Relaxin 1 and Relaxin 2 are differentially expressed in the tumors of breast cancer patients treated with trastuzumab.

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Trastuzumab (Herceptin) is a monoclonal antibody targeting the extracellular domain of the human epidermal growth factor receptor 2 (HER2)¹ utilized for the treatment of adjuvant and metastatic breast cancer² in the United States and worldwide. We mined published microarray data³⁻⁵ to discover in an unbiased manner the most significant transcriptional changes associated with trastuzumab treatment. We identified both relaxin 1 and relaxin 2, encoded by RLN1 and RLN2 as among the genes most differentially expressed in the primary tumors and tumor cells of patients with breast cancer treated with trastuzumab. The primary tumors of breast cancer patients treated with trastuzumab expressed higher levels of RLN1 and RLN2 messenger RNA than did patients not treated with trastuzumab; a single administration of trastuzumab was sufficient to result in differential expression of RLN1 in primary tumors of the breast, demonstrating increased expression of a peptide hormone important in the central nervous system and for reproduction⁶⁻⁸ as a transcriptional consequence of treatment with trastuzumab.

Keywords: trastuzumab, breast cancer, RLN1, RLN2, relaxin 1, relaxin 2, systems biology of breast cancer, targeted therapeutics in breast cancer.

The mechanism of action ascribed to trastuzumab function is thought to be binding of the F_{ab} domain of trastuzumab to the extracellular region of the human epidermal growth factor receptor 2 (HER2)¹, but the transcriptional consequences resulting from signaling downstream of trastuzumab binding to HER2 is less well understood. Through unbiased, systems-level comparative transcriptome analysis of published microarray data^{3,4}, we report here that RLN1 and RLN2 are differentially expressed genes in the tumors of trastuzumab-treated patients: patients treated with trastuzumab expressed significantly higher levels of RLN1 and RLN2 than did those of patients not treated with trastuzumab.

Methods

We used datasets GSE130788³, GSE114082⁴ and GSE20194⁵ for this differential gene expression analysis of the transcriptional response to trastuzumab treatment in patients with breast cancer. GSE130788 was generated using Agilent-014850 Whole Human Genome Microarray 4x44K G4112F technology with *n*=11 primary breast tumors from patients treated with trastuzumab and *n*=36 primary tumors from patients treated with trastuzumab, analysis performed using platform GPL6480. GSE114082 was generated using Illumina HumanHT-12 WG-DASL V4.0 R2 expression beadchip technology with *n*=17 primary breast tumors from patients before trastuzumab treatment and *n*=17 primary tumors from patients after trastuzumab treatment, analysis performed using platform GPL14951. GSE20194 was generated using Affymetrix Human Genome U133A Array technology with *n*=256 samples of fine needle aspiration isolated breast cancer cells of patients not treated with trastuzumab and *n*=12 samples of fine needle aspiration isolated breast cancer cells of patients treated with trastuzumab, analysis performed using platform GPL96. The Benjamini and Hochberg method of *p*-value adjustment was used for ranking of differential expression but raw *p*-values were used to assess statistical significance of global or multiplexed differential expression. Log-transformation of data was auto-detected, and the NCBI generated category of platform annotation was used. A statistical test was performed to evaluate whether RLN1 or RLN2 gene expression was significantly between primary breast tumors of patients receiving trastuzumab or not using a two-tailed, unpaired t-test with Welch's correction. We used PRISM for all statistical analyses of differential gene expression in human breast cancer (Version 8.4.0)(455).

Results

We performed unbiased comparative transcriptome analysis using published microarray and gene expression data^{3,4} to identify the most significant transcriptional features of the primary breast tumors of patients treated with trastuzumab (Herceptin).

RLN1 is differentially expressed in the tumors of patients treated with trastuzumab.

