bHLHb40 (HES-2) is differentially expressed in brain metastatic breast cancer.

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Metastasis to the brain is a clinical problem in patients with breast cancer¹⁻³. We mined published microarray data^{4,5} to compare primary and metastatic tumor transcriptomes to discover genes associated with brain metastasis in patients with metastatic breast cancer. We found that the transcription factor bHLHb40, also known as HES-2 (Hairy / Enhancer of Split 2), encoded by the HES2 gene was among those whose expression was most different in the brain metastases of patients with metastatic breast cancer as compared to primary tumors of the breast. bHLH transcription factors and hairy-related proteins are regulators of neurogenesis and developmental patterning⁶. HES2 may be relevant to processes underlying metastasis of primary tumor-derived cancer cells to the brain in humans with metastatic breast cancer.

Keywords: breast cancer, brain metastases, central nervous system metastases, HES2, bHLHb40, systems biology of breast cancer, targeted therapeutics in breast cancer.

One report described a 34% incidence of central nervous system metastases in trastuzumab-treated patients with breast cancer². This alarmingly high frequency of CNS metastasis events demands an enhanced understanding of the transcriptional makeup of brain metastatic tissues to support identification of therapeutic targets, whether they are treatment related or not. We performed a global comparative analysis of primary and metastatic tumors in patients with brain metastatic breast cancer^{4,5}. We discovered significant differential and decreased expression of HES2 in brain metastatic tissues of patients with metastatic breast cancer.

Methods

We used datasets GSE125989⁴, GSE52604⁵ and GSE43837⁶ for this global differential gene expression analysis of brain metastatic breast cancer in conjunction with GEO2R. GSE125989 was generated using Affymetrix Human Genome U133A 2.0 Array technology with $n=16$ primary tumors from patients with breast cancer and $n=16$ brain metastases from patients with brain metastatic breast cancer. GSE52604 was generated using Whole Human Genome Microarray 4x44K G4112F technology with $n=10$ normal breast tissues and $n=35$ brain metastases from patients with breast cancer, analysis performed using platform GPL6480. GSE43837 was generated using Affymetrix Human X3P Array technology with $n=19$ primary breast tumors and $n=19$ brain metastases from patients with breast cancer, analysis performed using platform GPL1352. 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 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 HES2 gene expression was significantly between primary breast tumors and brain metastases in humans with breast cancer 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). For Kaplan-Meier survival analysis, we used the Kaplan-Meier plotter online tool⁷ for correlation of HES2 mRNA expression levels with relapse (recurrence)-free survival (RFS) in $n=3951$ breast cancer patients.

Results

We performed global comparative transcriptome analysis of the primary and metastatic tumor tissues of patients with brain metastatic breast cancer using published microarray data⁴⁻⁶ to describe the transcriptional landscape of brain metastasis in human breast cancer in an unbiased fashion and for the discovery of novel therapeutic targets.

HES2 is differentially expressed in the brain metastases of patients with brain metastatic breast cancer.

We identified HES2 as among the genes whose expression was most significantly different in the brain metastases of patients with brain metastatic breast cancer⁴ (Table 1). When sorting each of the genes expressed in brain metastases based on significance of difference as compared to the primary tumors of patients with brain metastatic breast cancer, HES2 ranked 181 out of 22297 total transcripts (Table 1), equating to 99.2% differential expression. Differential expression of HES2 in the brain metastases of patients with brain metastatic breast cancer was statistically significant (Table 1; $p=1.26E-03$).

Cross-validation of differentially expressed genes across datasets in cancer can be difficult and more challenging than when cross-validating across organs and sorted cell populations due to higher levels of heterogeneity in tumors, and differing methods of tumor sampling between laboratories. Thus, we asked whether we could validate differential expression of HES2 when comparing brain metastases from patients with breast cancer to normal breast tissue as opposed to primary tumors of the breast. Comparison of global gene expression profiles of 10 normal breast tissues to 35 brain metastases⁵ revealed that HES2 was among the genes whose expression was most significantly different transcriptome-wide between the breast and brain metastases in patients with breast cancer (Table 2). When sorting each of the genes expressed in brain metastases based on significance of difference in expression between brain metastases and normal breast tissues, HES2 ranked 5603 out of 41093 total transcripts (Table 2), equating to 86.4% differential expression. Differential expression of HES2 in brain metastases from patients with brain metastatic breast cancer in this dataset was statistically significant (Table 2; $p=3.76E-04$).

HES2 is expressed at significantly lower levels in the brain metastases of patients with brain metastatic breast cancer.

We obtained exact mRNA expression levels for HES2, in primary tumors of the breast and in brain metastasis of patients with brain metastatic breast cancer to determine magnitude and direction of change in HES2 expression in brain metastatic tissues. HES2 was expressed at lower levels in the brain metastases of patients with breast cancer as compared to primary tumors of the breast, and this difference was statistically significant (Figure 1; $p=0.0012$). We calculated a mean fold change of 0.65 ± 0.27 in HES2 expression when comparing primary tumors of the breast to brain metastatic tissues in patients with breast cancer (Table 1).

