Comparative Circulating Tumor DNA Levels for *KRAS* Mutations in Non-Resectable Pancreatic Cancer Patients

Julia S. Johansen[1], Cecile Rose T. Vibat[2], Dan Calatayud[3], Benny Vittrup Jensen[1], Jane Preuss Hasselby[3], Eric Andrew Collisson[4], Timothy T. Lu[2], Jason C. Poole[2], Vlada Melnikova[2], and Mark G. Erlander[2]

[1]Herlev Hospital, Copenhagen University Hospital, Herlev, Denmark; [2]Trovagene, San Diego, CA; [3]Rigshospitalet, University of Copenhagen, Copenhagen, Denmark; [4]University of California, San Francisco, San Francisco, CA

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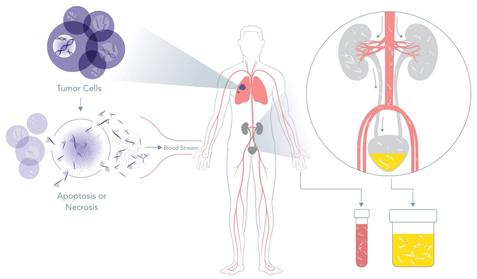
Background

Patients with non-resectable advanced/metastatic pancreatic cancer have a wide range of median time for overall survival (OS). Currently there is a lack of diagnostic tools to predict patient outcome at diagnosis. *KRAS* mutations are present in the vast majority of pancreatic tumors. The study objective was to determine if quantitative baseline and longitudinal monitoring of *KRAS* mutations from plasma circulating tumor DNA (ctDNA) could be used to stratify patients for predicting outcome.



Liquid biopsy: plasma and urine as sources of ctDNA

Clinical Study Design

- Archived plasma samples (up to 6 years) were prospectively collected from 182 patients with locally advanced or metastatic pancreatic cancer in the Danish BIOPAC study.
- Cohort consisted of non-resectable patients undergoing palliative treatment with gemcitabine or FOLFIRINOX.
- 640 longitudinal plasma samples were analyzed for *KRAS* mutational burden in circulating tumor (ctDNA) using the Trovagene quantitative *KRAS* ctDNA assay.
- Potential clinical utility of ctDNA *KRAS* burden to predict patient survival was investigated.
- Timepoints assayed: baseline, before cycle 2 of chemotherapy, and subsequent samples collected approximately every 2-3 months.

KRAS ctDNA Assay Design

- We developed a highly sensitive mutation enrichment assay for the detection of *KRAS* codon 12/13 mutations in highly fragmented plasma ctDNA.
- The assay is comprised of a mutant allele enrichment PCR step followed by massively parallel deep sequencing using MiSeq.
- To achieve greater sensitivity in fragmented ctDNA, the enrichment PCR assay utilizes a 31bp footprint and selectively amplifies mutant DNA fragments while suppressing wild-type (WT) sequence amplification using kinetically-favorable binding conditions for a WT blocking oligonucleotide.
- Barcoded adaptor primers are added for compatibility with massively parallel deep sequencing.
- Following sequencing, a proprietary analysis algorithm allows accurate quantitation of input level of mutant DNA. Results are standardized by reporting number of copies detected per 10^5 genome equivalents (GE).

Assay Performance

- Assay analytical characteristics were developed on a separate dataset using DNA blends from cell lines.
- *KRAS* ctDNA assay Lower Limit of Detection (LLOD) was determined using experimental design based on the Poisson distribution model for rare events.

Performance Characteristics	Performance
ctDNA Source	Plasma (BD Vacutainer – Sodium citrate blood collection tube)
Input DNA	Recommended input of 30-60 ng plasma ctDNA Accurate quantitation of mutant alleles with any DNA input
Analytical Sensitivity	Single copy detection LOD = 0.0055% mutant DNA in a background of wildtype DNA
Analytical Specificity	100%

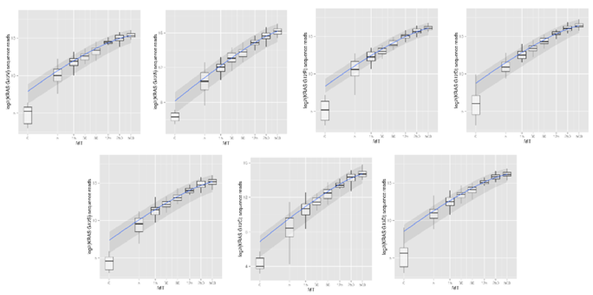
Lower Limit of Detection = 0.0055%

- To demonstrate that the *KRAS* ctDNA assay has a true single copy detection ability, DNA blends with defined mutant spike-in levels of 10, 20, and 40 copies were distributed over 20 wells to obtain 0.5, 1 and 2 mutant copies/well, respectively.
- The expected number of mutant copies present in a perfect Poisson distribution using 20 replicates at each mutant copy spike-in level (0.5, 1 and 2) is indicated.
- The actual positive/negative hit distribution very closely follows the theoretical model for a Poisson distribution, demonstrating the single copy sensitivity of the assay.

Average Number of Mutant Copies Spiked/Well	0.5	1.0	2.0
Expected (95% CI)	8 (3-12)	13 (8-17)	17 (14-20)
Observed G12A	9	10	17
Observed G12C	8	10	14
Observed G12D	5	11	19
Observed G12R	7	12	14
Observed G12S	5	11	15
Observed G12V	8	8	17
Observed G13D	9	12	16

Standard Curves for Quantitation

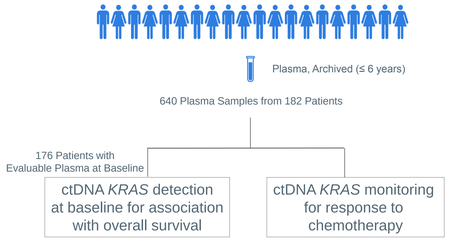
- Standard curves were developed for each mutation using 288 independent enrichment reactions per curve with different amounts of spiked DNA input from 0-500 copies. Master standard curves for *KRAS* Exon 2 codons G12A/C/D/R/S/V and G13D are shown below.



