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Global quantitative measures using next-generation sequencing for breast cancer presence outperform individual tumor markers in plasma

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Introduction & Objectives

Noninvasive means of detecting, characterizing and monitoring breast cancer are highly desirable and will improve our understanding of tumor biology and disease management for patients. Many groups have successfully detected tumor markers in blood using a variety of technologies, including next generation sequencing (NGS). We performed a series of studies on a small number of patients to evaluate the value of global versus individual markers for the quantitation of tumor-derived cell free DNA (cfDNA) in plasma. Three classes of tumor-derived markers were studied: single nucleotide variants (SNV), copy number aberrations (CNA) and differentially methylated regions (DMR). Tumor-specific alterations were identified by comparing profiles from tumor and buffy coat (BC) DNA samples from the same patient. To assess the feasibility of non-invasive detection of tumor-specific alterations, we looked for the ability to identify these tumor-specific alterations in DNA isolated from the patient's plasma. Our goal is to select markers and a technology that allows sensitive and specific detection of tumor signal in plasma for patients at different disease stages.

Methods

Whole Genome Bisulfite Sequencing
Sample sets from two patients, including primary tumor (fixed paraffin embedded tissue, FPET) and blood collected after diagnosis of metastatic disease, were analyzed. The blood was processed to obtain buffy coat cells and plasma. DNA was isolated from FPET, buffy coat cells (normal) and plasma, and treated with sodium bisulfite. Whole genome libraries were prepared and sequenced on a HiSeq (Illumina) sequencer with ~1.7 billion reads per sample (~29X average depth of coverage). Data were analyzed to obtain both copy number and methylation status[1].

Single Nucleotide Variation Sequencing
Sequencing libraries were prepared from the DNA isolated from 9 patient sample sets (blood and primary FPET) using a custom 280 amplicon AmpliSeq panel covering 346 hotspots in 191 genes selected from TCGA data to cover breast cancer variants observed in the TCGA dataset[2]. Libraries from the FPET and buffy coat DNA were sequenced on the PGM (Ion Torrent) sequencer. Libraries from plasma were sequenced on the Proton (Ion Torrent) sequencer. Data were analyzed to identify tumor-specific single nucleotide variants and to estimate their allelic fraction in plasma. Target sequencing coverage was ≥1000x for FPET and BC and ≥10,000x for cfDNA

Methylation-Specific Quantitative PCR
Sample sets (FPET and blood) were collected from 8 patients at various disease stages. DNA was isolated from FPET, buffy coat cells and plasma and treated with sodium bisulfite. Bisulfite converted DNA was preamplified followed by quantitative PCR amplification. Ten methylation-specific quantitative PCR assays were designed to target regions previously reported to be hypermethylated in some breast cancers[4-6] or identified as differentially methylated in the TCGA methylation array dataset[2]. Data were normalized using a methylation independent control and analyzed to obtain differential methylation signals.

Target Enriched Bisulfite Sequencing
Sample sets were collected from 8 patients at various disease stages. DNA was isolated from FPET, buffy coat cells and plasma, treated with sodium bisulfite and enriched using the NimbleGen SeqCap Epi Enrichment System. Paired end sequence was generated on the HiSeq (Illumina), approximately 1 billion reads were generated per sample. Data were analyzed using methods similar to WGBS analysis to obtain methylation status[1].

References

1. Chan CA et al., 2013. Noninvasive detection of cancer-associated genomewide hypomethylation and copy number aberrations by plasma DNA bisulfite sequencing. Proc. Natl. Acad. Sci. 110: 18761-18768
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5. Weisenberger DJ et al., 2008. DNA methylation analysis by digital bisulfite genomic sequencing and digital MethyLight. Nucleic Acids Res. 36:4689-98
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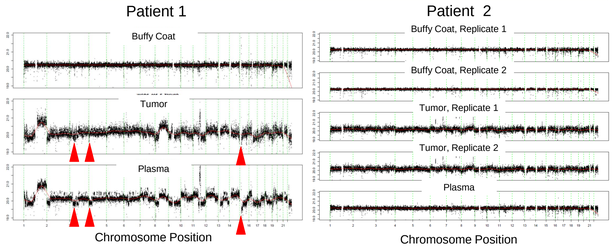
Differential Methylation (NGS – WGBS)

DMR detection in two patients with metastatic breast cancer. Differentially methylated regions were discovered by comparing methylation status in 100kb bins between the buffy coat DNA and the tumor DNA in WGBS data. Only those regions that were above our confidence threshold were considered differentially methylated in the tumor. Plasma samples were interrogated for their methylation status at each site identified as tumor specific. Generalized tumor fraction estimates were computed at each site as the ratio of the differences in methylation status (Plasma – BC) / (FPET – BC). The overall tumor fraction estimate is the weighted average of site-specific estimates, with weights determined by the empirical Bayes posterior probability that a DMR <-10% or >10% was present at each site, thresholded at probability 0.5, and multiplied by the inverse variance of the site-specific estimate.

