P3-07-57

Development of a 6-gene qPCR RUO-validated assay as a predictive biomarker for response of vantictumab (OMP-18R5; anti-frizzled) in HER2- breast cancer patients

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San Antonio Breast Cancer Symposium - December 8-12, 2015

Abstract

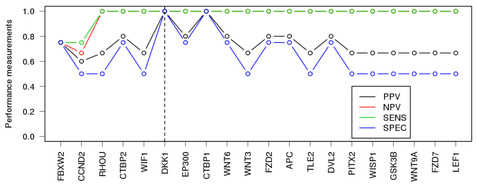
Background: We have developed a monoclonal antibody, vantictumab that blocks canonical WNT/β-catenin signaling through binding of five FZD receptors (1, 2, 5, 7, 8). This antibody inhibits the growth of several tumor types, including breast. Vantictumab reduces tumor-initiating cell frequency and exhibits synergistic activity with standard-of-care (SOC) agents (Gurney et al, 2012). To target breast cancer patients most likely to respond to vantictumab, we undertook a predictive biomarker study.

Methods: We have identified a 6-gene Wnt pathway-related signature, *FBXW2, CCND2, RHOU, CTBP2, WIF1,* and *DKK1,* based on microarray gene expression data from 8 breast cancer patient derived xenograft (PDX) models with established *in vivo* response to vantictumab plus SOC. This signature successfully predicted the response of 8 additional and independent PDX breast tumors. We further developed a qPCR Research Use Only (RUO) assay for the 6 genes to be used on FFPE human breast tumor samples. Multiple assays targeting different regions spanning each mRNA transcript were tested and selected based on PCR efficiency, specificity and sensitivity. We compared assay sensitivity under different cDNA synthesis and pre-amplification conditions: random vs. gene-specific priming, number of pre-amplification cycles, pre-amplification reaction volumes, and cDNA synthesis kits. A repeatability study was performed to test assay performance. The pre-amplification and PCR were repeated over three separate days and across two independent labs.

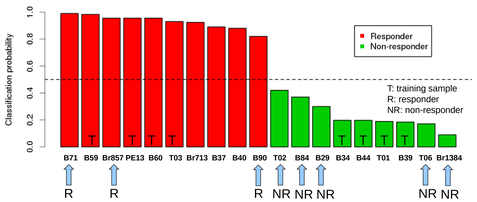
Results: Our results showed that cDNA synthesis by gene-specific priming followed by 18 cycles of pre-amplification performed the best and the assay is robust with minimal starting FFPE RNA input. The results of the repeatability study were consistent among the different days and the different labs (<5% CV). Using the 6-gene qPCR RUO assay, the signature score from the microarray data was further refined using 12 PDX HER2- breast tumors with known *in vivo* response to vantictumab with SOC. The prevalence of the 6-gene signature was established using ~100 HER2- breast cancer samples.

Conclusions: A robust 6-gene RUO-validated assay was developed as a predictive biomarker for vantictumab in HER2- breast cancer. The assay is currently being evaluated in a Phase 1b study of vantictumab with paclitaxel in HER2- breast cancer.

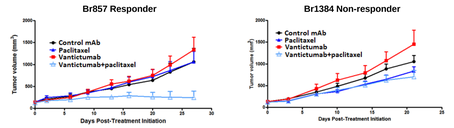
Identification of the 6-gene signature



Microarray gene expression data from 8 PDX breast tumors with established in vivo efficacy to vantictumab plus paclitaxel were used to identify the 6-gene signature. 6 gene signature achieved the best performance NPV=PPV=Sensitivity=Specificity=1



6-gene signature successfully predicted the response of 8 independent PDX breast tumors run at two independent facilities.



Assay design and selection

Six qPCR assays were designed for each of the 6 genes: DKK1, FBXW2, CCND2, RHOU, CTBP2, WIF1.

