Quantitative Mutational Assessment of Circulating Tumor DNA Using Massively Parallel Deep Sequencing in Plasma and Urine from Advanced Colorectal Cancer Patients

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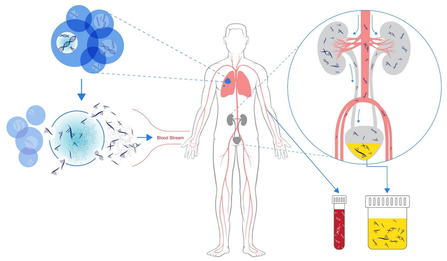
Background

- Technologies enabling the assessment of circulating tumor DNA (ctDNA) in urine and plasma expand the clinical utility to detect and monitor cancer patient oncogenic mutations by minimally invasive and non-invasive liquid biopsy methods.
- Mutational tumor load quantification with high clinical sensitivity is vital for robust individualized assessment of systemic therapeutic responsiveness and resistance.

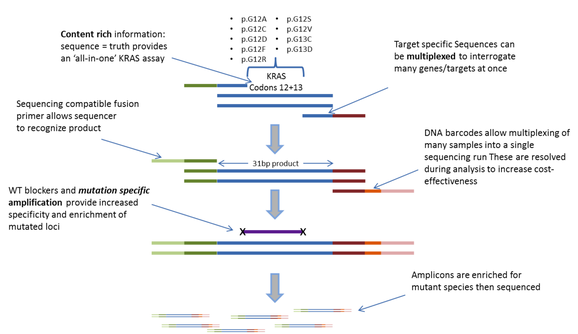


Clinical study

- Samples were collected from 20 patients with Stage IV colorectal cancer and *KRAS* positive primary tumor.
- All patients were undergoing surgical treatment in combination with various systemic medical therapies including neoadjuvant radio/chemotherapy, adjuvant targeted therapy and adjuvant chemotherapy.
- Archived, matched longitudinal plasma and urine samples were collected and evaluated prior to surgery (baseline) as well as time intervals throughout the course of the disease.
- In a blinded retrospective study, concordance between archived tissue/plasma/urine samples was studied for baseline and urine and plasma KRAS was monitored longitudinally.

Assay Design

- We developed a highly sensitive mutation enrichment assay for the detection of *KRAS* codon 12/13 mutations in highly fragmented urinary and plasma ctDNA.
- To achieve greater sensitivity in fragmented cfDNA, the assay utilizes a 31bp footprint and contains a selective enrichment step for mutated DNA fragments while suppressing wild-type (WT) sequence amplification with a blocker.
- Barcoded adaptor primers are added for compatibility with massively parallel deep sequencing.
- Following sequencing , our proprietary analysis algorithm allows accurate quantitation of input level of mutant DNA.



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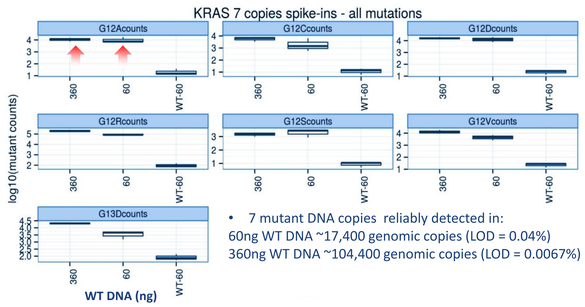
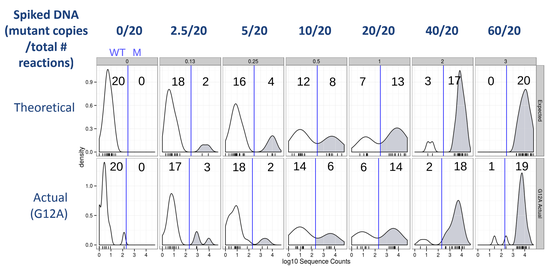
Assay Performance

Limit of Detection* = 0.0067%



- 7 mutant DNA copies reliably detected in:
  60ng WT DNA ~17,400 genomic copies (LOD = 0.04%)
  360ng WT DNA ~104,400 genomic copies (LOD = 0.0067%)

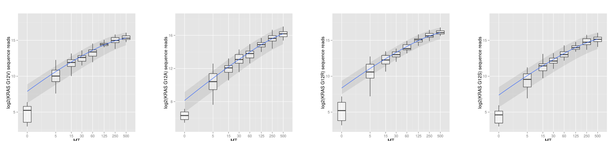
Single Copy Detection*



- Poisson distribution model: The theoretical model shows the number of positive and negative samples that would be present in a perfect Poisson distribution of our test using 20 replicates at each spike-in level. The actual positive/negative hit distribution follows the theoretical model for a Poisson distribution very closely, demonstrating the single copy sensitivity of the assay.

Standard Curves for Quantitation*

- Standard curves were developed for each mutation using 288 independent enrichments reactions/curve with different amounts of spiked DNA input from 0-500 copies. Examples of master standard curves for G12V/A/R/S are shown below.

