Her2 expression in Gastroesophageal Cancer (GEC) FFPE Tissue using Mass Spectrometry (MS) and correlation with *HER2* gene amplification

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Overview

- Trastuzumab had a survival benefit in HER2 positive GEC. Two companion diagnostics, IHC and FISH, are currently used to test HER2 status to determine patients' eligibility for the treatment.
- However, both IHC and FISH have limitations. IHC is semi-quantitative, subjective, and sensitive to antigen instability in FFPE; FISH is laborious, expensive, and subjective. Moreover these are low throughput assays.
- We developed a clinically-validated multiplex MS assay (selected reaction monitoring – SRM) on GEC FFPE tissues for HER2 status evaluation compared to IHC and FISH, along with multivariate analysis of other oncoprotein expression levels including Met-SRM, Egfr-SRM, Her3-SRM.

Methods

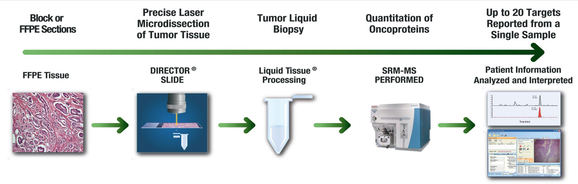


Figure 1: Liquid Tissue®-SRM workflow for analysis of proteins from FFPE tissue.

Analytical Performance of HER2-SRM Assay

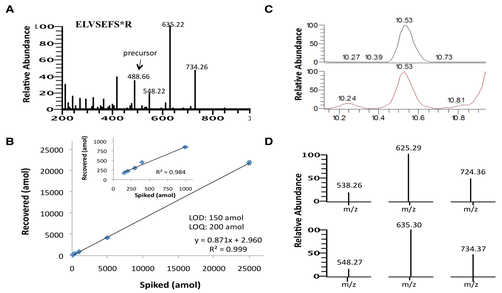


Figure 2. Development of HER2-SRM assay. (A) The fragmentation spectrum for heavy ELVSEFSR peptide and **(B)** the standard curve generated in human PC3 cell lysate; inset: the standard curve generated without the highest two spiking points (5000 and 25000 amol). **(C)** The total ion chromatograms for the light and heavy isotopically labeled peptides, with **(D)** the transition ions used to identify and quantitate each peptide.

SRM Quantitative Reproducibly From Archived Sections

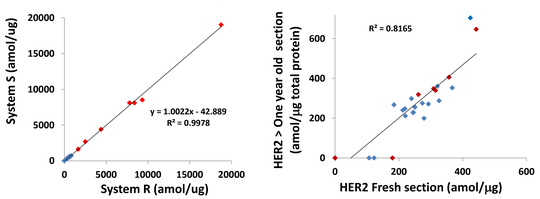
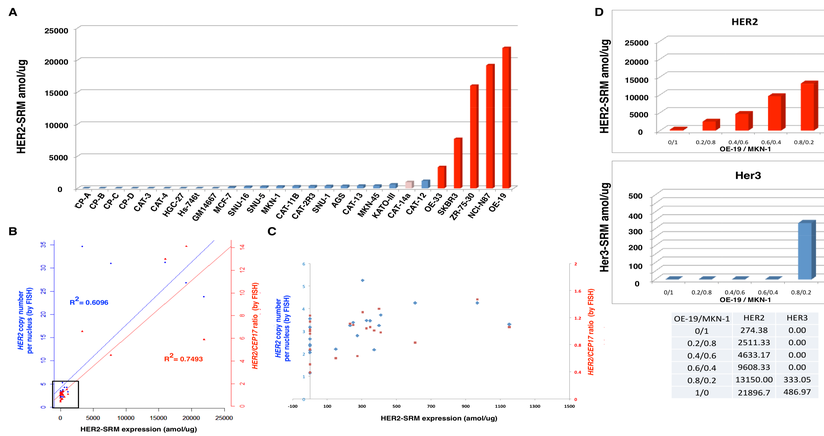


Figure 3. Precision assessment and temporal reproducibility of FFPE sections processed and analyzed using Liquid Tissue-SRM. (A) Precision assessment for measuring HER2 level in 8 breast cancer (red) and 11 GEC (blue) FFPE tissues. **(B)** Temporal reproducibility of FFPE sections processed and analyzed using LT-SRM at two time points over one year apart (blue, GEC (n=18); red, NSCLC (n=9)).