Assay evaluation criteria:

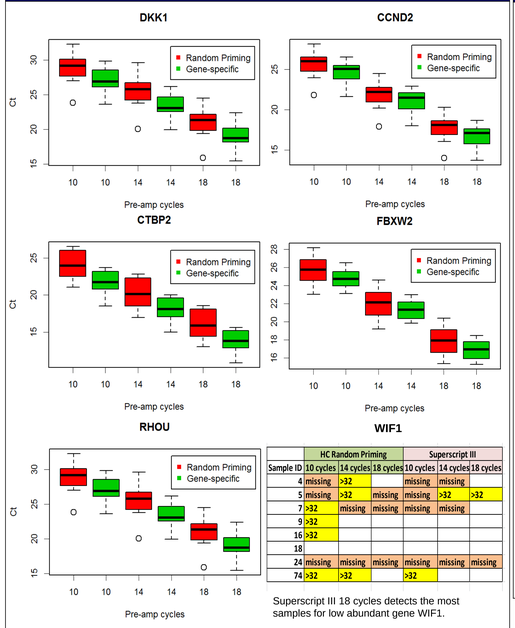
Exon spanning:
- The primer and/or probe cross an exon boundary in the gene sequence

Assay efficiency:
- At least 4 data points from a 5 fold dilution to generate a standard curve
- Range 90-110% efficiency (1.9-2.1)

Specificity:
- Only 1 PCR product amplified, and 15% difference between expected and actual product size

Sensitivity:
- The assay that generated the lowest threshold cycle (Ct) is the most sensitive for that gene.

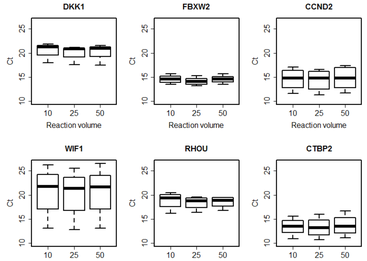
Assay optimization



	HC Random Priming			Superscript III		
Sample ID	10 cycles	14 cycles	18 cycles	10 cycles	14 cycles	18 cycles
4	missing	>32		missing	missing	
5	missing	>32	missing	missing	>32	>32
7	>32	missing	missing	missing	missing	
9	>32					
16	>32					
18						
24	missing	missing	missing	missing	missing	missing
74	>32	>32		>32		

Superscript III 18 cycles detects the most samples for low abundant gene WIF1.

High-capacity (HC) random priming, gene-specific priming, superscript III methods were compared using 8 FFPE samples. Superscript III with gene-specific priming at 18 pre-amplification cycles was the most sensitive method and performed the best. .



Performed superscript III pre-amplification using volumes 10 uL, 25 uL and 50 uL with 128 ng RNA input (concentration: 58 ng/uL, 23.3 ng/uL and 11.6 ng/uL). Using a reaction volume of 50ul enabled 90% of the 117 tested human breast tumors to pass concentration QC.

Repeatability study

10 FFPE samples were run 3 times (each in a separate day) by two different labs. In each run, pre-amp was processed in duplicates, PCR was done in triplicates. Human reference RNA was used as a positive control. Standard curves were used to evaluate assay efficiencies. %CV was calculated across the three runs for each gene in each sample. Consistent data observed across different days and different labs (most of the %CV values are below 5%).

%CV of the three runs for each target gene assay in each sample:

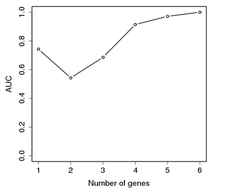
	Lab1	Lab2	Lab1	Lab2	Lab1	Lab2	Lab1	Lab2	Lab1	Lab2	Lab1	Lab2
SampleName	CCND2	CCND2	CTBP2	CTBP2	DKK1	DKK1	FBXW2	FBXW2	RHOU	RHOU	WIF1	WIF1
1	2.76	1.97	0.09	2.1	2.78	3.89	1.03	2.22	0.49	1.39	ND	ND
2	4.99	2.6	5.47	1.61	4.58	1.95	5.62	2.04	4.85	1.64	4.37	1.68
3	0.4	1.32	0.38	1.24	0.52	1.31	1.33	1.06	0.45	1.07	0.58	0.68
4	0.39	1.45	0.22	1.42	0.33	1.73	0.62	1.64	0.65	0.82	1.05	2.96
5	0.26	1.76	0.26	0.95	0.45	1.19	0.95	1	0.47	0.9	0.82	1.44
6	0.37	0.66	0.26	1.25	0.55	1.19	0.93	0.96	0.36	1.38	2.19	12.96
7	0.28	0.87	0.17	0.96	0.46	1.12	0.72	0.92	0.72	0.87	0.58	0.55
8	0.24	0.99	0.28	1.09	1.4	1.74	1.18	0.75	0.7	0.85	2.4	6.83
9	0.56	0.82	0.19	0.91	1.02	1.93	0.76	0.77	0.75	1.13	0.52	0.78
10	0.39	1.6	0.22	1.14	1.17	1.83	0.97	0.86	0.87	0.74	2.54	2.75
Reference RNA	1.13	0.62	0.64	0.76	0.89	0.41	1.9	1.05	0.6	0.52	1.38	1.03