We identified relaxin 1, encoded by RLN1 as among the genes whose expression changed most significantly in the tumors of breast cancer patients treated with trastuzumab in a multiplexed gene expression dataset³ (Table 1). When sorting each of the genes expressed in the primary tumors of breast cancer patients treated with trastuzumab based on significance of change as compared to that of patients not treated with trastuzumab, RLN1 ranked 37 out of 41000 total transcripts (Table 1), equating to 99.9% differential expression. RLN1 differential expression in the tumors of patients treated with trastuzumab trended towards statistical significance (Table 1; *p*=9.86E-72).

We turned to a second microarray dataset to validate our findings of RLN1 differential expression in the patients treated with trastuzumab. The tumors compared in this dataset were sampled from the same patient before and after a single, brief exposure to trastuzumab, not comparing tumors from different patients treated with a course of trastuzumab or not. Thus, while differentially expressed genes when comparing tumors based on trastuzumab treatment can result from a variety of sources, including

gene expression changes resulting from tumor evolution over the course of treatment, changes identified in this dataset, occurring after only a single treatment of trastuzumab, are more likely to be a direct transcriptional result of trastuzumab binding events. We found again that RLN1 was among the genes most differentially expressed in the tumors of patients administered a single treatment of trastuzumab (Table 2). When sorting each of the genes expressed in the tumors of patients administered a single treatment of trastuzumab, RLN1 ranked 4880 out of 29377 total transcripts (Table 2), equating to 83.4% differential expression. Differential expression of RLN1 in the primary tumors of breast cancer patients following treatment with trastuzumab was statistically significant (Table 2; $p=6E-03$).

RLN1 is expressed at significantly higher levels in the tumors of breast cancer patients treated with trastuzumab than in the tumors of those not treated with trastuzumab.

We obtained exact mRNA expression levels for RLN1 from tumors of patients treated with trastuzumab and from the tumors of patients not treated with trastuzumab to directly evaluate direction and statistical significance of change in expression of RLN1 based on history of trastuzumab treatment. RLN1 was expressed at higher levels in the tumors of patients treated with trastuzumab as compared to those of patients not treated with trastuzumab, and this difference was statistically significant (Figure 1; $p<0.0001$).

RLN2 is differentially expressed in the tumors and tumor cells of patients treated with trastuzumab.

We also identified relaxin 2, encoded by RLN2 as among the genes whose expression changed most significantly in the tumors of breast cancer patients treated with trastuzumab in a multiplexed gene expression dataset³ (Table 3). When sorting each of the genes expressed in the primary tumors of breast cancer patients treated with trastuzumab based on significance of change as compared to that of patients not treated with trastuzumab, RLN2 ranked 34 out of 41000 total transcripts (Table 3), equating to 99.9% differential expression. RLN2 differential expression in the tumors of patients treated with trastuzumab trended towards statistical significance (Table 3; $p=4.01E-73$).

We turned to a second microarray dataset to validate our findings of RLN2 differential expression in the patients treated with trastuzumab. We found again that RLN2 was among the genes most differentially expressed patients in patients treated with trastuzumab when compared to those not treated with trastuzumab, here in the fine needle aspiration-obtained primary tumor cells of breast cancer patients (Table 4). When sorting each of the genes expressed in the primary tumor cells of patients treated with trastuzumab as compared to that of patients not treated with trastuzumab, RLN2 ranked 1843 out of 22283 total transcripts (Table 4), equating to 91.7% differential expression. Differential expression of RLN2 in the primary tumor cells of breast cancer patients treated with trastuzumab approached statistical significance (Table 4; $p=5.54E-02$).

RLN2 is expressed at significantly higher levels in the tumors of breast cancer patients treated with trastuzumab than in the tumors of those not treated with trastuzumab.

We obtained exact mRNA expression levels for RLN2 from tumors of patients treated with trastuzumab and from the tumors of patients not treated with trastuzumab to directly evaluate direction and statistical significance of change in expression of RLN2 based on history of trastuzumab treatment. RLN2 was expressed at higher levels in the tumors of patients treated with trastuzumab as compared to those of patients not treated with trastuzumab, and this difference was statistically significant (Figure 2; $p<0.0001$).