Results

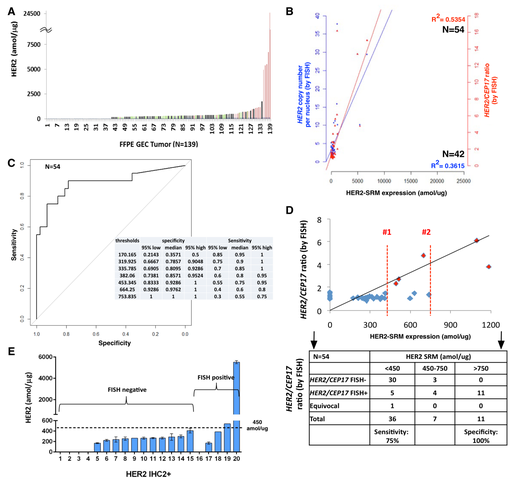
Quantitation of HER2 in Cell Lines & Correlation with Amplification



OE-19/MKN-1	HER2	HER3
0/1	274.38	0.00
0.2/0.8	2511.33	0.00
0.4/0.6	4633.17	0.00
0.6/0.4	9608.33	0.00
0.8/0.2	13150.00	333.05
1/0	21896.7	486.97

Figure 4: HER2 expression levels using LT-SRM and correlation with *HER2* gene amplification (by FISH) in 27 cell lines. (A) Quantification of HER2 (amol/ug) for 27 cell lines including GM15677 lymphoblast control. Dark red: *HER2* amplified cell lines (*HER2/CEP7* ratio ≥2); pink: heterogenous *HER2* amplification. **(B)** HER2-SRM and FISH gene copy number (GCN) univariate correlations (multivariate analysis including Egfr, Her3, and Met SRM coexpression, GCN R^2=0.8829 and Ratio R^2= 0.9824) and **(C)** scatter plot of *HER2* GCN/ratio (by FISH) and HER2-SRM expression in samples where HER2 expressions are < 1500 amol/ug. A preliminary HER2-SRM cut-off, from these cell line data, correlating with *HER2* amplification was determined to be ≥1150 amol/ug. **(D)** HER2-SRM (red) and Her3-SRM (blue) levels in a cell line mixing study (OE-19: *HER2* amplified / MKN-1: *HER2* non-amplified), modeling intra-tumor clonal heterogeneity.

Quantitation of HER2 in FFPE GEC Tumors



HER2 IHC vs SRM

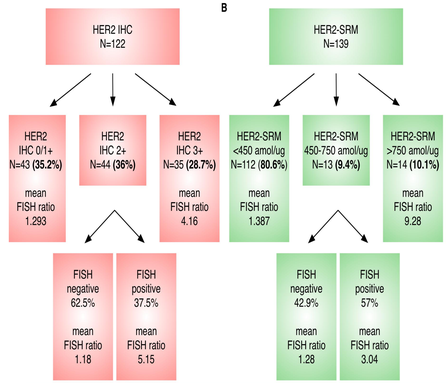
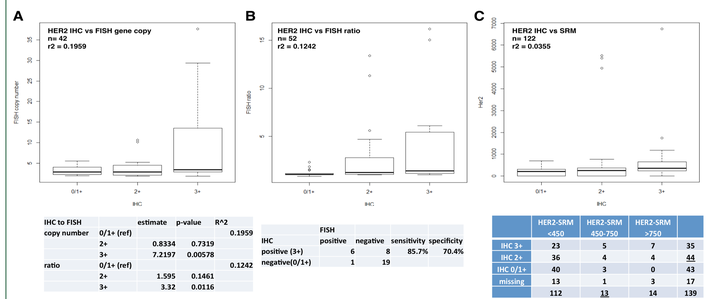


Figure 6. HER2 status assessment of GEC cases by IHC (A) and HER2-SRM (B) to identify HER2 'positive' and 'negative' cases, as determined by underlying FISH *HER2/CEP17* ratio. Both assays resulted in an equivocal zone in identifying underlying *HER2* amplification (by ratio>2).

Figure 5: Absolute levels of HER2 in GEC tissues and correlation of HER2 levels with *HER2* gene amplification. (A) SRM analysis of clinical FFPE GEC tissues (n=139) ranging <150-24617 amol/ug. Red: *HER2* amplified (HER2/CEP17 ratio>2); green: non-amplified; black: not FISH tested. (B) Univariate correlation of HER2 SRM and *HER2* FISH GCN and ratio. Multivariate analysis incorporating Met-SRM, Egfr-SRM, Her3-SRM and *HER2* FISH heterogeneity in the model: HER2-SRM:*HER2* GCN r2 = 0.7345 and HER2-SRM:*HER2/CEP17*ratio r2=0.7643. (C) Optimal HER2-SRM cutoff values determined by receiver operating characteristics (ROC) curve with respect to *HER2/CEP17* ratio >2 . Using one cut-off level, a value of 450 amol/ug was 75% sensitive and 93% specific to identify 'amplification'; alternatively, a cut-off level of 750 amol/ug was 55% sensitive and 100% specific. (D) Using two cut-points (analogous to IHC 0/1+ = HER2 negative, and IHC 3+ = positive), with values in between (analogous to IHC 2+) = 'equivocal', an upper SRM level bound of 750 amol/ug and lower bound of 450 amol/ug created an equivocal range 450-750 amol/ug. The two red lines (1 and 2) represent the HER2 expression falling into this equivocal range (n=9/54, 16%). (E) Among tumors exhibiting HER2 IHC 2+ with FISH results (n=20), 15 tumors (75%) were FISH- and 5 (25%) were FISH+. HER2-SRM expression levels are superimposed, demonstrating that the majority (18, 90%) of these IHC2+ samples were below the 450 amol/ug SRM cut-off.

