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Original Article

Real-world analysis of treatment sequencing and late-stage liquid biopsy genomic profiling in advanced pancreatic cancer

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Abstract

Background Real-world evidence on treatment sequencing and the molecular features of terminal-stage pancreatic cancer is limited. We evaluated treatment sequences and late-stage liquid biopsy genomic profiles using a nationwide clinico-genomic database.

Methods We retrospectively analyzed 16,782 patients with pancreatic ductal adenocarcinoma registered in the Center for Cancer Genomics and Advanced Therapeutics database in Japan. Overall survival was compared according to first-line regimen and first- to second-line sequence. Liquid biopsy data were analyzed in relation to the time before death, and a gradient-boosting survival model was developed to identify prognostic genomic features.

Results As first-line therapy, gemcitabine plus nab-paclitaxel was associated with longer overall survival than FOLFIRINOX (hazard ratio 0.89, 95% confidence interval 0.84–0.93), despite comparable time to treatment failure. Gemcitabine plus nab-paclitaxel followed by FOLFIRINOX was associated with the most favorable survival, measured from either first-line (hazard ratio 0.84) or second-line initiation (hazard ratio 0.68). Liquid biopsies were performed closer to death than tissue-based tests (median 140 vs. 473 days). The *KRAS* variant allele frequency increased as death approached and was the most influential prognostic feature; a *KRAS*-only model performed comparably to the full model (concordance index 0.67 for both), and survival worsened with increasing *KRAS* variant allele frequency. Alterations in chromatin-remodeling and epigenetic regulatory genes, including *SMARCA4*, *ARID1A*, *KDM6A*, and *KMT2D*, were associated with poor prognosis.

Conclusion Treatment sequencing may influence survival in advanced pancreatic cancer, and the *KRAS* variant allele frequency in late-stage liquid biopsy is a robust prognostic biomarker.

Keywords Pancreatic cancer · Real-world data · Treatment sequencing · Liquid biopsy · Epigenetic alteration

Abbreviations AUC, area under the curve; C-CAT, Center for Cancer Genomics and Advanced Therapeutics; CGP, comprehensive genomic profiling; CHIP, clonal hematopoiesis of indeterminate potential; CI, confidence interval; ctDNA, circulating tumor DNA; FFX, FOLFIRINOX; GnP, gemcitabine plus nab-paclitaxel; HR, hazard ratio; HRD, homologous recombination deficiency; mFFX, modified FOLFIRINOX; MSI, microsatellite instability; nal-IRI, nanoliposomal irinotecan; OS, overall survival; PFS, progression-free survival; ROC, receiver operating characteristic; SHAP, SHapley Additive exPlanations; SMD, standardized mean difference; TMB, tumor mutational burden; TTF, time to treatment failure; VAF, variant allele frequency

Introduction

Pancreatic cancer remains one of the most lethal malignancies worldwide. Although combination regimens such as FOLFIRINOX and gemcitabine-based therapies have improved response rates and survival in selected patients, the overall prognosis remains unsatisfactory [1]. In advanced or recurrent disease, the selection and sequencing of first- and second-line treatments critically affect outcomes [2]; however, robust evidence on optimal treatment sequencing in routine practice is scarce, and treatment decisions are often guided by limited data or physician preference.

Clinical trials are conducted under strict eligibility criteria, and patients with poor performance status, comorbidities, or rapidly progressive disease are frequently underrepresented [3]. Real-world data therefore play an important complementary role.

In Japan, the Center for Cancer Genomics and Advanced Therapeutics (C-CAT) database integrates detailed clinical and genomic information from patients undergoing comprehensive genomic profiling (CGP) in routine practice [4]. However, how treatment sequencing across multiple lines relates to genomic alterations remains incompletely elucidated.

Tumor evolution during disease progression, particularly in the terminal stage, also remains poorly characterized because tumor tissue is rarely obtained at late time points. Liquid biopsy enables minimally invasive, longitudinal monitoring of tumor-derived genetic alterations [5] and can capture dynamic molecular changes throughout the disease course [6]. Nevertheless, analyses of samples obtained shortly before death are extremely limited, and the molecular features and prognostic implications of the terminal phase remain unclear.

In this study, we used the C-CAT database to evaluate the association between first- and second-line treatment sequencing and clinical outcomes in pancreatic cancer. We

also analyzed liquid biopsy profiles obtained close to death to characterize late-stage tumor biology and applied machine learning to identify key prognostic factors in the advanced phase.

Materials and methods

Data source and study population

This study used the C-CAT database, a nationwide registry that integrates clinical and genomic information from patients who undergo CGP in routine clinical practice in Japan. The database primarily includes patients with unresectable, advanced, or recurrent solid tumors who are eligible for CGP. Data for 16,782 patients with pancreatic ductal adenocarcinoma were downloaded in May 2026. Patients with missing essential clinical information or incomplete genomic data were excluded from each analysis (Online Resource 1).

Treatment and survival analyses

First- and second-line therapies were identified from treatment records and categorized by drug combination. First-line analyses compared FOLFIRINOX (FFX) with gemcitabine plus nab-paclitaxel (GnP). Time to treatment failure (TTF) was calculated only for patients with documented treatment start and end dates. Overall survival (OS) was calculated from the initiation of each treatment line. For sequence analyses, patients were classified into three first- to second-line sequences: GnP followed by FFX (GnP→FFX), FFX followed by GnP (FFX→GnP), and GnP followed by 5-fluorouracil plus nanoliposomal irinotecan (GnP→nal-IRI). Baseline characteristics were compared between first-line regimens and across the three sequence groups using standardized mean differences (SMDs). Survival was estimated using the Kaplan–Meier method and compared with the log-rank test, and hazard ratios (HRs) were estimated using Cox proportional hazards models. Only patients with confirmed

follow-up or death dates were included in the prognostic analyses.

Gene alteration analysis

For analyses aligned to the time of death, only FoundationOne Liquid CDx data were used to minimize variability in detection sensitivity across platforms. Because paired germline sequencing was unavailable, somatic and germline variants could not be definitively distinguished. As an approximate filter, we excluded variants with VAF ≥ 0.4, based on the observation that, in this dataset, epigenetic-related and other non-driver gene alterations clustered near a variant allele frequency (VAF) of 0.5 (typical of germline variants), whereas somatic driver alterations were distributed at lower VAFs [7]; this threshold is an imperfect surrogate. For each gene, the temporal change in VAF toward death was quantified by fitting a linear regression model to all mutation observations from deceased patients:

$$\log(VAF_{ij} + \varepsilon) = \beta_0 + \beta_1 t_{ij}$$

where VAF_{ij} is the VAF of gene *i* in sample *j*, and t_{ij} is the time (days) before death, expressed as a negative value. A small constant ($\varepsilon = 1 \times 10^{-5}$) was added to avoid taking the logarithm of zero. The slope β_1 was used as a measure of temporal change in VAF toward death; a positive slope indicates an increase in VAF as death approaches. VAFs in primary and metastatic lesions were compared using the Wilcoxon rank-sum test.

Machine learning and statistical analysis

Circulating tumor DNA (ctDNA) data obtained with FoundationOne Liquid CDx were restricted to small-scale somatic variants. Patient-level features included maximum VAF, mean VAF, total number of mutations (somatic small-variant calls passing the VAF filter across all panel genes), the proportion of high-VAF (>10%) variants, and the maximum VAF of *KRAS*, *TP53*, *SMAD4*, and *CDKN2A*. Event time was defined as the interval from blood sampling to the clinical endpoint, with right censoring. The dataset

was randomly divided at the patient level into training (60%), validation (20%), and test (20%) cohorts. Survival models were developed using gradient boosting with a Cox proportional hazards objective (XGBoost) [8], with fixed hyperparameters (learning rate 0.05, maximum tree depth 3, subsample 0.8, colsample_bytree 0.8) and early stopping based on validation performance. A *KRAS*-only model and the full model were compared in the independent test cohort using the concordance index (C-index) [9] and time-dependent receiver operating characteristic (ROC) curves, with the area under the curve (AUC) calculated using the timeROC package [10]. Differences in model performance were assessed by bootstrap resampling (n = 300) of the test set. Feature importance was assessed using SHapley Additive exPlanations (SHAP) values calculated from the training dataset [11]. Potential non-linear effects of *KRAS* VAF were modeled using restricted cubic splines with three knots applied to log-transformed *KRAS* VAF (reference, VAF = 0). All tests were two-sided, and $p < 0.05$ was considered statistically significant. Analyses were performed using R version 4.5.2.