HES2 expression associates with survival outcomes in breast cancer.

We performed Kaplan-Meier survival analysis to evaluate correlation between HES2 mRNA expression levels with survival outcomes in patients with breast cancer. In 3951 breast cancer patients, we found a statistically significant correlation between HES2 expression and relapse (recurrence)-free survival (RFS) (Figure 2; log rank p -value: 0.0012 for relapse-free survival, hazard ratio: 0.84 (0.75 - 0.93) (Fig. 2)). Lower HES2 mRNA levels in primary tumors of the breast, like which we observed in metastases to the brain, were a negative prognostic indicator in patients with breast cancer. Median RFS was 191.21 months for patients with low tumor expression of HES2 while median RFS was 216.66 months for patients with high tumor expression of HES2 (Table 3).

Thus, by mining published microarray data⁴⁻⁶ in an unbiased and systematic fashion, we identified HES2 as among the genes whose expression was most different in the brain metastases of patients with breast cancer both when compared to primary tumors of the breast and to the normal breast; HES2 was expressed at significantly lower levels in brain metastases as compared to primary tumors of the breast. HES2 expression in primary tumors of the breast in a large cohort of patients demonstrated a significant correlation with relapse-free survival, with higher expression of HES2 correlating with improved survival in humans with breast cancer.

Discussion

HES-2 encodes a protein of 173 amino acids with expression described in the placenta, and in cancers of the cervix, pancreas, head and neck, and colon and rectum⁸. HES-2 from adult brain shares 59% homology with *Drosophila h* (hairy)⁹. Expression of HES-2 in rat was observed starting embryonic

1 day 9.5 and in adults expression could be observed in the adrenal glands, skin, heart, lungs, and to a lesser
2 extent in the central nervous system. HES-2 from rat contains a basic HLH domain as well as a carboxy-
3 terminal WRPW sequence¹⁰. HES-2 can bind E-boxes through the CANNTG nucleotide sequence and
4 can suppress transcription from E-boxes and N-boxes¹⁰. In mice, HES-2 transcriptional targets can be
5 classified into two broad groups - those also regulated by Notch, and those not regulated by Notch. While
6 bHLH transcription factors and HES proteins in specific are important for neurogenesis^{12,13}, specific
7 functions for HES-2 in this context have not yet been described, but based on its transcriptional capacity,
8 ability to co-regulate Notch targets and homology to other HES and bHLH family proteins, likely exists.

We report here that HES2 is among the genes whose expression is most different in the brain
metastases of patients with metastatic breast cancer and that its expression in primary tumors of the breast
correlated with patient survival. Understanding the transcriptional functions of HES2 in the breast and in
the brain in greater detail may provide insights into molecular mechanisms underlying metastasis to the
brain in patients with metastatic breast cancer.

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Rank	ID	p-value	t	B	FC	Gene	Gene name
181	216674_at	1.26E-03	-3.513359	-0.9356	0.65 ± 0.27	HES2	hes family bHLH transcription factor 2

Table 1: HES2 is differentially expressed in brain metastases in brain metastatic breast cancer.

Rank of differential expression, probe ID, *p*-value with respect to differential expression, t, a moderated t-statistic, B, the log-odds of differential expression between the two groups compared, fold change (FC) of HES2 expression between the brain metastases of patients with breast cancer and primary tumors of the breast, gene and gene name are listed in this chart.

Rank	ID	p-value	t	B	Gene	Gene name
5603	A_24_P214198	3.76E-04	3.8330866	-0.303792	HES2	hes family bHLH transcription factor 2

Table 2: HES2 is differentially expressed in brain metastases in brain metastatic breast cancer.

Rank of differential expression, probe ID, *p*-value with respect to 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: HES2 is expressed at significantly lower levels in the brain metastases of patients with brain metastatic breast cancer.

The mRNA expression level of HES2 in primary tumors of the breast (left) and in the brain metastases of brain metastatic breast cancer patients (right) is graphically represented here with mean HES2 levels marked and the result of a statistical test evaluating difference in mRNA expression level between brain metastases and primary tumors of the breast, a *p*-value, listed above.

Figure 2: HES2 expression correlates with relapse-free survival in patients with breast cancer.

Depicted in this Kaplan-Meier plot is the probability of relapse (recurrence)-free survival (RFS) for $n=3951$ total patients stratified into two groups, based on low or high expression of HES2 in patient primary tumors. The log rank p -value denoting statistical significance of difference in relapse-free survival when comparing the two groups, as well as hazard ratio for this comparison is listed above. Listed below is the number of patients at risk (number of patients alive) per interval, after stratification based on HES2 expression; in the first interval, number at risk is number of patients alive; in each subsequent interval, number at risk is the number at risk less those who have expired or are censored.

Low expression cohort (months)	High expression cohort (months)
191.21	216.66

Table 3: Median relapse-free survival is superior in breast cancer patients with high primary tumor expression of HES2.

The relapse-free survival of $n=3951$ breast cancer patients based on stratification into low or high tumor expression of HES2 is listed in this chart.