		Clinical Information					Methylation Derived Tumor Fraction			
Patient	Replicates (BC/FPET)	Disease Status at Blood Collection	Primary Tumor Grade	Positive Nodes at Diagnosis	Primary Tumor Size (cm)	ER / PR / HER2 Status at Diagnosis	Tumor Fraction Estimate	95% CI Lower Limit	95% CI Upper Limit	Bins with weight >0
1	-	Metastatic	2	0	4	+ / + / -	59.6%	57.9%	61.4%	4,492
2	1/1	Metastatic	3	0	2.2	- / - / -	7.3%	5.9%	8.7%	1,835
2	1/2	Metastatic	3	0	2.2	- / - / -	0.8%	0.1%	1.5%	1,076
2	2/1	Metastatic	3	0	2.2	- / - / -	2.5%	1.7%	3.2%	1,354
2	2/2	Metastatic	3	0	2.2	- / - / -	2.0%	1.3%	2.8%	1,240

Whole Genome Copy Number (NGS - WGBS)



CNA detection in two patients with metastatic breast cancer. The log ratio of counts from WGBS was plotted to depict copy number aberrations. The plasma-derived sample from patient 1 shows aberrations that are more extreme than those seen in the tumor, suggesting tumor heterogeneity and/or evolution compared to the primary tumor FPET sample (examples indicated by red arrows). A summary of the results is presented below.

		Clinical Information					CNA Derived Tumor Fraction			
Patient	Replicates (BC/FPET)	Disease Status at Blood Collection	Primary Tumor Grade	Positive Nodes at Diagnosis	Primary Tumor Size (cm)	ER / PR / HER2 Status at Diagnosis	Tumor Fraction Estimate	95% CI Lower Limit	95% CI Upper Limit	Bins with weight >0
1	-	Metastatic	2	0	4	+ / + / -	65.2%	65.1%	65.4%	1,265,763
2	1/1	Metastatic	3	0	2.2	- / - / -	11.8%	11.7%	11.9%	426,935
2	1/2	Metastatic	3	0	2.2	- / - / -	10.9%	10.8%	11.0%	500,487
2	2/1	Metastatic	3	0	2.2	- / - / -	10.9%	10.8%	11.0%	500,487
2	2/2	Metastatic	3	0	2.2	- / - / -	10.3%	10.2%	10.3%	516,969

WGBS Results Summary:
- WGBS data can be used to detect both CNA and methylation-derived tumor signal in cfDNA from patients with metastatic breast cancer
- These methods give consistent, but not identical, estimates for % tumor DNA in plasma
- Visualization of the CNA results indicates that some CNA are only observed in the plasma sample

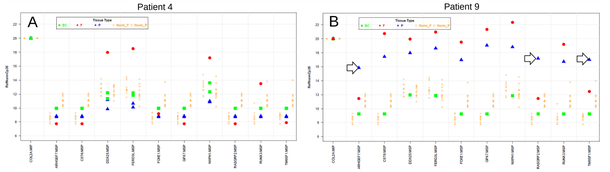
Enriched Methylation (NGS)

	Clinical Information					Enriched DMR Derived Tumor Fraction			
Patient	Disease Status at Blood Collection	Primary Tumor Grade	Positive Nodes at Diagnosis	Primary Tumor Size (cm)	ER / PR / HER2 Status at Diagnosis	Tumor Fraction Estimate	95% CI Lower Limit	95% CI Upper Limit	% Sites (240,131 total)
6	Early Stage	2	0	3.2	+ / + / -	7.3%	7.1%	7.4%	38.3%
11	Early Stage	2	2	4.2	+ / + / -	12.6%	12.3%	12.9%	22.5%
12	Early Stage	3	0	2.2	- / - / -	6.3%	6.1%	6.5%	28.4%
7	Early Stage	3	3	3.4	+ / + / -	5.4%	5.2%	5.7%	13.9%
13	Metastatic	2	2	-	N/A	11.3%	11.0%	11.5%	21.4%
9	Metastatic	2	2	8.5	+ / + / -	76.4%	76.0%	76.8%	30.7%
10	Metastatic	3	-	-	+ / + / -	38.6%	37.8%	39.5%	19.0%
14	Metastatic	3	0	1.7	+ / - / -	17.9%	17.7%	18.1%	27.1%

Differentially methylated regions were discovered by comparing methylation status in 100kb bins between tumor and normal. The plasma-derived samples were subsequently interrogated for the methylation status at sites identified as tumor specific.

