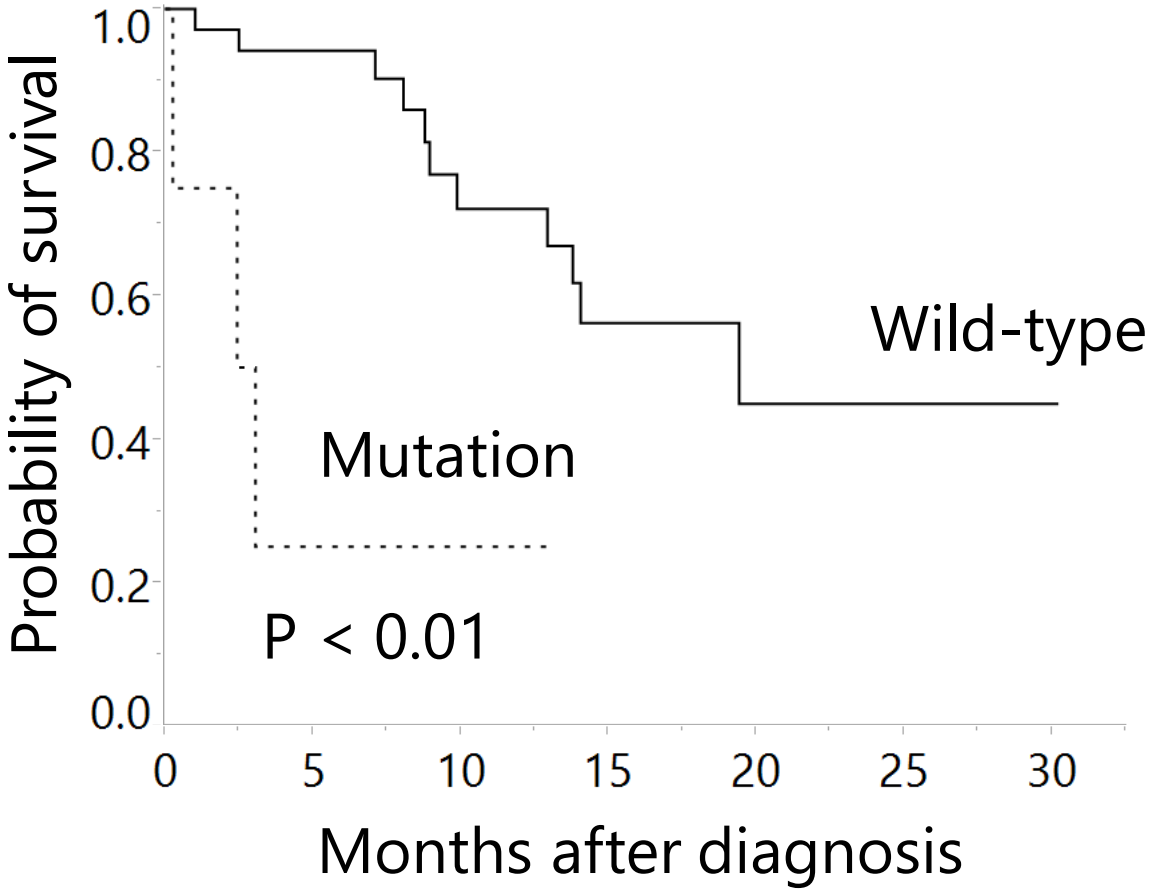

Probability of survival
1.0
0.8
0.6
0.4
0.2
0.0
Mutation
Wild-type
P = 0.70
0
5
10
15
20
25
30
Months after diagnosis

Figure 3d

G12V in serum


Probability of survival
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
30
Months after diagnosis
Wild-type
Mutation
P < 0.01