Results

Overview of comprehensive genomic profiling in pancreatic cancer

Among tissue-based panels, FoundationOne CDx, NCC Oncopanel, and GenMineTOP were used in 8,507 (50.7%), 2,448 (14.6%), and 587 (3.5%) patients, respectively; among liquid biopsy panels, FoundationOne Liquid CDx and Guardant360 CDx were used in 4,077 (24.3%) and 1,163 (6.9%) patients, respectively (Fig. 1a). The median interval from sampling to death was substantially shorter for liquid than for tissue samples (140 days [IQR 77.8–250] vs. 473 days [IQR 272–770]) (Fig. 1b).

The so-called "BIG4" genes (*KRAS*, *TP53*, *CDKN2A*, and *SMAD4*) were frequently altered in tissue samples (Fig. 1c). *KRAS* mutations were identified in over 90% of tissue samples but in only approximately 50% of liquid samples, suggesting limited

sensitivity of liquid biopsy (Online Resource 2a). In contrast, *ATM*, *DNMT3A*, *CHEK2*, and *TET2* mutations were more frequent in liquid samples, possibly reflecting clonal hematopoiesis of indeterminate potential (CHIP) rather than tumor-derived mutations [12, 13] (Fig. 1c; Online Resource 2b).

Clinical utility of comprehensive genomic profiling

Actionable alterations with approved biomarker-matched therapies included *BRCA1/BRCA2* mutations (4.1%), *ERBB2* amplification (1.7%), high TMB (≥10 and ≥20 mutations/Mb: 1.6% and 0.6%, respectively), MSI-high (0.4%), fusion genes (*ALK*, *FGFR2*, *NTRK*, *RET*, and *ROS1*; 0.3%), and *BRAF* V600E (0.2%) (Fig. 1d). Although each alteration was rare, patients with at least one "evidence level A" biomarker had longer survival than those without (median 530 days [95% CI 419–NA] vs. 289 days [95% CI 273–304]; $p < 1 \times 10^{-4}$). Fusion genes (HR 0.27, 95% CI 0.12–0.61), MSI-high (HR 0.39, 95% CI 0.22–0.69), TMB ≥20 (HR 0.54, 95% CI 0.35–0.83), TMB ≥10 (HR 0.69, 95% CI 0.53–0.90), and *BRCA1/BRCA2* mutations (HR 0.75, 95% CI 0.64–0.88) were associated with improved survival, whereas *BRAF* V600E (HR 0.74, 95% CI 0.40–1.38) and *ERBB2* amplification (HR 1.03, 95% CI 0.82–1.30) were not (Fig. 1e; Online Resource 3a–g). No *ERBB2*-targeted therapy was reimbursed for pancreatic cancer in Japan before March 2026. Among MSI-negative patients, prognosis did not differ substantially between TMB ≥10 and TMB <10, indicating that MSI status was the dominant prognostic factor (Online Resource 3h).

First-line treatment selection, treatment duration, and survival

Baseline characteristics were well balanced between the FFX and GnP groups (SMD < 0.1) except for age and sex; the GnP group included more older patients and women (Table 1). TTF was comparable between the FFX and GnP groups (153 days [95% CI 148–154] vs. 161 days [95% CI 154–169]; HR 1.03, 95% CI 0.98–1.08; p =

0.21), whereas median OS was longer with GnP than with FFX (656 days [95% CI 641–673] vs. 616 days [95% CI 592–636]; HR 0.89, 95% CI 0.84–0.93; $p < 0.0001$) (Fig. 2a).

In subgroup analyses of homologous recombination deficiency (HRD)-related genes, core (*BRCA1/2* and *PALB2*) and non-core (*ATM* and *CHEK2*) HRD genes were analyzed separately. In patients with *BRCA1/BRCA2* mutations, TTF was significantly longer with FFX (HR for GnP vs. FFX 1.43, 95% CI 1.16–1.75; $p < 0.001$), whereas OS did not differ significantly (Fig. 2b). In patients with *PALB2* mutations, TTF did not differ significantly ($p = 0.078$), whereas OS was worse with FFX than with GnP ($p = 0.036$) (Fig. 2c). In patients with *ATM* or *CHEK2* alterations, neither TTF nor OS differed significantly between regimens (Fig. 2d; Online Resource 4).

Survival according to the sequence of first- and second-line therapies

The sequence analysis included 3,533 patients (GnP→nal-IRI, n = 1,638; FFX→GnP, n = 1,072; GnP→FFX, n = 823). Baseline characteristics differed between the sequence groups, most notably age (SMD 0.444) and the number of regimens before genomic testing (SMD 0.209) (Table 2). Using GnP→nal-IRI as the reference, median OS from the start of first-line therapy was 656 days (95% CI 608–714) for GnP→FFX, 577 days (95% CI 550–608) for FFX→GnP, and 606 days (95% CI 570–644) for GnP→nal-IRI (log-rank $p < 0.001$) (Fig. 3a). GnP→FFX was associated with significantly improved survival (HR 0.84, 95% CI 0.75–0.93; $p = 1.36 \times 10^{-3}$), whereas FFX→GnP was associated with marginally worse survival (HR 1.10, 95% CI 1.00–1.21; $p = 0.048$).

To account for potential immortal time bias, we repeated the analysis using the start of second-line therapy as the time origin. Median OS was 436 days (95% CI 411–462) for GnP→FFX, 331 days (95% CI 312–350) for FFX→GnP, and 333 days (95% CI

317–352) for GnP→nal-IRI (log-rank $p < 0.001$) (Fig. 3b). GnP→FFX remained significantly associated with improved survival (HR 0.68, 95% CI 0.61–0.76; $p = 4.47 \times 10^{-12}$), whereas FFX→GnP was not (HR 0.95, 95% CI 0.87–1.05; $p = 0.31$).

KRAS VAF as a prognostic biomarker in advanced pancreatic cancer

Because liquid biopsies were frequently performed near the end of life (Fig. 1b), we analyzed VAF changes in late-stage FoundationOne Liquid CDx samples aligned to the time of death. VAF increased progressively as death approached, with marked expansion of variability within 100 days before death, suggesting ongoing clonal evolution in the terminal phase (Fig. 4a). These patterns differed according to *TP53* mutation status (Online Resource 5a). *KRAS* showed the most pronounced time-dependent increase in VAF (Fig. 4b), a pattern distinct from that of germline or CHIP-associated variants (Online Resource 5b).