Methylome Enrichment Summary:
- Tumor derived DMR were detected in cfDNA from all patients tested
- Patients with metastatic disease generally had higher tumor fraction estimates than those with earlier disease
- Additional studies are required to understand the relationship between tumor fraction estimates and clinical pathology measures and to more completely control for potential tissue-specific differential methylation signals

Targeted Methylation (MS-PCR)



Representative methylation-specific PCR results are shown in top 2 graphs. Panel A shows patient with early stage, grade 1, disease and Panel B shows a patient with metastatic disease. A methylation signal of 20 represents 100% methylation, a signal of 14 represents 1.5% and a 12 represents 0.4% methylation. The buffy coat signal is shown in green squares, FPET signal is shown in red circles, plasma signal is illustrated in blue triangles, unrelated normal breast tissue and plasma samples are shown in yellow circles and triangles, respectively. Hypermethylation signals seen only in plasma samples are indicated with an arrow. The table below summarizes the results for all eight patients.

	Clinical Information						Methylation Specific PCR Tumor		
Patient ID	Disease Status at Blood Collection	Primary Tumor Grade	Positive Nodes at Diagnosis	Primary Tumor Size (cm)	ER / PR / HER2 Status at Diagnosis	Tumor DMR Detected in cfDNA	Tumor DMR Count	% Sites
3	Early Stage	1	0	0.6	+ / + / -	No	2	20.0%
4	Early Stage	1	0	0.6	+ / + / -	No	3	30.0%
5	Early Stage	2	0	2.8	+ / + / -	No	9	90.0%
6	Early Stage	2	0	3.2	+ / + / -	No	5	50.0%
7	Early Stage	3	3	3.4	+ / + / -	No	7	70.0%
8	Early Stage	3	0	3.9	+ / + / +	No	3	30.0%
9	Metastatic	2	2	8.5	+ / + / -	Yes	7	70.0%
10	Metastatic	3	-	-	+ / + / -	Yes	1	10.0%

Methyl-Specific PCR Results Summary:
- MS-PCR assays identify differential methylation in all tumor/normal pairs
- The assays selected for this study were only able to identify tumor-derived signal in the plasma of patients with metastatic disease, more sensitive assay methods will be required for early stage disease
- DMRs not observed in the tumor/normal profile were seen in both patients with metastatic disease raising the possibility we can see evidence of tumor heterogeneity or evolution in the plasma samples from these patients

Enriched SNV (NGS)

SNV detection in nine patients at different disease stages. Tumor-derived SNV were identified by comparing variant calls between the FPET and BC in targeted sequencing data. The allelic fraction of tumor derived SNV in cfDNA was then calculated using a dilution formula. Four of 9 patients did not have any tumor-derived SNV, so these samples are uninformative. As expected, patients with tumor derived SNV in our amplicon panel had only a small number of variants. Tumor fraction estimates for each SNV were not consistent within a patient, so we did not attempt to calculate a summary tumor fraction value.

	Clinical Information					SNV Derived Tumor Fraction	
Patient	Disease Status at Blood Collection	Primary Tumor Grade	Positive Nodes at Diagnosis	Primary Tumor Size (cm)	ER / PR / HER2 Status at Diagnosis	Tumor-derived SNV Count	cfDNA % Tumor Estimate for Each SNV
15	Early Stage	-	-	-	N/A	0	N/A
22	Early Stage	1	0	1.2	+ / + / -	0	N/A
17	Early Stage	2	0	0.4	+ / + / -	0	N/A
21	Early Stage	2	0	2.2	+ / + / -	2	0%, 33%
20	Early Stage	2	4	3.5	+ /+ / +	3	0%, 3%, 0%
19	Early Stage	3	0	2.4	+ / + / -	2	100%. 0%
18	Early Stage	3	0	9.0	+ /+ / +	0	N/A
1	Metastatic	2	0	3.7	+ / + / -	1	84%
16	Metastatic	2	-	3.6	+ / + / Eq	1	0%

SNV Analysis Summary:
- Tumor derived SNV can be detected in cfDNA
- It is difficult to design a small targeted sequencing panel for breast cancer that identifies several tumor-derived SNV for most patients
- When multiple SNV could be measured and each SNV was used to calculate a tumor fraction estimate, individual measures did not generate a consistent tumor fraction estimate for a patient

Conclusions

- Robust detection of tumor in the plasma from patients with active disease requires sensitive, digital detection methods, such as NGS
- While SNVs represent important biomarkers for treatment selection, copy number and methylation markers may be more appropriate for detection and monitoring of tumor-derived cfDNA levels
- Copy number and methylation-based tumor fraction estimates provide similar, but not identical, estimates of tumor fraction
- Profiling many tumor specific markers in both the primary tumor and cfDNA may be useful for studying tumor heterogeneity and/or evolution
- Much more work is required to determine what relationships exist between tumor fraction estimates and clinical pathology measures

We Thank

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