HAGE in Triple-Negative Breast Cancer Is a Novel Prognostic, Predictive, and Actionable Biomarker: A Transcriptomic and Protein Expression Analysis

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Abstract

Purpose: The expression of HAGE as a novel prognostic and predictive tool was assessed in 1,079 triple-negative breast cancers (TNBC).

Experimental Design: HAGE protein expression was investigated in an early primary TNBC (EP-TNBC; n = 520) cohort who received adjuvant chemotherapy (ACT) and in a locally advanced primary TNBC cohort who received anthracycline combination Neo-ACT (n = 110; AC-Neo-ACT). HAGE-mRNA expression was evaluated in the METABRIC-TNBC cohort (n = 311) who received ACT and in a cohort of patients with TNBC who received doxorubicin/cyclophosphamide Neo-ACT, followed by 1:1 randomization to ixabepilone (n = 68) or paclitaxel (n = 64) as part of a phase II clinical trial. Furthermore, a cohort of 128 tumors with integrated HAGE gene copy number changes, mRNA, and protein levels were analyzed.

Results: In patients with EP-TNBC, who were chemotherapy-naïve, high HAGE protein expression (HAGE⁺) was associated with a higher risk of death [HR, 1.3; 95% confidence interval (CI), 1.2–1.5; P = 0.000005] when compared with HAGE⁻ cases. Patients who received ACT and expressed mRNA-HAGE⁺ were at a lower risk of death than those who were mRNA–HAGE⁻ (P = 0.004). The expression of HAGE was linked to the presence of tumor-infiltrating lymphocytes (TIL), and both features were found to be independent predictors for pathologic complete response (pCR, P < 0.001) and associated with prolonged survival (P < 0.01), following AC-Neo-ACT. In patients with residual disease, HAGE⁺ had a 2-fold death risk increase (P = 0.018) compared with HAGE⁻.

Conclusions: HAGE expression is a potential prognostic marker and a predictor of response to anthracycline treatment in TNBC. A prospective clinical trial to examine the therapeutic value of HAGE for TNBC cases is warranted. Clin Cancer Res; 22(4); 905-14. ©2015 AACR.

Introduction

Of the 1.38 million newly diagnosed breast cancer cases each year, 12% are defined as being triple negative (TNBC; refs. 1, 2). The management of TNBC remains a significant clinical challenge, and is hindered by the inability of these tumors to respond to traditional hormone therapies and targeted agents. Research efforts to discover specific prognostic and predictive molecular signatures that can guide individualized therapy for this subgroup of breast cancer patients are therefore urgently needed (3). Recent studies suggest that clinical outcomes in TNBC are particularly influenced by immune responses to the tumor (4–7), and data have revealed that the basal-like, immunomodulatory, and mesenchymal stem-like molecular subtypes, are characterized by (i) tumor-infiltrating lymphocytes (TIL) with high PD1 and PDL1 expression, (ii) elevated expression of genes that are involved in T-cell function, immune transcription, IFN response, and antigen processing (8) and (iii) high pathologic complete response (pCR) rate and favorable clinical outcome after anthracycline combination Neo-ACT (AC-Neo-ACT; refs. 6–8). Moreover, recent data suggest that some patients with TNBC may benefit from immune-based therapies (4, 5) such as immunostimulating therapies that might act synergistically when combined with chemotherapy, and tumor vaccines targeting cancer-specific antigens (CSA) that might be highly expressed in TNBC (5, 9). However, there is a general lack of information underpinning the expression of CSAs in breast cancer, especially in the TNBC subtype.

We have previously shown that Helicase antigen (HAGE, DDX43) protein expression is (i) commonly expressed in ER-negative breast cancer, (ii) that expression is significantly associated with aggressive clinicopathologic features in breast cancer, and (iii) that its expression is a potential predictor for response to chemotherapy (10). Consequently, HAGE could be a novel prognostic biomarker and a surrogate predictive marker of response to the standard chemotherapy prescribed for primary TNBC in the
Translational Relevance

Helicase antigen (HAGE, DDX43) is a cancer-specific antigen that is required for cancer cell proliferation and has immunogenic properties. This is the first study to report HAGE expression as a promising prognostic biomarker that predicts benefit from adjuvant and neoadjuvant anthracycline-based chemotherapy in triple-negative breast cancer (TNBC). The primary potential clinical significance of our results is the ability to identify those patients with TNBC who are likely to benefit from the standard neoadjuvant/adjuvant anthracycline chemotherapy, and spare other patients whose response would be poor from enduring the unnecessary serious cytotoxic side effects. Considering the limited treatment options for TNBC, the potential of combining chemotherapy with immunotherapy as a therapeutic option should be explored in more detail, and a prospective clinical trial of such an approach is warranted.