1 Thus, we found using published microarray data^{3,4} that both RLN1 and RLN2 was among the
2 genes most differentially expressed in the tumors of patients treated with trastuzumab, that RLN1 and
3 RLN2 were both expressed at higher levels in the tumors of patients treated with trastuzumab as
4 compared to those not treated with trastuzumab, and that this transcriptional change, at least for RLN1,
was likely a direct result of trastuzumab treatment as opposed to a gene expression change associated with
clonal selection and evolution of the tumor across the course of treatment.

5 **Discussion**

6 We provided evidence here that the peptide hormones RLN1 and RLN2 are differentially
7 expressed genes in the tumors of patients treated with trastuzumab, and expressed at significantly higher
8 levels in the primary tumors of breast cancer patients treated with trastuzumab as compared to patients not
9 treated with trastuzumab. These data demonstrate increased primary tumor expression of peptide
hormones with functions in the central nervous system and in male and female reproduction⁶⁻⁸ as a
transcriptional consequence of treatment with trastuzumab in patients with breast cancer.

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Rank	ID	p-value	t	B	Gene	Gene name
37	A_23_P60210	9.86E-72	28.288542	152.96821	RLN1	relaxin 1

Table 1: RLN1 is differentially expressed in the primary tumors of breast cancer patients treated with trastuzumab.

The rank of differential expression, probe ID, p-value with respect to global differential expression, t, a moderated t-statistic, B, the log-odds of differential expression between the two groups compared, gene and gene name are listed in this chart.

Rank	ID	p-value	t	B	Gene
4880	ILMN_1796775	6E-03	2.9208245	-2.51109	RLN1

Table 2: A single exposure to trastuzumab is sufficient to drive differential expression of RLN1 in the primary tumors of patients with breast cancer.

The rank of differential expression, probe ID, p-value with respect to global differential expression, t, a moderated t-statistic, B, the log-odds of differential expression between the two groups compared, gene and gene name are listed in this chart.

Figure 1: RLN1 is expressed at significantly higher levels in the primary tumors of patients treated with trastuzumab.

The mRNA expression level of RLN1 is graphically depicted in this chart in the primary tumors of breast cancer patients not treated with trastuzumab (left) and in the primary tumors of breast cancer patients treated with trastuzumab (right), with mean mRNA expression level marked and the result of a statistical test evaluating significance of difference between RLN1 expression in the primary tumors of patients treated with trastuzumab or not, a *p*-value, listed above.

Rank	ID	p-value	t	B	Gene	Gene name
34	A_23_P216455	4.01E-73	28.859013	156.145286	RLN2	relaxin 2

Table 3: RLN2 is differentially expressed in the primary tumors of breast cancer patients treated with trastuzumab.

The rank of differential expression, probe ID, p-value with respect to global differential expression, t, a moderated t-statistic, B, the log-odds of differential expression between the two groups compared, gene and gene name are listed in this chart.

Rank	ID	p-value	t	B	Gene	Gene name
1843	214519_s_at	5.54E-02	-1.9239763	-4.2459	RLN2	relaxin 2

Table 4: RLN2 is differentially expressed in the fine needle aspiration-derived tumor cells of breast cancer patients treated with trastuzumab.

The rank of differential expression, probe ID, p-value with respect to global differential expression, t, a moderated t-statistic, B, the log-odds of differential expression between the two groups compared, gene and gene name are listed in this chart.

Figure 2: RLN2 is expressed at significantly higher levels in the primary tumors of patients treated with trastuzumab.

The mRNA expression level of RLN2 is graphically depicted in this chart in the primary tumors of breast cancer patients not treated with trastuzumab (left) and in the primary tumors of breast cancer patients treated with trastuzumab (right), with mean mRNA expression level marked and the result of a statistical test evaluating significance of difference between RLN2 expression in the primary tumors of patients treated with trastuzumab or not, a *p*-value, listed above.