We therefore constructed prognostic models incorporating *KRAS*, *TP53*, *SMAD4*, and *CDKN2A* VAFs, the high-VAF ratio (proportion of mutations with VAF > 10%), total mutation count, and maximum VAF. SHAP analysis identified *KRAS* VAF as the most influential feature (Fig. 4c, d). In the independent test cohort, a *KRAS*-only model showed discriminative ability comparable to that of the full model (C-index 0.67 for both; bootstrap mean difference 0.002, 95% CI −0.014 to 0.017; $p = 0.79$) (Online Resource 5c). Time-dependent ROC analysis showed similar AUC values for the two models (Fig. 4e, f). Spline analysis revealed a non-linear association between *KRAS* VAF and hazard, with a particularly steep increase in risk in the low-VAF range (Fig. 4g). When patients were stratified by *KRAS* VAF (undetectable [VAF = 0], 0–0.5%, 0.5–5%, and ≥5%), median OS was 252 days (95% CI 232–305), 197 days (174–226), 138 days (130–150), and 84 days (76–92), respectively (Fig. 4h). Compared with undetectable *KRAS*, HRs were 1.42 (95% CI 1.19–1.70) for 0–0.5%, 2.26 (95% CI

1.93–2.66) for 0.5–5%, and 4.21 (95% CI 3.57–4.96) for ≥5% (all $p < 0.001$). These findings indicate that *KRAS* VAF derived from liquid biopsy is a robust prognostic biomarker that likely reflects disease burden.

Epigenetic regulatory genes in terminal-stage pancreatic cancer

We next evaluated the 30 most frequently mutated genes in liquid biopsy samples, including the rate of change in VAF toward death for each gene as a covariate in Cox proportional hazards models. In addition to the canonical driver genes (*KRAS*, *TP53*, *CDKN2A*, and *SMAD4*), several genes involved in chromatin remodeling and epigenetic regulation were significantly associated with poor prognosis, including the SWI/SNF complex components *SMARCA4* and *ARID1A* [14] and the epigenetic modifiers *KDM6A* and *KMT2D* [15, 16] (Fig. 5a, b).

Kaplan–Meier analyses based on VAF demonstrated significant survival differences for these genes (Fig. 5c; Online Resource 6a), and truncating mutations, which are likely to result in loss of function, were more frequent in patients with poor outcomes (Fig. 5d; Online Resource 6b). Because liquid biopsy in advanced pancreatic cancer may predominantly reflect tumor DNA from metastatic lesions [6], we compared VAFs between primary and metastatic tumors using FoundationOne CDx data. *ARID1A* and *KDM6A* mutations showed significantly higher VAFs in metastatic lesions than in primary tumors (Online Resource 6c).

Discussion

In Japan, GnP and FFX are the standard first-line treatments [17]; NALIRIFOX had not been approved as of April 2026. In the phase II PASS-01 trial, progression-free survival was similar between GnP and modified FOLFIRINOX (mFFX), while OS and safety trends favored GnP [18]. In the phase II/III JCOG1611 (GENERATE) trial conducted in Japan, neither mFFX nor S-1, irinotecan, and oxaliplatin improved OS

compared with GnP [19]. Consistent with these trials, GnP was not associated with improved TTF compared with FFX in our study but was associated with more favorable survival.

BRCA1/2-mutated pancreatic cancers are sensitive to platinum-based therapies [20]. In this subgroup, FFX was associated with longer TTF than GnP, although OS did not differ. This may reflect the use of platinum-based regimens in later lines among patients initially treated with GnP, suggesting that treatment sequence, rather than first-line choice alone, may influence survival. In the *PALB2* subgroup, a core HRD gene similar to *BRCA1/2* [21], OS was worse with FFX despite similar TTF. We interpret this cautiously given the small subgroup (n = 45 for FFX, n = 104 for GnP), wide confidence intervals, multiple comparisons, and the influence of subsequent therapy on OS; it requires confirmation in larger cohorts. For non-core HRD alterations, including *ATM* and *CHEK2*, FFX showed no advantage over GnP, consistent with weaker evidence for platinum sensitivity in non-core HRD genes [22, 23].

For second-line treatment, gemcitabine-based therapy after FFX or 5-fluorouracil plus nal-IRI after GnP is commonly used, supported by the NAPOLI-1 trial [24], but randomized evidence to guide sequencing after GnP is limited. In our study, patients who received FFX after GnP had significantly better survival than those who received 5-fluorouracil/nal-IRI after GnP or GnP after FFX, and this trend persisted when survival was measured from second-line initiation to account for immortal time bias [25]. Introducing FFX after GnP may therefore improve survival in selected patients, although the sequence groups differed in baseline characteristics, particularly age (Table 2), and confounding by indication cannot be excluded.

Another important finding is the clinical relevance of liquid biopsy in the terminal phase. Liquid biopsy was performed later than tissue-based profiling, likely when rapid

results were required or tissue was inadequate [26], and may therefore capture the molecular landscape close to the end of life. The detection rate of *KRAS* mutations was lower in liquid biopsy (approximately 50%) than in tissue (>90%), likely reflecting differences in assay sensitivity [26]. However, detectable *KRAS* mutations may themselves reflect tumor burden and ctDNA shedding. Given that *KRAS* mutations are present in over 90% of pancreatic cancers [27], *KRAS* VAF in liquid biopsy may serve as an integrated biomarker of tumor fraction and clonal composition.

Alterations in epigenetic regulatory genes, including *SMARCA4*, *ARID1A*, *KDM6A*, and *KMT2D*, although less frequent than canonical drivers, were also associated with poor prognosis. These genes are involved in chromatin remodeling and tumor evolution [15, 28]. *ARID1A* and *KDM6A* alterations showed higher VAFs in metastatic than in primary lesions, suggesting evolved, aggressive subclones.

Our findings should be considered alongside established prognostic factors. Performance status, tumor markers, metastatic burden, and inflammatory indices are robust pretreatment prognostic factors [29], and transcriptomic subtyping is a robust molecular classifier [28] but requires RNA profiling. *SMAD4* loss and *CDKN2A* alterations have been linked to poorer outcomes [30, 31], whereas *BRCA1/2* and other core HRD alterations have been linked to platinum sensitivity and more favorable survival [20]. Our study adds that *KRAS* VAF in late-stage liquid biopsy and epigenetic regulatory gene alterations provide additional prognostic information.

Our findings also underscore the importance of CGP in pancreatic cancer. Patients harboring actionable biomarkers, including *BRCA1/2* mutations, high TMB, MSI-high, and fusion genes, had significantly better outcomes. MSI status had a stronger prognostic impact than TMB.

This study has several limitations. As a retrospective database analysis, it is subject

to selection bias, including that from complete-case analysis, and unmeasured confounding. Patients undergoing CGP generally have better performance status, limiting generalizability. Patients able to receive a more intensive regimen (i.e., FFX after GnP) may have had more favorable baseline characteristics, warranting cautious interpretation of the observed survival benefit [32]. In Japan, FFX is often administered in a modified form [33], which may affect outcomes. TTF excluded patients without a documented discontinuation date, which may underestimate absolute TTF. Molecular analyses were based on single time-point samples and do not directly capture intra-patient clonal evolution. Finally, because paired germline sequencing was unavailable, the VAF < 0.4 threshold cannot fully distinguish somatic from germline variants, and the epigenetic-gene associations should be interpreted with caution.

In conclusion, this large-scale real-world analysis demonstrated that first-line treatment selection, treatment sequencing, and advanced-phase molecular profiles are associated with survival in pancreatic cancer. The sequence of GnP followed by FFX was linked to improved survival, and liquid biopsy in the advanced phase, particularly *KRAS* VAF, may provide prognostic information.

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Statements and declarations

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Competing interests

NN has received honoraria from Chugai Pharmaceutical Co., Ltd.; Daiichi Sankyo

Company, Limited; GenMine Labs Corp.; Guardant Health Japan Corp.; Kyowa Kirin Co., Ltd.; Takeda Pharmaceutical Company Limited; and MSD K.K., and other remuneration from M3, Inc. EI has received grants from Gadelius Medical K.K. KO has received honoraria from Daiichi Sankyo Co., Ltd.; MSD K.K.; Tsumura & Co.; Johnson & Johnson; Taiho Pharmaceutical Co., Ltd.; and Viatris Pharmaceuticals Japan Inc., and grants from Johnson & Johnson; Covidien Japan Inc.; and Chugai Pharmaceutical Co., Ltd.