Materials and Methods

Study populations

All patients were consented as per hospital standard of care. The study was approved by the Institutional Review Board or Independent Ethics Committee and the Hospital Research and Innovations Department at all participating sites.

Adjuvant cohorts. For HAGE protein expression: Nottingham Early Primary TNBC cohort (EP-TNBC; n = 520). We retrospectively identified 520 consecutive patients with early primary invasive TNBC who were diagnosed between 1986 and 2010 (13), and whose tissues were suitable for the analysis of HAGE expression by immunohistochemistry. Nottingham-EP-TNBC patients were all female and their median age was 51 years (range 28–71 years). Their median follow-up was 107 months (range 2–243 months). Each patient’s tumor was confirmed as being estrogen receptor (ER) negative, progesterone receptor (PR) negative, and human epidermal growth factor receptor 2 (HER2) negative. Patients received standard breast surgery (mastectomy and axillary node clearance) 4 weeks after surgery (mastectomy and axillary node clearance) 4 weeks after mastectomy and axillary node clearance for node positive patients did not receive ACT. RNA was extracted from fresh frozen tumors and subjected to transcriptional profiling on the Illumina HT-12 v3 platform.

For HAGE mRNA level: METABRIC-TNBC cohort. HAGE mRNA and its clinicopathologic significance were evaluated in 311 of TNBC cases derived from the Molecular Taxonomy of BC International Consortium (METABRIC) cohort. The METABRIC study protocol, detailing the molecular profiling methodology in a cohort of breast cancer samples has been described previously (14). Patient demographics are summarized in Supplementary Table S2. ACT was given to 167 patients, whereas 144 patients did not receive ACT. RNA was extracted from fresh frozen tumors and subjected to transcriptional profiling on the Illumina HT-12 v3 platform.

Neoadjuvant cohorts. For HAGE protein expression and response to chemotherapy: Nottingham Locally Advanced Primary TNBC (n = 110). The expression of HAGE protein and the presence of TILs were evaluated in pair-matched prechemotherapy core biopsies and postchemotherapy surgical specimens from 110 patients with locally advanced primary breast cancer (LAP-TNBC; stage IIIA–C) treated with neoadjuvant chemotherapy (Neo-AC-T). The median follow-up was 60 months. The relationship (s) of these parameters with the response to treatment and survival were investigated. These patients were diagnosed at Nottingham University City Hospital between 1996 and 2012. All LAP-TNBC patients were female and their mean age was 50 years (range 25–75 years). Patients either received AC-Neo-ACT alone (72/110; 65.5%) or with Taxane (38/110; 34.5%). Seventy of 110 (63.6%) of patients received six cycles of an anthracycline-based therapy [FEC: 5-fluourouracil (5-FU) 500 mg/m2, epirubicin 75–100 mg/m2, cyclophosphamide 500 mg/m2, on day 1 of a 21-day cycle]. All patients underwent surgery (mastectomy and axillary node clearance) 4 weeks after the last cycle of chemotherapy, followed by radiotherapy to the chest wall. Additional ACT (docetaxel 75 to 100 mg/m2 every 3 weeks, for 3 weeks) was given to patients with significant residual tumor (20.8%). The pCR was defined as the absence of any residual invasive carcinoma at both the primary site and in axillary lymph nodes. On average, 16 breast blocks and all submitted lymph nodes were examined for each case before a diagnosis of pCR was reached. Detailed patient demographics and clinicopathologic characteristics were routinely assessed and regularly updated (Supplementary Table S3).

Histopathologic analysis of the TILs was performed on hematoxylin and eosin–stained sections by adopting the Denkert and colleagues protocol (10, 15). In summary, intratumoral lymphocytes (iTu-Ly) were defined as intraepithelial mononuclear cells within tumor cell nests or in direct contact with tumor cells; and were reported as the percentage of the tumor epithelial nests that contain infiltrating lymphocytes. Stromal lymphocytes (str-Ly) were defined as the percentage of tumor stroma area that contains a lymphocytic infiltrate without direct contact to tumor cells. Histopathologic analysis was performed by two pathologists (T.M.A. Abdel-Fatah and L.O. Ellis) who were blinded to clinical and response data; the mean was used for analysis. TILs were classified into three categories: (i) predominant TIL infiltrate, defined as the presence of either iTu-Ly in >60% of tumor cell nests or lymphocytes in >60% of the stromal area, (ii) focal TIL infiltrate, included detectable mononuclear cells >10%, but <60% of
str-ly or iTu-ly, and (iii) no or minimal TILs, defined as no detectable or presence of ≤10% mononuclear cells in tumor cell nests and tumor stroma.