Correlation of HER2-SRM with *HER2* FISH & HER2 IHC in FFPE GEC Tissues



IHC to FISH copy number		estimate	p-value	R^2
	0/1+ (ref)			0.1959
	2+	0.8334	0.7319	
	3+	7.2197	0.00578	
ratio	0/1+ (ref)			0.1242
	2+	1.595	0.1461	
	3+	3.32	0.0116	

IHC	FISH positive	FISH negative	sensitivity	specificity
positive (3+)	6	8	85.7%	70.4%
negative(0/1+)	1	19		

	HER2-SRM <450	HER2-SRM 450-750	HER2-SRM >750	
IHC 3+	23	5	7	35
IHC 2+	36	4	4	44
IHC 0/1+	40	3	0	43
missing	13	1	3	17
	112	13	14	139

Total sample# (n=122)	SRM≥LOD (>150 amol)	SRM (ND)
IHC HER2 negative (0)	9	5
IHC HER2 ≥ 1+	80	28
	Sensitivity: 89.9%	Specificity: 15.2%

	SRM≥450 (amol/ug)	SRM<450 (amol/ug)
IHC 2+	12	21
IHC <2+	2	16
	Sensitivity: 85.7 %	Specificity: 43.2%

	FISH positive (HER2/CEP17>2)	FISH negative (HER2/CEP17<2)
IHC 2+	12	21
IHC <2+	1	17
	Sensitivity: 92.3 %	Specificity: 44.7%

Figure 7. HER2 IHC correlations with FISH assay or SRM. Correlation of HER2 IHC to **(A)** FISH GCN in 42 GEC tumors (r^2=0.1959), **(B)** *HER2/CEP17* ratio in n=52 GEC tumors (r^2=0.1242) and **(C)** HER2 SRM (amol/μg) in 122 GEC tumors (r^2=0.0355). **(D)** IHC compared to HER2-SRM with inset 2X2 table; and (E) three-way comparison of HER2-IHC, HER2-SRM and *HER2/CEP17* ratio by FISH in 51 GEC tumors where all three values were available. Inset tables demonstrate the comparison of the lower boundary for each assay (left, IHC2+ versus SRM 450amol/ug) and IHC2+ versus FISH (right). Inset tables assess sensitivity/specificity of IHC assuming SRM (D,E) and FISH (B,E) as the comparative standards.

Multiplex Proteomics

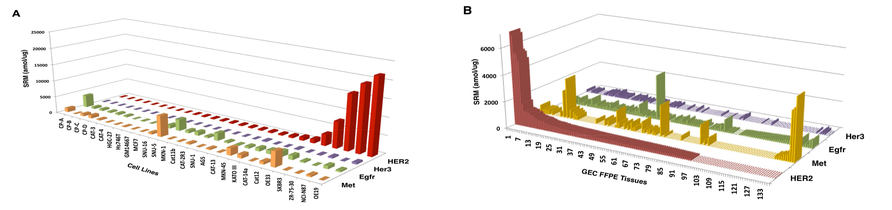


Figure 8. GEC cell lines (N=27) (A) and tissues (N=139) (B) multiplex expression for HER2, Met, Egfr, and Her3 within the 'GEC-Plex'. Samples sorted by HER2 expression. When adjusting for Her3-, Met-, and Egfr-SRM covariates, the correlation between HER2-SRM and *HER2* FISH significantly improved for both sample sets . (Catenacci *et al. PLoS One* July 2014 – Correlation of *MET* amplification with Met-SRM.

Conclusions

- **HER2-SRM is a quantitative assay in clinical FFPE tissues with high specificity and sensitivity.**
- **The *HER2/CEP7* FISH ratio is linear with the level of HER2-SRM, particularly when adjusting for *HER2* FISH heterogeneity and Her3-SRM and Met-SRM.**
- **HER2 expression (any level) was seen in 71.2% of GEC cases. 10.1% (14/139) of samples had HER2 >750amol/ug → all were *HER2* FISH amplified (with an observed wide expression range within FISH+/IHC3+ cases).**
- **SRM/IHC/FISH correlation results suggested that HER2 overexpression determined by SRM is more closely correlated with FISH *HER2* status than IHC HER2 score.**
- **"Equivocal" HER2-SRM (450-750amol/ug) occurred only 9.4% (13/139) vs IHC2+ 36% (44/122), with better FISH+ PPV.**
- **Correlation of SRM HER2 level to clinical outcome on anti-HER2 therapy is ongoing, compared to IHC and FISH scoring.**
- **The ability to concurrently mutiplex HER2 and other relevant proteins via SRM testing represents a refined clinical tool for efficient/expedient tumor expression profiling for clinical application.**