Ethics approval

This study was conducted in accordance with the ethical guidelines for medical research involving human subjects and the principles of the Declaration of Helsinki and its later amendments. The study protocol was approved by the Research Ethics Committee of Tokai University (approval no. 25R050) and the Information Utilization Review Board of C-CAT (approval no. AP20251128-01N). This study did not involve a clinical trial; trial registration was not applicable.

Consent to participate

All patients provided informed consent for inclusion in the C-CAT database and for the secondary use of their data for research purposes.

Consent to publish

Not applicable.

Data availability

The data that support the findings of this study are available from C-CAT, but restrictions apply to their availability. The data were used under approval for the current study and are not publicly available; they can be accessed through the C-CAT data utilization application process.

363 ***Author contributions***

364 NN: Conceptualization, Data curation, Formal analysis, Funding acquisition,
365 Methodology, Visualization, Writing – original draft. EI: Investigation, Writing – review
366 & editing. KO: Supervision, Writing – review & editing. All authors read and approved
367 the final manuscript.

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Figure legends

Fig. 1 Overview of comprehensive genomic profiling and its clinical utility in pancreatic cancer. **a** Pie chart showing the types of genomic panels used in this study; liquid biopsy panels are labeled in red. **b** Timing and number of liquid- and tissue-based genomic profiling tests performed during the clinical course; day 0 indicates the date of death. **c** Frequency of genomic alterations in the overall cohort. Red and blue bars represent alterations identified by liquid- and tissue-based panels, respectively. Variants of uncertain significance were included. **d** Proportion of patients harboring "evidence level A" biomarkers (excluding *ERBB2*), indicating the availability of approved targeted therapies. Only clinically significant alterations were included. **e** Kaplan–Meier curves comparing patients with and without evidence level A biomarkers (top) and forest plot showing hazard ratios for overall survival according to each biomarker category (bottom)

Fig. 2 Clinical outcomes according to first-line therapy. Kaplan–Meier curves comparing time to treatment failure (TTF; left) and overall survival (OS; right) between gemcitabine plus nab-paclitaxel (GnP; blue lines) and FOLFIRINOX (FFX; red lines) as first-line therapy. **a** Overall cohort. **b** Patients harboring *BRCA1* or *BRCA2* mutations. **c** Patients harboring *PALB2* mutations. **d** Patients harboring *ATM* mutations

Fig. 3 Overall survival according to the sequence of first- and second-line therapies. Kaplan–Meier curves for gemcitabine plus nab-paclitaxel (GnP) followed by FOLFIRINOX (FFX), FFX followed by GnP, and GnP followed by 5-fluorouracil plus nanoliposomal irinotecan (nal-IRI), measured from the initiation of first-line therapy (**a**)

and second-line therapy (**b**). Line colors are indicated within the figure

Fig. 4 Clonal expansion in late-stage pancreatic cancer detected by liquid biopsy. **a** Temporal changes in variant allele frequency (VAF) of all profiled genomic alterations in patients who died during follow-up; the rightmost time point indicates the date of death. **b** Temporal changes in *KRAS* VAF according to sampling timing. **c** SHapley Additive exPlanations (SHAP) analysis using maximum VAF, mean VAF, total number of mutations, proportion of high-VAF (>10%) variants, and the maximum VAF of *KRAS*, *TP53*, *SMAD4*, and *CDKN2A* as features; the bar plot on the left shows feature importance. **d** Scatter plot showing the correlation between *KRAS* VAF and SHAP values. **e** Receiver operating characteristic curves for 90-day survival prediction by the *KRAS*-only model (red) and the full model (blue). **f** Time-dependent area under the curve for the *KRAS*-only model (red) and the full model (blue). **g** Restricted cubic spline curve showing the association between log-transformed *KRAS* VAF and hazard ratios (HRs) for survival (reference, VAF = 0); the gray area indicates 95% confidence intervals. **h** Kaplan–Meier curves according to four *KRAS* VAF groups (undetectable [VAF = 0], 0–0.5%, 0.5–5%, and ≥5%) (top; line colors are indicated within the figure) and forest plot showing HRs for each group with VAF = 0 as the reference (bottom)

Fig. 5 Alterations in epigenetic regulatory genes in late-stage pancreatic cancer. **a** Forest plot showing hazard ratios for survival associated with the 30 most frequently altered genes in patients who died during follow-up. **b** Bubble plot showing the association between temporal changes in variant allele frequency (VAF) toward death (log scale; x-axis) and hazard ratios for survival (y-axis) (see Materials and methods); circle size

represents the frequency of alterations in each gene. **c** Temporal changes in VAF according to sampling timing (top) and Kaplan–Meier curves (bottom) for *SMARCA4*, *ARID1A*, and *KMT2D*; groups were stratified using the upper quartile of VAF as the cutoff. **d** Temporal changes in VAF for *SMARCA4* (left) and *KMT2D* (right); day 0 indicates the date of death. Truncating mutations, deletions, and rearrangements were classified as loss-of-function (LOF) alterations. Blue and red dots indicate missense mutations and LOF alterations, respectively. Labels are shown only for LOF variants observed in at least two samples or with a maximum VAF > 0.03

Table 1 Baseline patient characteristics according to first-line regimen

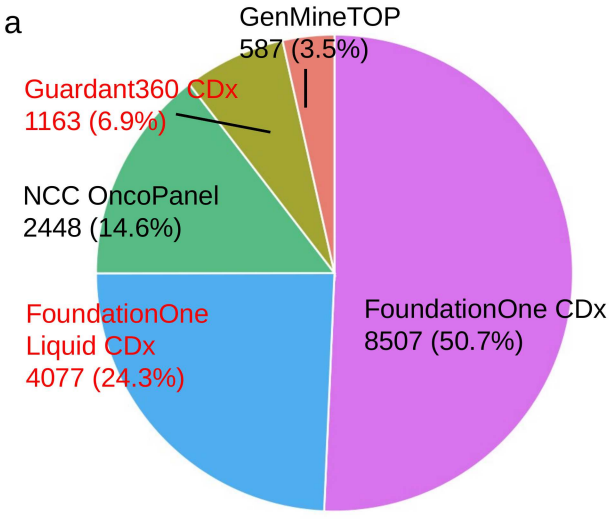
Characteristic	Level	FFX (n = 2,916)	GnP (n = 8,374)	p	SMD
Age (years), mean (SD)		62.11 (9.39)	67.01 (9.59)	<0.001*	0.517
Sex (%)	Female	1192 (40.9)	3855 (46.0)	<0.001*	0.104
	Male	1724 (59.1)	4518 (54.0)		
PS (%)	0-2	2875 (99.8)	8237 (99.5)	0.037*	0.054
	3-4	5 (0.2)	40 (0.5)		
Panel type (%)	Liquid	978 (33.5)	2608 (31.1)	0.018*	0.051
	Tissue	1938 (66.5)	5766 (68.9)		
Metastasis (any) (%)		2573 (89.1)	7290 (87.7)	0.05	0.044
Lung metastasis (%)		559 (21.7)	1701 (23.3)	0.101	0.038
Liver metastasis (%)		1543 (60.0)	4110 (56.4)	0.002*	0.073
Metastasis (other sites) (%)		1496 (58.1)	4165 (57.1)	0.386	0.02
Regimens before gene testing (%)	1-2	1621 (62.0)	4833 (64.2)	0.121	0.046
	3-4	875 (33.5)	2396 (31.8)		
	>5	117 (4.5)	300 (4.0)		
BRCA1/2 mutation (%)		145 (5.8)	345 (4.7)	0.03*	0.05
KRAS mutation (%)		2265 (90.8)	6711 (91.3)	0.496	0.017
TP53 mutation (%)		1948 (78.1)	5784 (78.7)	0.565	0.014
CDKN2A mutation (%)		438 (17.6)	1238 (16.8)	0.425	0.019
SMAD4 mutation (%)		450 (18.0)	1440 (19.6)	0.096	0.04
MSI-high (%)		16 (0.7)	28 (0.4)	0.191	0.034
TMB-high (>10 mut/Mb) (%)		65 (2.6)	126 (1.8)	0.024*	0.053