For HAGE mRNA expression: Multicentre phase II trial. Relationship between HAGE mRNA expression and response to chemotherapy. The relationship between HAGE mRNA expression and the response to Neo-AC was explored in a randomized, open-label, multicentre, phase II trial (NCT00455533) which enrolled previously untreated women with histologically confirmed early primary breast cancer (T2–3, N0–3, M0, tumor size 2.0 cm). This randomized phase II trial was designed to compare the rate of pCR induced by neoadjuvant cyclophosphamide plus doxorubicin (AC), followed by ixabepilone or paclitaxel in this cohort. Patients received sequential Neo-AC starting with 4 cycles of AC (doxorubicin 60 mg/m² intravenously and cyclophosphamide 600 mg/m² intravenously) given every 3 weeks, followed by 1:1 randomization to either ixabepilone (40 mg/m² 3-hour infusion) every 3 weeks for 4 cycles, or paclitaxel (80 mg/m² 1-hour infusion) weekly for 12 weeks. All patients underwent de-duction scheme and using a machine learning model, which was applied to the TNBC cohort dataset (NCT00455533). The data mining algorithm comprised a three layer multilayer perception architecture modified with a feed forward back-propagation algorithm and a sigmoidal transfer function. The top 100 predictive genes identified were then applied to an ANN-based network inference algorithm, as has been described previously (19). This model predicted a weighted link (direction and magnitude) between each of the top 100 gene probe markers that are associated with HAGE expression and every other marker in the top 100. The 100 strongest interactions, based on the magnitude, were then visualized as a map with Cytoscape software (20).

In a second bioinformatics analysis step, we sought to obtain a robust ranking of genes that are differentially expressed between the mRNA HAGE⁺ and the mRNA HAGE⁻ cases, and show a high predictive power. This was achieved by applying an ensemble sample classification method within a leave-one-out cross-validation scheme and using a machine learning model, which was evaluated on the basis of the left-out sample (a procedure known as "external cross-validation"), as previously described (21). To rank the genes based on the cross-validation results, the frequency of occurrence in the list of significantly differentially expressed genes (P < 0.05) across different cross-validation cycles was recorded, and genes received higher scores the more often they had been selected. All steps of the analysis were conducted using an in-house web application for microarray analysis.

Tissue microarrays and immunohistochemistry

Tumors from the Nottingham EP-TNBC cohorts and post-surgical specimens from patients with LAP-TNBC were incorporated into tissue microarrays (TMA). These were constructed using six replicate 0.6 mm cores from the centre and periphery of the tumors of each patient. In addition, full-face sections derived from formalin-fixed paraffin-embedded (FFPE) diagnostic pre-chemotherapy core biopsy were used for those LAP-TNBC cases that were treated with Neo-AC. The TMA sections and full face sections were immunohistochemically profiled using antibodies for HAGE and other markers (Supplementary Table S4), as previously described (10). Immunohistochemical staining was performed using a Novolink Detection kit according to the manufacturer's protocol (Leica Microsystems). For this, sections were incubated overnight at 4°C with 1/175 dilution of custom-made rabbit anti-HAGE monospecific polyclonal antibody (22). The IHC was validated as previously described (10) using a commercially available antibody, originally employed in the Human Protein Atlas (HPA) project (anti-DDX43; HPA031381, Sigma-Aldrich); in this case sections were incubated overnight at 4°C at 1/100 dilution.

Expressions of HER2, ER, and PR were reassessed according to the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines (23, 24), as summarized in Supplementary Table S5. For ER status, the EP1 clone has been used (Dako-Cytomation). ER and PR assays were considered negative if there were <1% positive tumor nuclei in the presence of the expected reactivity of internal and external controls. To validate the use of TAMs for immunophenotyping, full-face sections of 40 cases were stained and the protein expression levels were compared. The concordance between TAMs and full-face sections was excellent using Cohen kappa statistical test for categorical variables (κ = 0.8). Positive and negative (omission of the primary antibody and IgG-matched serum) controls were included in each run.

Evaluation of HAGE immunohistochemical staining

Tumor cores were evaluated by two pathologists (T.M.A. Abdel-Fatah and I.O. Ellis) who were blinded to the clinicopathologic characteristics of patients in two different settings, as previously described (10). High HAGE (HAGE⁺) expression was defined as the presence of strong cytoplasmic and/or nuclear staining in >10% of malignant cells (Fig. 1A), as described previously (10). Intra- (κ > 0.8; Cohen kappa test) and inter- (κ < 0.8; using multirater kappa tests) observer agreements were excellent. In cases where discordant results were obtained, the slides were re-evaluated by both pathologists together and a consensus was reached.