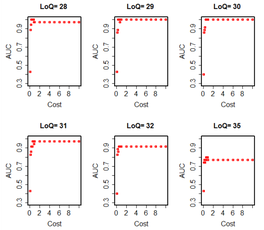
%CV of the three runs for each reference gene assay in each sample:

	Lab 1	Lab 2	Lab 1	Lab 2	Lab 1	Lab 2	Lab 1	Lab 2
SampleName	GUSB	GUSB	PUM1	PUM1	SDHA	SDHA	TOP1	TOP1
1	0.98	1.42	0.26	1.37	1.5	4.64	0.45	1.39
2	4.16	2.63	5.23	1.47	6.1	4.95	3.76	2
3	1.08	0.84	0.45	0.95	0.67	2.43	0.41	1.18
4	0.43	2.93	0.51	0.75	0.51	2.97	0.8	0.97
5	1.1	1.41	0.43	1.12	0.85	2.75	0.45	0.75
6	1.55	1.39	0.34	1.1	0.32	2.16	0.41	1.3
7	1.03	0.79	0.72	0.83	0.82	3.54	1.14	1.39
8	0.85	1.11	0.4	0.94	0.82	1.59	0.47	2.25
9	0.99	0.92	0.28	0.94	0.81	1.41	0.33	1.64
10	0.62	1.23	0.43	0.7	0.27	2.69	1.08	1.4
Reference RNA	1.7	0.58	1.1	0.62	0.79	2.97	1.64	1.68

6-gene signature score refinement

TumorName	Classification	Histology
B71-p3	Responder	ER+PR+HER2-
B59-p2	Responder	ER-PR-HER2-
B60-p2	Responder	ER-PR-HER2-
B90-p2	Responder	ER-PR-HER2-
PE13-p5	Responder	ER-PR-HER2-
B84-p3	Non-responder	ER-PR-HER2-
B39-p2	Non-responder	ER-PR-HER2-
B29-p3	Non-responder	ER-PR-HER2-
T6-p7	Non-responder	ER+PR+HER2-
B44-p3	Non-responder	ER-PR-HER2-
T2-p6	Non-responder	ER-PR-HER2-
T1-p7	Non-responder	ER-PR-HER2-



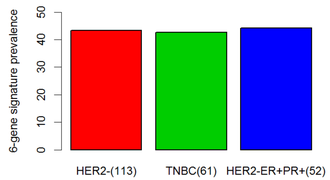


6 gene signature score was refined by using the qPCR assay results and the in vivo response to vantictumab plus paclitaxel in 12 PDX breast tumors.

LoQ was set to be 30 based on the in silico performance with LoQ=28~35.

AUC achieved 100% at 6 genes using leave-one-out cross-validation.

Prevalence estimation



Using the refined 6-gene signature score, prevalence was estimated to be 43.4% in HER2- breast cancer FFPE samples (n=113), 42.6% in TNBC (HER2-ER-PR-, n=61) and 44.2% in HER2- hormone receptor positive (HER2-ER+PR+, n=52).

6-gene signature was not significantly associated with any clinical or technical factors tested in the 113 HER2- breast cancer cohort: primary vs. met, resection vs. biopsy, TNBC vs. HER2-ER+PR+, patient age, % tumor, disease stage, or tumor age.

Summary

- 6-gene signature was identified as a potential predictive biomarker for the response to vantictumab plus paclitaxel in breast tumors using microarray gene expression data.
  - Biomarker was tested in18 PDX models total including validation in 8 independent models
- qPCR-based RUO-validated assay was developed for the 6 gene biomarker.
  - cDNA synthesis by gene-specific priming followed by 18 cycles of pre-amplification with SuperScript III performed the best. Robust results were obtained with minimal RNA concentrations, which maximized the number of samples that passed concentration QC.
  - Repeatability study demonstrated consistent data for the 6-gene assay run across different days and in different labs.
  - 6-gene biomarker score was refined by using the qPCR assay results in 12 PDX breast tumors.
- Prevalence of the 6-gene signature was estimated to be ~43% in HER2- patient samples with a similar distribution between TNBC and HER2- hormone positive breast cancer.
- A robust 6-gene RUO-validated assay has been developed as a predictive biomarker for vantictumab in HER2- breast cancer. The assay is currently being evaluated in a Phase 1b study of vantictumab in combination with paclitaxel HER2- breast cancer (NCT01973309).