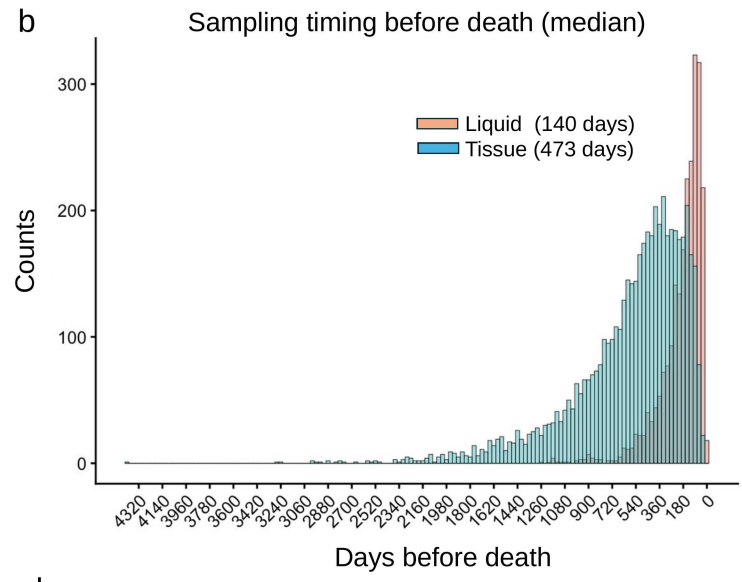
FFX, FOLFIRINOX; GnP, gemcitabine plus nab-paclitaxel; MSI, microsatellite instability; PS, performance status; SD, standard deviation; SMD, standardized mean difference; TMB, tumor mutational burden
** $p < 0.05$*

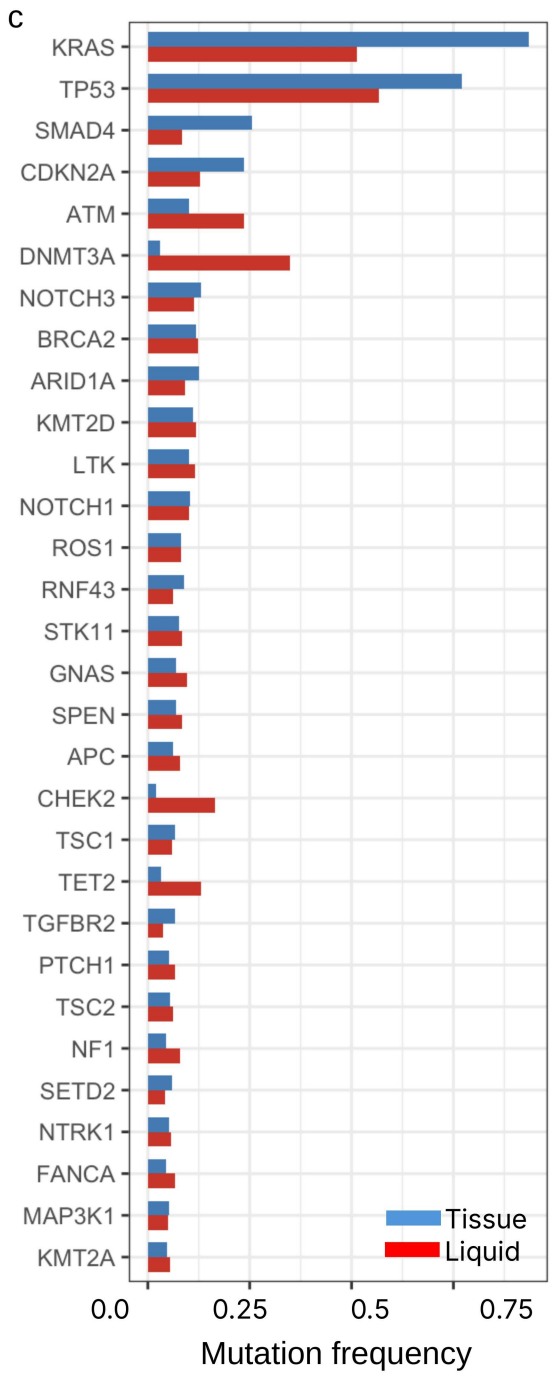
Table 2 Baseline characteristics according to second-line treatment sequence (n = 3,533)

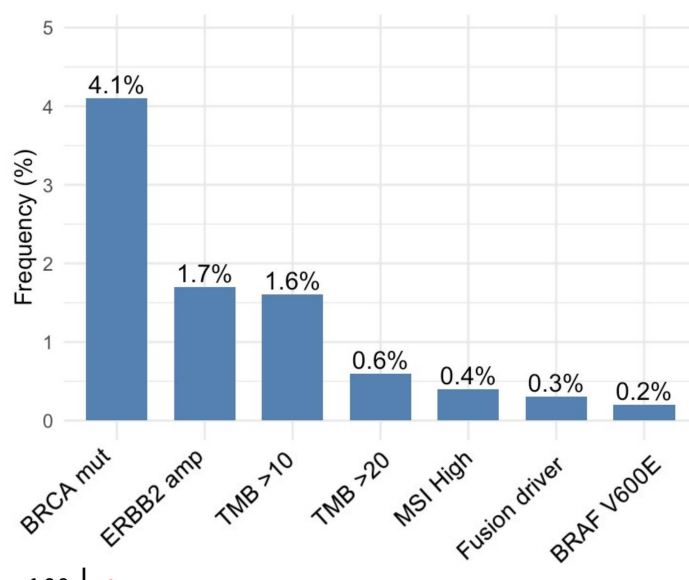
Characteristic	Level	GnP→nal-IRI (n = 1,638)	FFX→GnP (n = 1,072)	GnP→FFX (n = 823)	p	SMD
Age (years), mean (SD)		67.62 (8.85)	61.57 (9.03)	63.38 (9.55)	<0.001	0.444
Sex (%)	Female	741 (45.2)	430 (40.1)	377 (45.8)	0.013	0.077
	Male	897 (54.8)	642 (59.9)	446 (54.2)		
PS (%)	0-2	1607 (99.4)	1058 (99.9)	808 (99.6)	0.159	0.055
	3-4	9 (0.6)	1 (0.1)	3 (0.4)		
Panel type (%)	Liquid	732 (44.7)	478 (44.6)	317 (38.5)	0.008	0.084
	Tissue	906 (55.3)	594 (55.4)	506 (61.5)		
Metastasis (any) (%)		1459 (89.6)	926 (87.1)	713 (87.2)	0.074	0.052
Lung metastasis (%)		323 (22.1)	195 (21.1)	148 (20.8)	0.707	0.022
Liver metastasis (%)		943 (64.6)	612 (66.1)	442 (62.0)	0.224	0.057
Other metastasis (%)		784 (53.7)	527 (56.9)	409 (57.4)	0.166	0.049
Regimens before gene testing (%)	1-2	1257 (76.7)	747 (69.7)	533 (64.8)	<0.001	0.209
	3-4	372 (22.7)	306 (28.5)	261 (31.7)		
	>5	9 (0.5)	19 (1.8)	29 (3.5)		
BRCA1/2 mutation (%)		67 (4.8)	44 (4.9)	51 (7.3)	0.038	0.072
KRAS mutation (%)		1251 (88.8)	815 (90.9)	627 (89.8)	0.277	0.046
TP53 mutation (%)		1138 (80.8)	703 (78.4)	548 (78.5)	0.285	0.040
CDKN2A mutation (%)		244 (17.3)	165 (18.4)	126 (18.1)	0.790	0.019
SMAD4 mutation (%)		259 (18.4)	156 (17.4)	130 (18.6)	0.777	0.021
MSI-high (%)		7 (0.5)	8 (0.9)	4 (0.6)	0.550	0.030
TMB-high (≥10 mut/Mb) (%)		23 (1.6)	27 (2.9)	13 (1.8)	0.108	0.055