Integrated array CGH, mRNA gene expression, and protein expression analysis of HAGE

HAGE gene copy number changes (GCNC). and mRNA and protein expression, were derived from a set of 171 stage I and II primary invasive breast cancer that was previously described (25). The raw and mode-normalized data for GCNCs are available from the National Centre for Biotechnology Information Gene Expression Omnibus (NCBI-GEO) under the series accession number GSE8757 and the expression data are available at the EBI website with the accession number E-TABM-576. GCNCs at HAGE locus (6q13 spanning from 74104285 to 74127292 with size of 23008 bases) were retrieved from the oligonucleotide microarrays profiling. HAGE mRNA and protein expressions were available for 128 breast cancer samples. The correlation between HAGE mRNA expression log intensity values and HAGE protein expression (H-score) were evaluated using the Pearson correlation. The demographics of this patient cohort have been described previously (25).
Survival data
Survival data, including survival time, disease-free survival (DFS), and development of locoregional and distant metastases (DM), were maintained on a prospective basis. DFS was defined as the number of months from diagnosis to local–regional recurrence or DM relapse. Breast cancer–specific survival (BCSS) was defined as the number of months from diagnosis to the occurrence of breast cancer–related death. Survival was censored if the patient was still alive, lost to follow-up, or died from other causes.

Statistical analysis
Data analysis was performed using SPSS statistics software (SPSS, version 17). Where appropriate, Pearson χ², and Student tests were used. Positivity for HAGE protein both pre- and post-chemotherapy was calculated and compared using McNemar test. Significance was defined at P < 0.05. Cumulative survival probabilities were estimated using the Kaplan–Meier method, and differences between survival rates were tested for significance using the log-rank test. Multivariate analysis for survival was performed using the Cox proportional hazard model. The proportional hazards assumption was tested using standard log–log plots. Hazard ratios (HR) and 95% confidence intervals (95% CI) were estimated for each variable. All tests were two-sided with a 95% CI and P < 0.05 was considered to be indicative of statistical significance. A stringent P value < 0.01 was considered to indicate statistical significance for multiple comparisons.

The Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) criteria (26) were followed throughout this study.

Sample size and power analysis
The Power of the study, sample size, and effect-size were determined using PASS (NCSS, version 13). For the protein expression study, a two-sided log-rank test with an overall sample size of 520 subjects (443 in the HAGE⁺/C0 and 77 in the HAGE⁺ group) achieves 99.5% power at a 0.050 significance level to detect a HR of 2.13 when the proportion surviving in the HAGE⁺/C0 group is 0.70. For the mRNA study, a two-sided log-rank test with

Figure 1.
A, Photomicrographs showing negative expression of HAGE (top) and positive expression of HAGE in neoplastic cells (bottom; magnification × 200). B, Kaplan–Meier curves showing the relationship between HAGE expression and BCSS (top) and DFS (bottom) in the EP-TNBC. See text for details. C, Kaplan–Meier curves showing the relationship between HAGE expression and BCSS (top) and DFS (bottom) in EP-TNBC patients who did not receive any chemotherapy. D, Kaplan–Meier curves showing the relationship between HAGE expression and BCSS (top) and DFS (bottom) in EP-TNBC patients who received chemotherapy. See text for details.
Table 1. Multivariate analysis using Cox regression analysis confirms that HAGE protein expression, Bcl2, and lymph node stage are independent prognostic factors in TNBC

<table>
<thead>
<tr>
<th>Clinicopathologic variables</th>
<th>BCSS at 10 years</th>
<th>P</th>
<th>PFS at 10 years</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAGE expression</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>1</td>
<td>0.00007*</td>
<td>0.002*</td>
</tr>
<tr>
<td>High</td>
<td>2.1 (1.5–3.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bcl2 expression</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>1</td>
<td>0.01*</td>
<td>0.02*</td>
</tr>
<tr>
<td>Low</td>
<td>1.7 (1.1–2.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymph node stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td></td>
<td>6.7 × 10^-12*</td>
<td>1.2 × 10^-10*</td>
</tr>
<tr>
<td>Positive (1–3 nodes)</td>
<td>1</td>
<td>1.5 (1.0–2.2)</td>
<td>1.3 (0.9–1.8)</td>
</tr>
<tr>
<td>Positive (&gt;3 nodes)</td>
<td>4.5 (3.0–6.8)</td>
<td>3.8 (2.6–5.5)</td>
<td></td>
</tr>
<tr>
<td>Tumor grade</td>
<td></td>
<td>0.469</td>
<td>0.770</td>
</tr>
<tr>
<td>Grade 1 (low)</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Grade 2 (intermediate)</td>
<td>0.5 (0.1–4.1)</td>
<td>0.8 (0.1–6.2)</td>
<td></td>
</tr>
<tr>
<td>Grade 3 (high)</td>
<td>0.8 (0.1–5.4)</td>
<td>1.0 (0.1–7.2)</td>
<td></td>
</tr>
<tr>
<td>Tumor size (continuous)</td>
<td>1.0 (1.0–11)</td>
<td>1.0 (1.0–11)</td>
<td>0.661</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td></td>
<td>0.007*</td>
<td>0.03*</td>
</tr>
<tr>
<td>No</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0.6 (0.5–0.9)</td>
<td>0.7 (0.5–1.0)</td>
<td>0.092</td>
</tr>
<tr>
<td>Menopausal status</td>
<td></td>
<td>0.023*</td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Post</td>
<td>1.5 (1.1–2.1)</td>
<td>1.3 (1.0–1.7)</td>
<td></td>
</tr>
</tbody>
</table>

*Statistically significant P < 0.05.

an overall sample size of 311 subjects (134 in the HAGE+ group and 177 in the HAGE– group) achieves 79.6% power at a 0.05 significance level to detect a HR of 1.78 when the proportion surviving in the HAGE+ group is 0.7300.