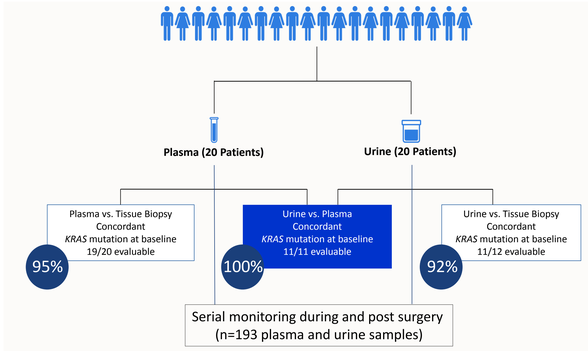


- Boxplots show 25 and 75% quartiles and bars indicate 1.5x of the interquartile ranges.

* Assay characteristics were developed on a separate dataset using DNA blends from cell lines

Performance Characteristics	Performance
Tissue Source	Urine (proprietary collection kit and DNA isolation technology) Plasma (BD Vacutainer EDTA, Streck Cell-Free DNA BCT tube)
Input DNA	2 – 480 ng, recommended input 60 ng Accurate quantitation of mutant alleles with any DNA input
Analytical Sensitivity	Single copy detection LOD = 0.0067% mutant DNA in a background of wildtype DNA
Analytical Specificity	100%

Concordance Study



PLASMA

- *KRAS* mutation concordant with tumor tissue detected in 19 of 20 evaluable baseline plasma samples (95%). In one patient, a *KRAS* mutation was detected consistently across all serial plasma and urine samples but not in tissue biopsy.

URINE

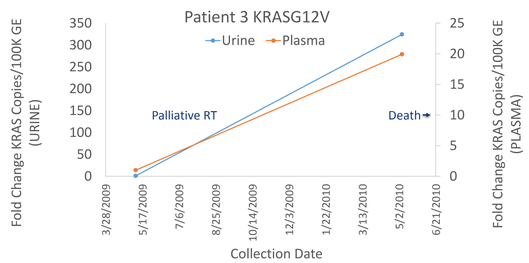
- *KRAS* mutation concordant with tissue detected in 11 of 12 evaluable baseline urine samples (92%).

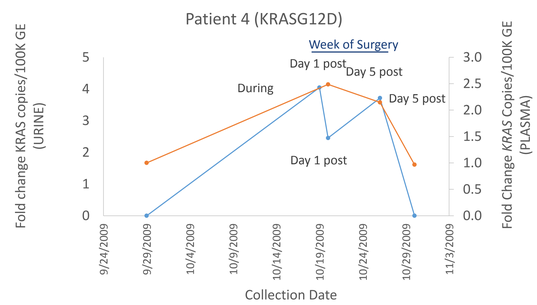
SAMPLE QUALITY

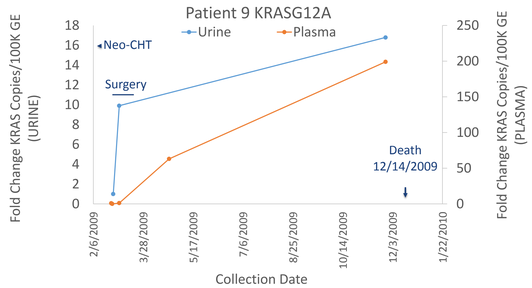
- Total of 190 plasma and urine samples archived for 3-5 years were tested.
- 101 of 101 plasma samples (100%) and 79 of 92 urine samples (86%) had sufficient DNA concentration and were deemed evaluable. The degree of DNA degradation in these archival urine samples is significantly elevated compared to typical clinical samples.

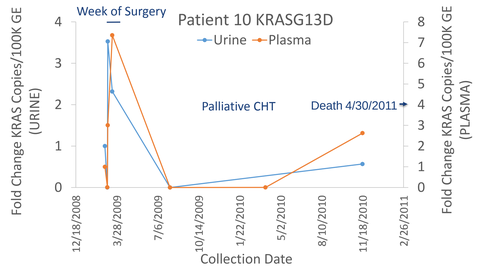
Monitoring during treatment (total n=193 plasma and urine samples)

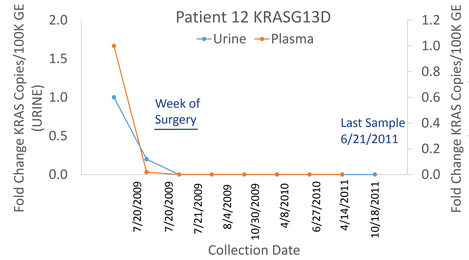
Representative cases of 20 patients shown











Conclusions

- A quantitative ctDNA assay using a massively parallel deep sequencing approach was developed to determine patient ctDNA status of *KRAS* (Exon 2) mutations in plasma and urinary ctDNA.
- We achieved an analytical sensitivity of 0.0067% in a background of WT DNA.
- In a blinded study of colorectal cancer patients with known *KRAS* mutational status in tumor tissue, a correct *KRAS* mutation was identified in 95% of archival plasma and 92% of archival urine specimens.
- Clear correlation and compatible fold change was observed between plasma and urinary ctDNA *KRAS* levels in course of surgery and adjuvant therapy (chemo or targeted).
- Analysis of association between the dynamics of ctDNA *KRAS* load and changes in clinical status is ongoing.
- The first clinical correlation between urinary and plasma ctDNA.

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