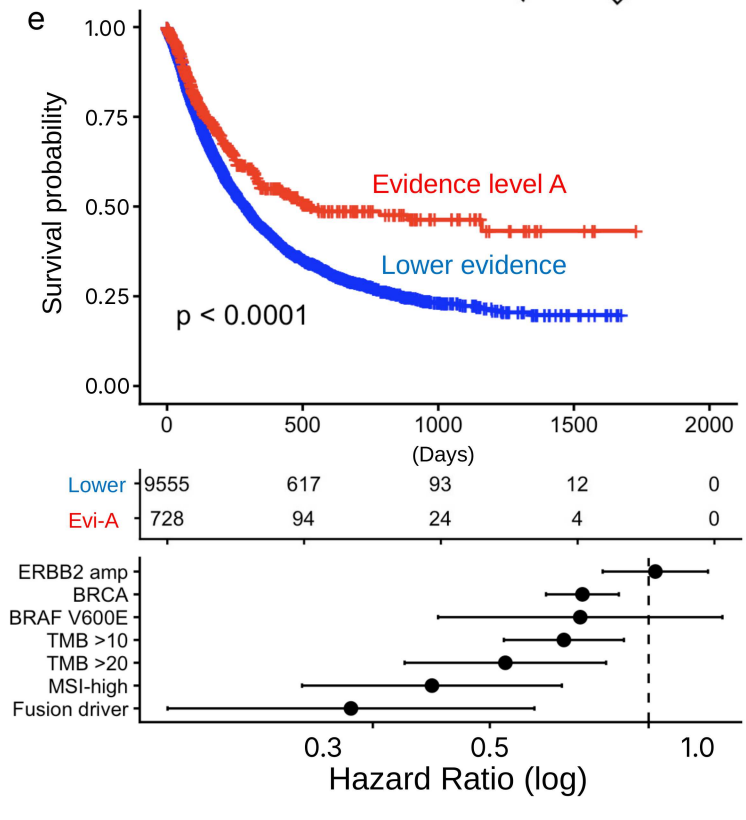
The sequence-analysis population comprises patients who received one of the three second-line sequences (GnP→nal-IRI, n = 1,638; FFX→GnP, n = 1,072; GnP→FFX, n = 823) and had evaluable survival data at both the first- and second-line time origins. SMD is the overall value across the three groups
FFX, FOLFIRINOX; GnP, gemcitabine plus nab-paclitaxel; MSI, microsatellite instability; nal-IRI, nanoliposomal irinotecan; PS, performance status; SD, standard deviation; SMD, standardized mean difference; TMB, tumor mutational burden

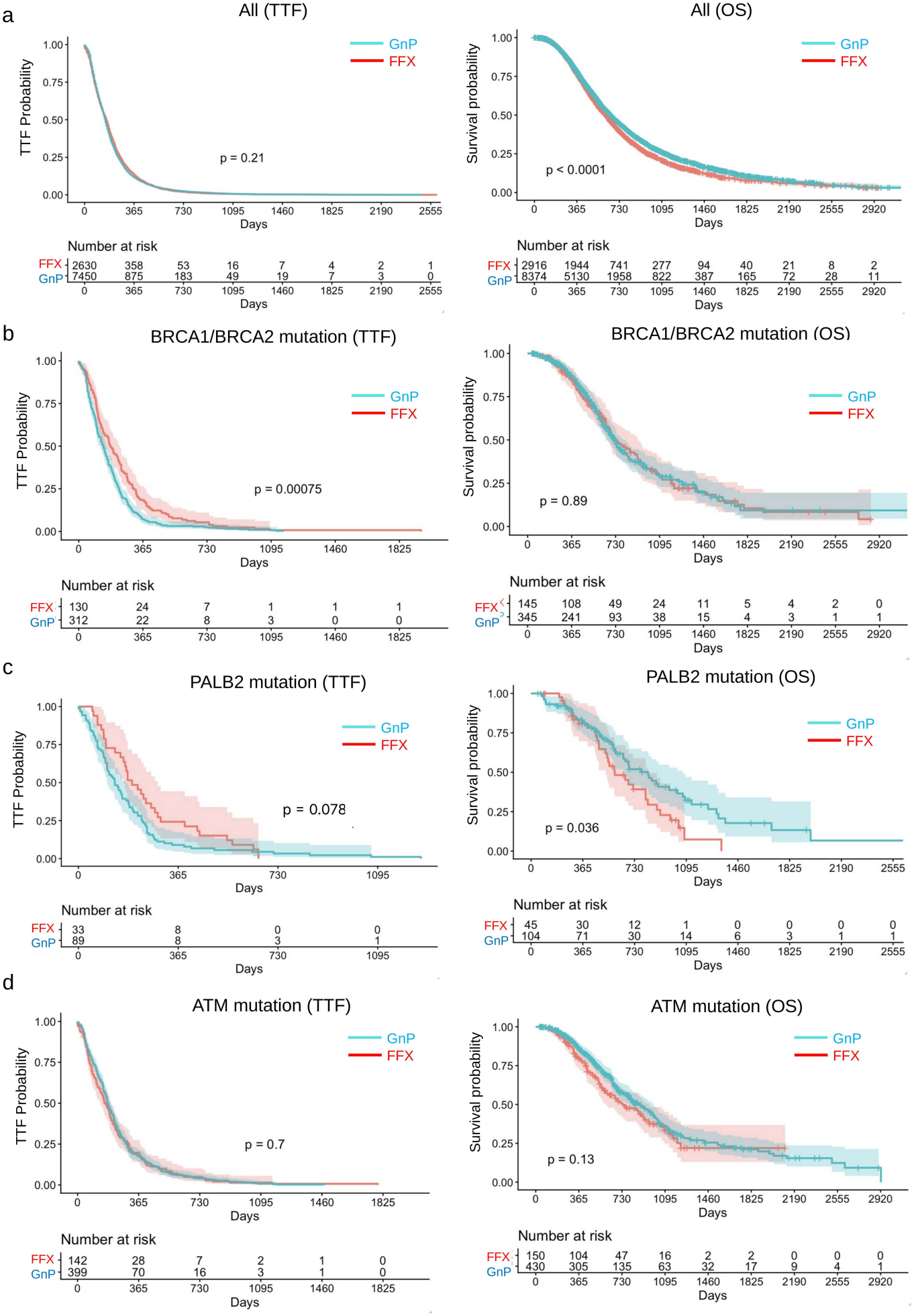

a
GenMineTOP
587 (3.5%)
Guardant360 CDx
1163 (6.9%)
NCC OncoPanel
2448 (14.6%)
FoundationOne
Liquid CDx
4077 (24.3%)
FoundationOne CDx
8507 (50.7%)


b
Sampling timing before death (median)
Liquid (140 days)
Tissue (473 days)
Counts
Days before death