Results

Nottingham-EP-TNBC cohort and HAGE protein expression

Positive HAGE protein expression (HAGE+, Fig. 1A) was observed in 77 of 520 (14.8%) of EP-TNBC tumors and was more common in postmenopausal patients with TNBC (P = 0.016). In univariate analysis, HAGE+ protein expression was strongly associated with an adverse outcome at 10 years and an increased risk of death from EP-TNBC (HR, 1.3; 95% CI, 1.2–1.5; P = 0.000005) and recurrence (HR, 1.2; 95% CI, 1.1–1.4; P = 0.0003; Fig. 1B). In multivariate Cox regression analysis, HAGE+ was confirmed as an independent prognostic factor after controlling for ACT and other validated prognostic factors (Table 1). HAGE+ cases were associated with a doubling of the risk of death (HR, 2.1; 95% CI, 1.5–3.1; P = 0.00007) and recurrence (HR, 1.6; 95% CI, 1.2–2.5; P = 0.002). Subgroup analysis of the EP-TNBC cohort cases that did not receive ACT revealed that HAGE+ protein expression was associated with a higher risk of death (HR, 1.4; 95% CI, 1.2–1.7; P = 0.00000004) and recurrence (HR, 1.3; 95% CI, 1.1–1.5; P = 0.00007; Fig. 1C). EP-TNBC patients who received either AC or CMF adjuvant chemotherapy with HAGE+ tumors exhibited a similar clinical outcome to those with HAGE+ tumors (Fig. 1D), with respect to BCSS (HR, 1.2; 95% CI, 0.9–1.47; P = 0.192) and DFS (HR, 1.1; 95% CI, 0.9–1.3; P = 0.338).

METABRIC-TNBC cohort and HAGE transcript levels

METABRIC-TNBC patients (134/311) exhibited a high HAGE mRNA expression [43.1% using median (5.41) as a cutoff for HAGE mRNA expression data in the entire METABRIC patient cohort (n = 1980)], which was significantly associated with medullary carcinoma (P = 0.026). High HAGE mRNA expression in tumors was associated with prolonged BCSS (log-rank = 7.2; P = 0.007) and a 44% lower risk of death from TNBC (HR, 0.56; 95% CI, 0.36–0.86; P = 0.007; Fig. 2A) compared with those with low HAGE mRNA, especially among those who received ACT (Fig. 2A). Patients exhibiting a high level of HAGE mRNA expression who received ACT exhibited a higher BCSS and lower risk of death compared with those with low HAGE mRNA level (HR, 0.46; 95% CI, 0.27–0.79; P = 0.005, and log rank = 8.3, P = 0.004; Fig. 2A). In multivariate Cox regression analysis that included NPI components, HAGE mRNA expression (HR, 0.53; 95% CI, 0.34–0.83; P = 0.005) and lymph node stage (HR, 3.8; 95% CI, 2.2–6.5; P = 0.000004) were the only independent predictors for clinical outcome.

Nottingham LAP-breast cancer patients treated with AC-Neo-ACT (n = 110)

To validate our observation regarding the effect of HAGE expression and response to ACT, we investigated its expression in LAP-TNBC patients treated with AC-Neo-ACT. Positive HAGE expression was observed in 43% and 18% of LAP-TNBC tumors before and after receiving AC-Neo-ACT, respectively. Following AC-Neo-ACT, after excluding cases that already have achieved pCR, a statistically significant loss of HAGE expression was found in paired pre- and post-chemotherapy samples, (P = 0.002). Forty-eight percent (48%, 20/42) of HAGE+ LAP-TNBC tumors; (Fig. 2A) compared with those with low HAGE mRNA level (HR, 0.46; 95% CI, 0.27–0.79; P = 0.005, and log rank = 8.3, P = 0.004; Fig. 2A). In multivariate Cox regression analysis that included NPI components, HAGE mRNA expression (HR, 0.53; 95% CI, 0.34–0.83; P = 0.005) and lymph node stage (HR, 3.8; 95% CI, 2.2–6.5; P = 0.000004) were the only independent predictors for clinical outcome.
receiving Neo-ACT. Similarly, TIL\textsuperscript{+} in postchemotherapy specimens was associated with decreased risk of death (HR, 0.13; 95% CI, 0.03–0.53; \(P = 0.005\)) and recurrence (HR, 0.23; 95% CI, 0.08–0.66; \(P = 0.006\)). Following Neo-ACT, patients with TIL\textsuperscript{+} tumors in prechemotherapy biopsies achieved higher pCR rates (53%) compared with those with a TIL\textsuperscript{−} status (15%); \(P = 0.00004\). Multivariate logistic regression analysis showed that prechemotherapy TIL status (OR, 9.2; 95% CI, 1.9–43.5; \(P = 0.005\)) and HAGE expression (OR, 5.1; 95% CI, 1.2–22.4; \(P = 0.03\)) are independent predictors of pCR in patients with LAP-TNBC (Table 2).