- Boxplots show 25 and 75% quartile. Bars indicate 1.5x of the interquartile ranges.

Clinical Study

Prospective-retrospective study of 182 patients with non-resectable, locally advanced or metastatic pancreatic cancer undergoing treatment with chemotherapy

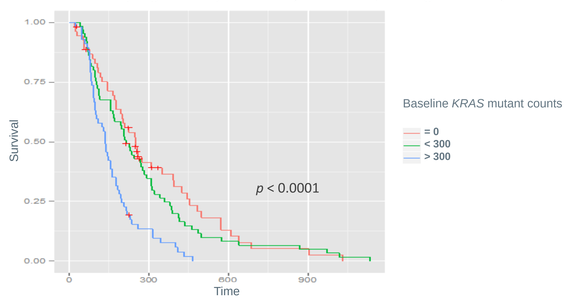


- 617 plasma samples were evaluable (archived for ≤ 6 years).
- 176 out of 182 patients had evaluable baseline samples.
- Of 182 patients, 143 (79%) had more than 1 copy of mutant *KRAS*.
- *KRAS* mutational load in 176 baseline plasma ctDNA samples was analyzed for association with the overall patient survival (OS).

Results

Association between Baseline ctDNA *KRAS* Counts and Overall Survival

- *KRAS* mutational load in 176 baseline plasma ctDNA samples was analyzed for association with OS using a Cox proportional hazards model.
- Statistically significant negative association between baseline *KRAS* counts and OS was observed indicating that patients with lower *KRAS* counts survive longer. This negative association was observed using:
  - log-transformed *KRAS* counts ($p < 0.0001$)
  - rank-transformed *KRAS* counts ($p < 0.0001$)
  - *KRAS* counts categorized into three groups: (1) zero baseline *KRAS* counts (n=58), (2) baseline *KRAS* counts greater than zero but less than or equal to 300 (n=65), and (3) counts greater than 300 (n=53) ($p < 0.0001$). The median survival rates in three groups were also statistically significantly different ($p < 0.0001$, the log-rank test).



Kaplan-Meier survival curves in patients categorized into 3 groups (*KRAS*=0, ≤ 300, >300) demonstrate that patients with lower ctDNA *KRAS* burden survive longer.

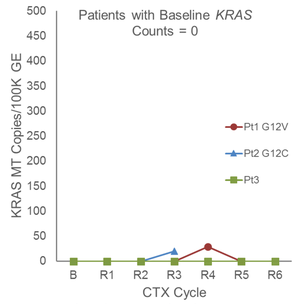
Baseline KRAS count	Number of patients	Median survival	95% CI for median survival
0	58	249 days	194 – 394 days
>0, ≤ 300	65	214 days	168 – 293 days
> 300	53	136 days	102 – 162 days

The median survival rates are statistically significantly different in patients categorized into three groups (*KRAS*=0, ≤ 300, >300) ($p < 0.0001$, the log-rank test). The hazard ratio was 1.624 (95% CI, 1.097 to 2.404) between the <=300 and >300 groups.

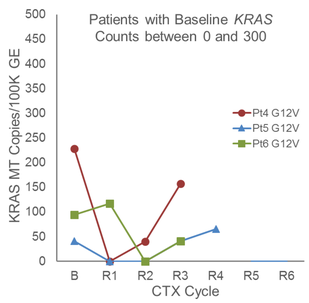
Results

Monitoring KRAS ctDNA Burden Over Time

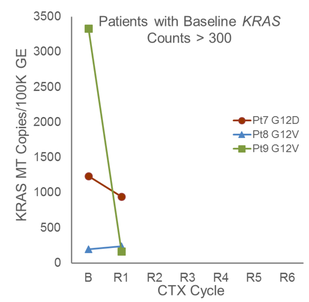
- Representative cases of 176 patients categorized into three groups (baseline plasma ctDNA *KRAS* = 0, <300, >300) demonstrate longitudinal dynamics of KRAS burden on chemotherapy.



KRAS burden remains low on treatment in patients with baseline plasma mutant ctDNA *KRAS* count = 0



KRAS burden decreases, then rises during treatment in patients with baseline plasma mutant ctDNA *KRAS* count ≤ 300



KRAS burden remains high during treatment in patients with baseline plasma mutant ctDNA *KRAS* count >300

Conclusions

- We developed a quantitative PCR-NGS mutation enrichment assay for the detection of *KRAS* codon 12/13 mutations in highly fragmented plasma ctDNA. *KRAS* ctDNA assay has a single copy sensitivity and the ability to detect 0.0055% mutant DNA in a background of WT DNA.
- In a prospective retrospective study of 182 patients with metastatic pancreatic cancer, a statistically significant negative association was found between baseline ctDNA *KRAS* counts and OS ($p < 0.0001$), indicating that patients with lower *KRAS* burden in ctDNA survive longer.
- Ability to monitor *KRAS* ctDNA burden over time is demonstrated.
- Longitudinal monitoring of *KRAS* ctDNA burden over time indicated that patients with longest survival were usually associated with a continued low copy number of ctDNA KRAS mutations over time.
- Stratification at baseline of non-resectable pancreatic cancer patients by ctDNA KRAS mutation copy number may be clinically useful for prognosis.

For more information, please contact
Mark Erlander, PhD
11055 Flintkote Avenue
San Diego, CA 92121
merlander@trovagene.com