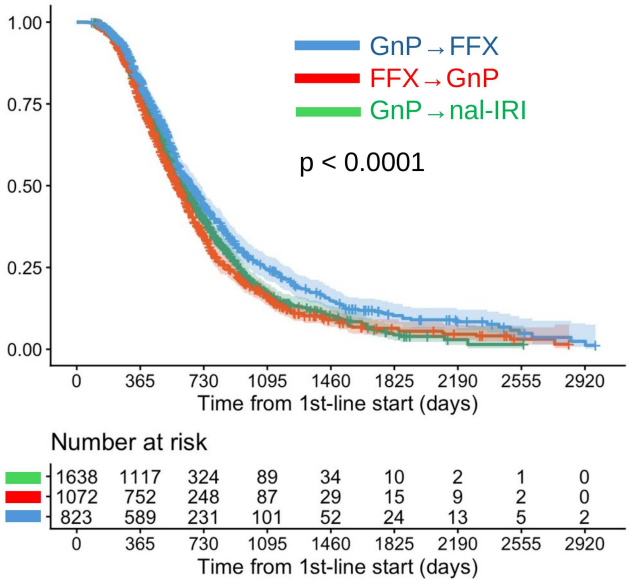

c
KRAS
TP53
SMAD4
CDKN2A
ATM
DNMT3A
NOTCH3
BRCA2
ARID1A
KMT2D
LTK
NOTCH1
ROS1
RNF43
STK11
GNAS
SPEN
APC
CHEK2
TSC1
TET2
TGFBR2
PTCH1
TSC2
NF1
SETD2
NTRK1
FANCA
MAP3K1
KMT2A
Tissue
Liquid
Mutation frequency


d
Frequency (%)
4.1%
1.7%
1.6%
0.6%
0.4%
0.3%
0.2%
BRCA mut
ERBB2 amp
TMB >10
TMB >20
MSI High
Fusion driver
BRAF V600E

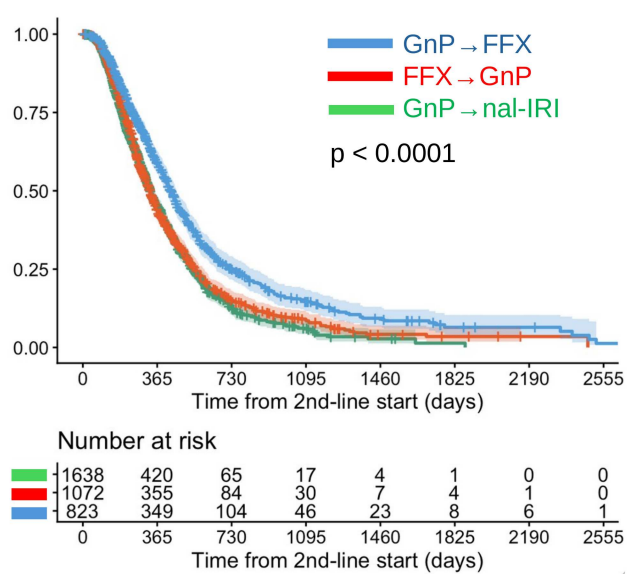

e
Survival probability
Evidence level A
Lower evidence
p < 0.0001
(Days)
Lower 9555 617 93 12 0
Evi-A 728 94 24 4 0
ERBB2 amp
BRCA
BRAF V600E
TMB >10
TMB >20
MSI-high
Fusion driver
Hazard Ratio (log)

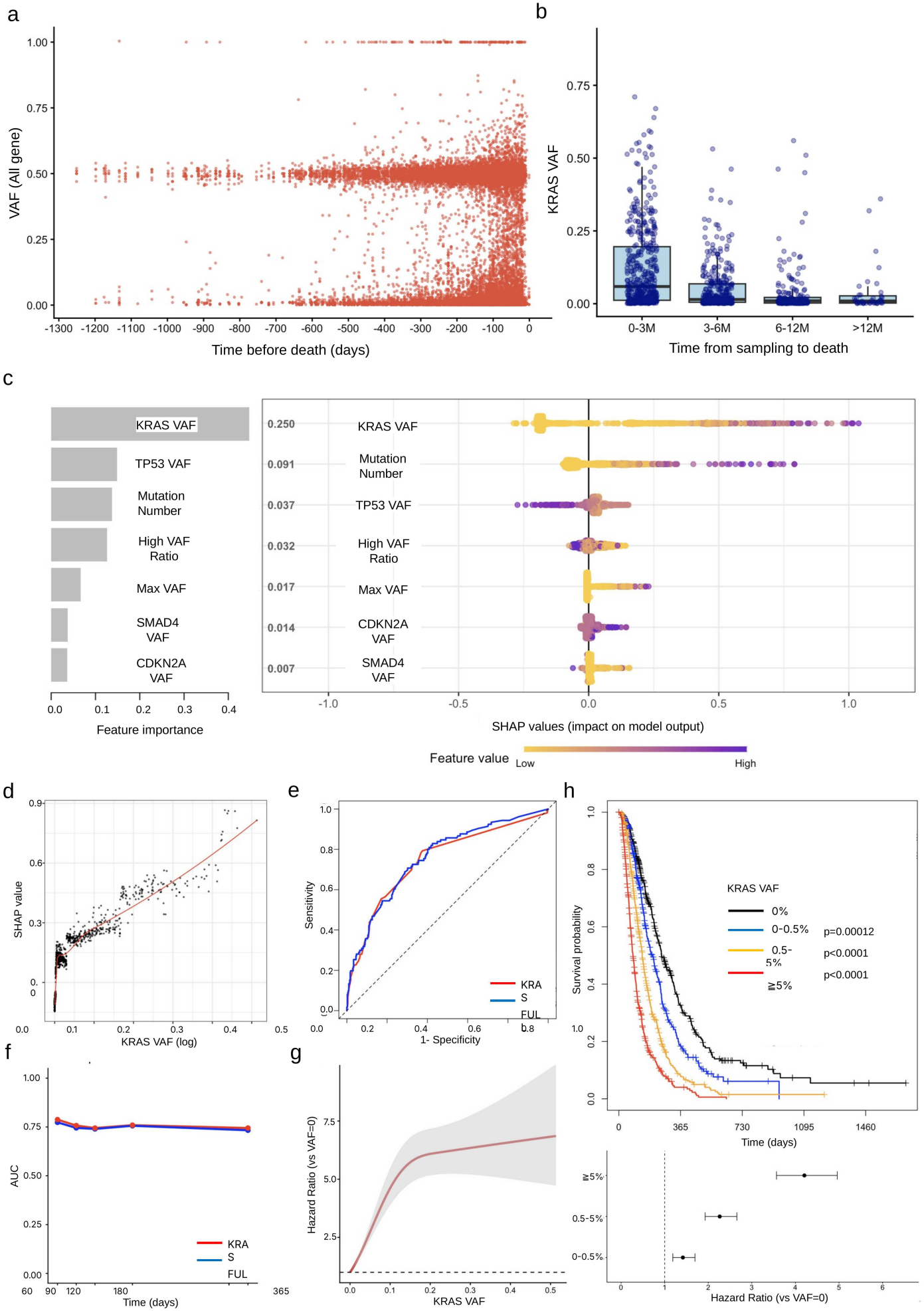

a
All (TTF)
TTF Probability
GnP
FFX
p = 0.21
Days
Number at risk
FFX 2630 358 53 16 7 4 2 1
GnP 7450 875 183 49 19 7 3 0
0 365 730 1095 1460 1825 2190 2555
All (OS)
Survival probability
p < 0.0001
FFX 2916 1944 741 277 94 40 21 8 2
GnP 8374 5130 1958 822 387 165 72 28 11
0 365 730 1095 1460 1825 2190 2555 2920
b
BRCA1/BRCA2 mutation (TTF)
p = 0.00075
FFX 130 24 7 1 1 1
GnP 312 22 8 3 0 0
0 365 730 1095 1460 1825
BRCA1/BRCA2 mutation (OS)
p = 0.89
FFX 145 108 49 24 11 5 4 2 0
GnP 345 241 93 38 15 4 3 1 1
c
PALB2 mutation (TTF)
p = 0.078
FFX 33 8 0 0
GnP 89 8 3 1
0 365 730 1095
PALB2 mutation (OS)
p = 0.036
FFX 45 30 12 1 0 0 0 0
GnP 104 71 30 14 6 3 1 1
d
ATM mutation (TTF)
p = 0.7
FFX 142 28 7 2 1 0
GnP 399 70 16 3 1 0
0 365 730 1095 1460 1825
ATM mutation (OS)
p = 0.13
FFX 150 104 47 16 2 2 0 0 0
GnP 430 305 135 63 32 17 9 4 1
0 365 730 1095 1460 1825 2190 2555 2920