In prechemotherapy core biopsies, HAGE\textsuperscript{−} was significantly associated with TIL\textsuperscript{−} (\(P = 0.00005\)). Following Neo-ACT, 64% of prechemotherapy TIL\textsuperscript{−}/HAGE\textsuperscript{−} TNBC achieved pCR and the remaining 36% of those tumors showed loss of HAGE expression in their paired postchemotherapy specimens (\(P = 0.00017\)). Similarly, after exclusion of tumors that have achieved pCR, 67% of TIL\textsuperscript{−}/HAGE\textsuperscript{−} tumors in prechemotherapy biopsies showed loss of HAGE expression after receiving Neo-ACT. Patients with HAGE\textsuperscript{−} residual disease after Neo-ACT had a 2-fold increase in the risk of recurrence (HR, 1.7; 95% CI, 1.1–2.5; \(P = 0.018\)) and death (HR, 1.5; 95% CI, 0.98–2.21; \(P = 0.062\)), compared with those in whom HAGE expression was absent in residual disease (Fig. 2B), and none of these cases showed any TIL\textsuperscript{+} in their postchemotherapy surgical specimens.

**Table 2. Multivariate regression analysis: TILs and HAGE expression were independent predictors for pCR in LAP-TNBC**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Expression</th>
<th>OR (95% CI)</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAGE</td>
<td>Low</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>5.114 (1.169–22.364)</td>
<td>0.030*</td>
</tr>
<tr>
<td>TILs</td>
<td>Low</td>
<td>1</td>
<td>0.005*</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>9.184 (1.939–43.507)</td>
<td></td>
</tr>
<tr>
<td>TOP2A</td>
<td>Low</td>
<td>1</td>
<td>0.023*</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>6.375 (1.290–31.500)</td>
<td></td>
</tr>
<tr>
<td>Neoadjuvant taxane</td>
<td>No</td>
<td>1</td>
<td>0.088</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>3.697 (0.821–16.640)</td>
<td></td>
</tr>
<tr>
<td>Age at diagnosis</td>
<td>Continuous</td>
<td>0.964 (0.902–1.031)</td>
<td>0.284</td>
</tr>
<tr>
<td>Maximum tumor diameter</td>
<td>Continuous</td>
<td>0.987 (0.958–1.016)</td>
<td>0.373</td>
</tr>
<tr>
<td>Tumor grade</td>
<td>GT-2</td>
<td>1</td>
<td>0.002*</td>
</tr>
<tr>
<td></td>
<td>G3</td>
<td>0.091 (0.021–0.402)</td>
<td></td>
</tr>
<tr>
<td>Tumor stage</td>
<td>T1–2</td>
<td>1</td>
<td>0.067</td>
</tr>
<tr>
<td></td>
<td>T3–4</td>
<td>7.889 (0.867–71.780)</td>
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</tbody>
</table>

\*Statistically significant \(P < 0.05\).
Multicentre phase II trial
There was a statistically significant inverse correlation between mRNA level and pCR rate in the ixabepilone arm \( (t(62) = -1.487, P = 0.007) \). In addition, the combined RCB-0/RCB-1 rate was significantly lower in patients with high HAGE mRNA level \( (t(62) = -1.8, P = 0.001) \), and patients with high mRNA HAGE had a lower RCB-0/RCB-1 rate (19%) compared with those with low HAGE transcript (50%); \( P = 0.028 \). In contrast, there was no statistically significance correlation between mRNA level and both pCR \( (t(59) = -0.81, P = 0.198) \) and RCB-0/RCB-1 rates \( (t(59) = -1.23, P = 0.061) \) in the paclitaxel arm. These results suggest that higher levels of HAGE transcript are associated with resistance to ixabepilone.

Mechanistic insights
ANN, ensemble classification and cross-validation analysis reveals novel HAGE interaction genes and function pathways (shown in Fig. 3 and summarized in Supplementary Table S6) that were involved in pathways related to antigen processing and presentation via MHC class I and MHC class II, innate immune response, response to drug, protein degradation, cell proliferation, cell migration, DNA replication, DNA transcription and RNA editing, processing, and splicing.

Integrated array CGH, mRNA gene expression, and protein analysis were conducted in 128 breast cancer samples (Nottingham cohort), for which HAGE GCNCs, mRNA expression and protein expression data were all available. None of 128 cases of breast cancer showed any GCNCs of the HAGE gene locus at chromosome 6q13. Although there was a trend towards a positive correlation between HAGE mRNA and HAGE protein expression, this was not statistically significant \( (P = 0.06) \). The data suggest that HAGE protein expression could be modified by epigenetic mechanisms rather than genomic alterations in a proportion of breast cancer.

Discussion
Although recent studies strongly suggest that CSA expression is a common feature of ER- breast cancer, especially TNBC (27), knowledge about their respective prognostic or predictive

Figure 3.
ANN analysis of HAGE expression, as visualized using Cytoscape software. Top pairwise interactions for gene probe markers associated with HAGE expression in TNBCs revealed novel HAGE interaction genes. Each gene probe is represented by a node and the interaction weight between them as an edge, the width being defined by the magnitude of the weight. Interactions are directed from a source gene to a target gene as indicated by arrows. Red interactions indicate an excitatory interaction and blue indicates an inhibitory interaction. Highly linked genes represent hubs that are indicated to be highly influential or highly regulated in the HAGE system.
implications remains largely unknown. To the best of our knowledge, this is the first study to report on HAGE expression as a promising prognostic biomarker in TNBC and it seems to predict benefit from adjuvant and neoadjuvant anthracycline-based chemotherapy. Considering the limited treatment options for TNBC, the therapeutic potential of combining chemotherapy and immunotherapy could be explored in more detail.

In our study, high HAGE protein expression was associated with poor clinical outcome in agreement with what has been found in leukemia (28), whereas high mRNA level was associated with a favorable prognosis. These apparently contradictory results could mainly be due to the difference in the use of anthracycline chemotherapy. The majority of the cohort tested for protein expression received no anthracycline chemotherapy because they presented before 2000, whereas most of those in which mRNA expression was observed were diagnosed after 2000 and did receive any adjuvant chemotherapy. This finding has been further confirmed in both adjuvant and neoadjuvant settings, when the analysis has been repeated in anthracycline chemotherapy-naive patients versus anthracycline-treated patients. However, we cannot exclude the possibility that the inconsistent effect of mRNA and protein on survival could also be due to the imperfect correlation between protein and mRNA levels, given that only about 40% of cellular protein levels can be predicted from mRNA measurements (29).

We also found that HAGE\(^+\) expression was highly associated with TIL\(^+\) compared with those with HAGE\(^-\) in TNBC, and that both HAGE expression and the presence of TILs are independent predictors for pCR after receiving AC-Neo-ACT. Moreover, there was a significant loss of HAGE expression in the residual tumors following AC-Neo-ACT. The explanation behind these findings is likely to be complex and further investigations of the triad relationship between HAGE, TILs, and response to chemotherapy are warranted.

The higher rate of response to a given chemotherapy could probably result from an accumulation of DNA damage, abnormal mitoses, and subsequent mitotic catastrophe (30, 31). The mechanisms of mitotic catastrophe are unknown, but it likely results from a combination of deficient cell-cycle checkpoints and cellular damage. Conversely, nonresponding tumors could escape the response through accumulation of genetic abnormalities that would not lead to a mitotic catastrophe, but rather to aneuploidy and subsequent growth advantage (30, 31). To kill tumor cells, CD8\(^+\) T cells must recognize specific peptides that are derived from CSAs such as HAGE that are presented by MHC class I molecules on tumor cells (5). In our study, HAGE\(^+\) expression was highly associated with TIL\(^+\) compared with those with HAGE\(^-\) in TNBC. This could suggest that a subset of patients with TNBC whose tumors express HAGE may have particularly high inherent immunogenicity. We have recently identified two HAGE-derived sequences (a 24 amino acids long and a 30 amino acids long) containing several epitopes exhibiting a broad HLA-binding spectrum (data not shown). Using these sequences, we have been able to demonstrate the immunogenicity and the endogenous processing of several of the predicted peptides for HLA-A2 and HLA-DR1 molecules. Moreover, ANN analysis suggests that HAGE is involved in antigen processing and presentation via MHC class I and MHC class II, innate immune responses, responses to drug, wound healing, protein degradation, apoptotic processes, cell proliferation, cell migration, cell adhesion, DNA replication and transcription, and RNA editing, processing, and splicing. This is in agreement with our previous findings, which have shown that HAGE is immunogenic and involved in promoting proliferation, tumor motility, and metastases via the Ras signaling pathway (10, 32).

There is abundant evidence indicating that the innate and adaptive immune systems play a crucial role in the efficacy of chemotherapy, and that the immune system itself can be activated by cytotoxic drugs (33). In addition, the correlation between intratumoral immune responses and clinical outcomes could be potentially related to the role of immune cells in the activity of cytotoxic chemotherapeutics (4, 5).