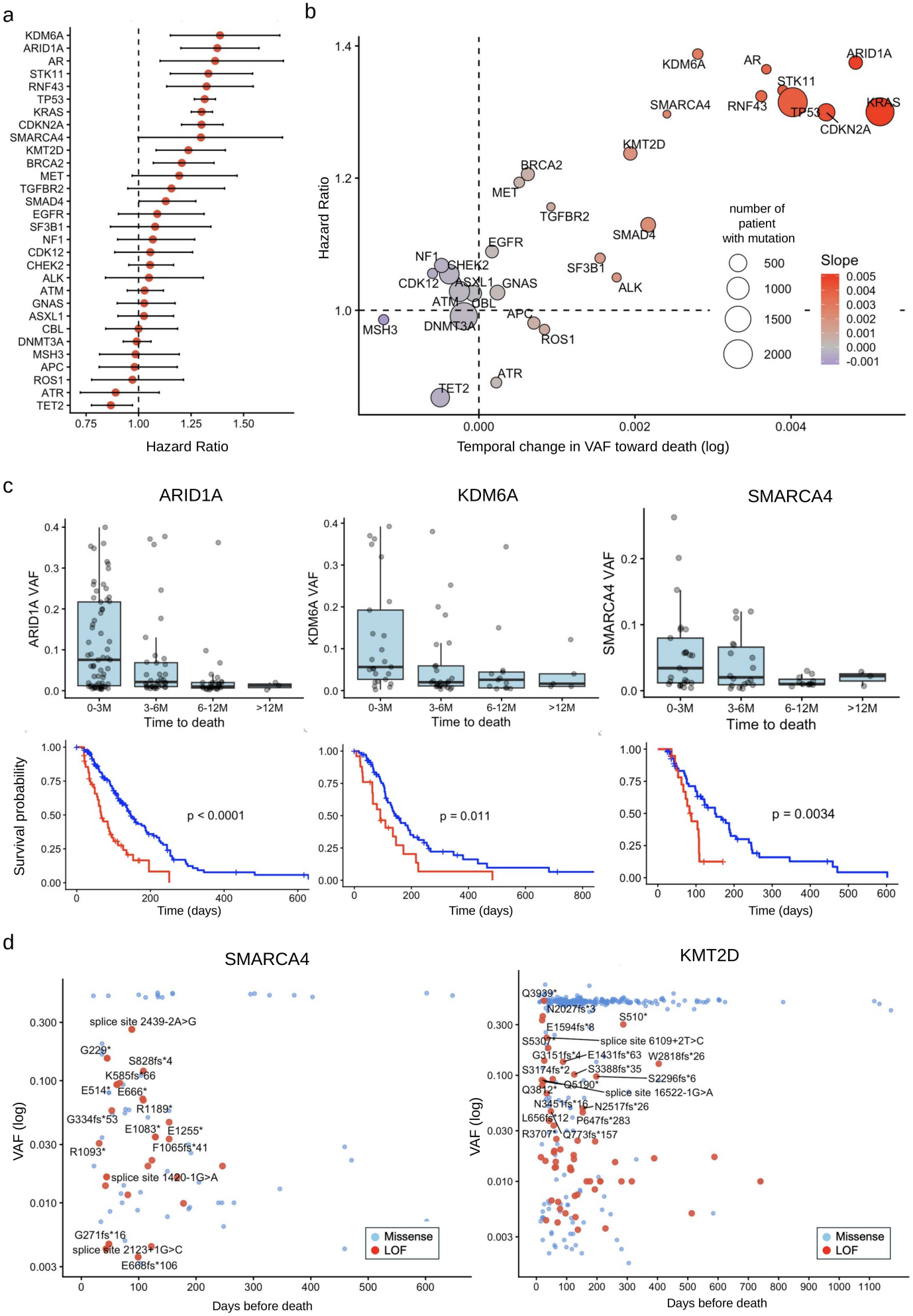
a


From 1st line start
1.00
0.75
0.50
0.25
0.00
GnP → FFX
FFX → GnP
GnP → nal-IRI
p < 0.0001
0
365
730
1095
1460
1825
2190
2555
2920
Time from 1st-line start (days)
Number at risk
1638 1117 324 89 34 10 2 1 0
1072 752 248 87 29 15 9 2 0
823 589 231 101 52 24 13 5 2
0
365
730
1095
1460
1825
2190
2555
2920
Time from 1st-line start (days)

b


From 2nd line start
1.00
0.75
0.50
0.25
0.00
GnP → FFX
FFX → GnP
GnP → nal-IRI
p < 0.0001
0
365
730
1095
1460
1825
2190
2555
Time from 2nd-line start (days)
Number at risk
1638 420 65 17 4 1 0 0
1072 355 84 30 7 4 1 0
823 349 104 46 23 8 6 1
0
365
730
1095
1460
1825
2190
2555
Time from 2nd-line start (days)


a
VAF (All gene)
Time before death (days)
b
KRAS VAF
0-3M
3-6M
6-12M
>12M
Time from sampling to death
c
KRAS VAF
TP53 VAF
Mutation Number
High VAF Ratio
Max VAF
SMAD4 VAF
CDKN2A VAF
Feature importance
0.250
0.091
0.037
0.032
0.017
0.014
0.007
SHAP values (impact on model output)
Feature value
Low
High
d
SHAP value
KRAS VAF (log)
e
Sensitivity
1- Specificity
KRA
S
FUL
h
Survival probability
KRAS VAF
0%
0-0.5%
0.5-5%
≥5%
p=0.00012
p<0.0001
p<0.0001
Time (days)
Hazard Ratio (vs VAF=0)
f
AUC
Time (days)
g
Hazard Ratio (vs VAF=0)
KRAS VAF


a
KDM6A
ARID1A
AR
STK11
RNF43
TP53
KRAS
CDKN2A
SMARCA4
KMT2D
BRCA2
MET
TGFBR2
SMAD4
EGFR
SF3B1
NF1
CDK12
CHEK2
ALK
ATM
GNAS
ASXL1
CBL
DNMT3A
MSH3
APC
ROS1
ATR
TET2
0.75
1.00
1.25
1.50
Hazard Ratio
b
Hazard Ratio
1.4
1.2
1.0
0.000
0.002
0.004
Temporal change in VAF toward death (log)
number of patient with mutation
500
1000
1500
2000
Slope
0.005
0.004
0.003
0.002
0.001
0.000
-0.001
c
ARID1A
KDM6A
SMARCA4
ARID1A VAF
KDM6A VAF
SMARCA4 VAF
0-3M
3-6M
6-12M
>12M
Time to death
Survival probability
p < 0.0001
p = 0.011
p = 0.0034
Time (days)
d
SMARCA4
KMT2D
VAF (log)
splice site 2439-2A>G
G229*
S828fs*4
K585fs*66
E514*
E666*
R1189*
G334fs*53
E1083*
E1255*
R1093*
F1065fs*41
splice site 1420-1G>A
G271fs*16
splice site 2123+1G>C
E668fs*106
Missense
LOF
Days before death
Q3939*
N2027fs*3
S510*
E1594fs*8
S5307*
splice site 6109+2T>C
G3151fs*4
E1431fs*63
W2818fs*26
S3174fs*2
S3388fs*35
S2296fs*6
Q3812*
Q5190*
splice site 16522-1G>A
N3451fs*16
N2517fs*26
L656fs*12
P647fs*283
R3707*
Q773fs*157

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