Accumulating data now suggest that anthracyclines mediate their anticancer activity not only by direct cytotoxic effects but also through activation of CD8\(^+\) T-cell responses (5), but also by eliciting immunogenic cell death (33). In fact, several studies have linked TILs to a chemotherapeutic response (4, 6), especially anthracycline-based chemotherapy in breast cancer (15). In addition, a recent report revealed that high lymphocyte gene expression is associated with a remarkably high rate (74%) of pCR in response to AC-Neo-ACT in ER\(^-\) breast cancer (4).

The main potential clinical significance of our results is the ability to identify patients with TNBC who are likely to benefit from the standard neoadjuvant/adjuvant anthracycline chemotherapy, and spare patients whose response would be poor from enduring the unnecessary serious cytotoxic side effects. However, identification of patients who are unlikely to benefit from anthracycline chemotherapy on the bases of evaluation of CSAs and TILs in the TNBC tumors either before initiation of neo-ACT or shortly after surgery and before starting the additional ACT may have even greater importance.

TNBC patients with low HAGE, TILs, or other low immune function indicators might be enrolled onto trials to test the efficacy of other therapeutic agents such as pertuzumab and cisplatin, particularly in HER2-enriched and BRCA1 mutation carrier subgroups, respectively. Alternatively, these patients might be the focus of future clinical trials that are designed to evaluate therapeutic approaches, which might enhance the immune activity within TNBC and thereby sensitize the tumors to biologic therapies. This could occur by lowering the defences of the tumor either by (i) targeting the immune system to reduce tumor-induced immunosuppressive cells; (ii) targeting the tumor to increase immunogenicity (increase MHC or antigen expression); (iii) directly stimulating effector response by activating T cells (reviewed by Weir GM; ref. 34) or via the use of "checkpoint inhibitors."

The fact that patients with HAGE\(^+\) residual disease (ACT-resistant cells) have a poorer outcome suggests that an alternative therapy, such as immunotherapy, may be warranted for this group. Given that the HAGE antigen is immunogenic and is highly associated with TILs, one should consider targeting HAGE along with other CSAs using immunotherapeutic interventions in conjunction with chemotherapy for TNBC. Although in the past, cancer vaccines have had a poor clinical track record due to poor clinical results (34), in recent trials using immunotherapy, clinical outcomes have shown improved progression-free survival, or overall survival (35–38). In fact, it is proposed that chemotherapies could be used to condition the immune system and tumor, and create an environment in which cancer vaccines have a better chance of success (34, 39).

The exact molecular mechanisms underlying the increase of HAGE expression in human cancer remain unclear. There is
substantial evidence that epigenetic events represent one mechanism which regulates the expression of HAGE, and global DNA methylation seems to play a major role (21). It has previously been shown that hypomethylation of the HAGE gene promoter correlates with increased HAGE expression and is strongly associated with advanced disease and poor prognosis of leukemia (28). Furthermore, the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine (decitabine), that has been shown to be beneficial for patients with chronic myeloid leukemia (28, 40) and to be capable of inducing HAGE expression, could be used to increase the eligibility of patients for CSA-targeted immunotherapy and improve the management of patients with recurrent disease.

Although we validated our findings in multiple independent cohorts, at both mRNA and protein levels, the clinical utility of HAGE requires further validation in larger patient populations that have received a uniform therapy, and in which both mRNA and protein expression data are available. Whereas our study highlighted the importance of HAGE as a novel biomarker in TNBC, a prospective study to demonstrate its role as a prognostic and protein expression data are available. Whereas our study highlighted the importance of HAGE as a novel biomarker in TNBC, a prospective study to demonstrate its role as a prognostic tool, and its potentiality to select patients for novel systemic therapies, including immunotherapy, following neoadjuvant chemotherapy would be required. Although our study would suggest HAGE as an immunotherapeutic target for TNBC, these data are preliminary and a further study that is designed to address its immunogenicity and patient selection to demonstrate clinical benefit is urgently needed.

In conclusion, this study demonstrates that the expression of HAGE is a potential prognostic marker of outcome as well as a predictor of response to anthracycline treatment in TNBC. Our findings may aid oncologists to identify a subgroup of patients who would benefit greatly from a certain type of chemotherapy. There is a need for further investigation of the molecular mechanisms in HAGEþ tumor cells and their integration with the local immune environment in cases treated with neoadjuvant chemotherapy. Development of, and a prospective trial for, an adjuvant chemotherapy/vaccine combination treatment, to confirm these findings is warranted.


HAGE in Triple-Negative Breast Cancer Is a Novel Prognostic, Predictive, and Actionable Biomarker: A Transcriptomic and Protein Expression Analysis

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