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Overexpression of the oncogenic signal transducer Gab2 occurs early in breast cancer development

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Gab2, a docking-type signaling protein with demonstrated oncogenic potential, is overexpressed in breast cancer, but its prognostic significance and role in disease evolution remain unclear. Immunohistochemical detection of Gab2 in a large cohort of primary human breast cancers of known outcome revealed that while Gab2 expression was positively correlated with increased tumor grade, it did not correlate with disease recurrence or breast cancer-related death in the total cohort or in patients stratified according to lymph node, estrogen receptor (ER) or *HER2* status. Interestingly, analysis of a "progression series" that included premalignant and preinvasive breast lesions as well as samples of metastatic disease revealed that Gab2 expression was significantly enhanced in the earliest lesion examined, usual ductal hyperplasia, with a further increase detected in ductal carcinoma *in situ* (DCIS). Furthermore, expression was less in invasive cancers and lymph node metastases than in DCIS, but still higher than in normal breast. These findings indicate that while Gab2 expression is not prognostic in breast cancer, its role in early disease evolution warrants further analysis, as Gab2 and its effectors may provide targets for novel strategies aimed at preventing breast cancer development.

Gab2 is a docking-type signaling protein that recruits the tyrosine phosphatase Shp2 and the p85 subunit of PI3K and thereby positively regulates the Ras/Erk and PI3K pathways in response to activation of a variety of receptor and receptor-associated tyrosine kinases.[1] Strong evidence has emerged supporting a functional role for Gab2 in development of several human malignancies. When uncoupled from negative feedback regulation, Gab2 is transforming in both fibroblasts[2] and MCF-10A mammary epithelial cells.[3] In addition, Gab2 overexpression has been detected in breast and gastric cancers, metastatic melanoma and acute myeloid leukemia (AML)/myelodysplastic syndrome (MDS).[4–8] One mechanism underpinning Gab2 overexpression in breast cancer, melanoma and AML/MDS is amplification of the *GAB2* gene at chromosome 11q14.1, which also occurs in ovarian cancer.[6–11] Importantly, use of transgenic/knock-out approaches has identified a critical role for Gab2 in particular mouse models of tumorigenesis. Gab2 ablation in mice prevents transformation of myeloid cells by Bcr-Abl or mutant Shp2.[12,13] In addition, Gab2 overexpression in the mammary gland accelerates tumorigenesis induced by active erbB2,[9] whereas Gab2 deficiency either attenuates erbB2-induced mammary tumorigenesis[9] or suppresses metastatic spread to the lungs without major effects on tumor growth,[14] possibly reflecting the use of different Gab2 gene-knockout alleles and/or oncogenic forms of erbB2.

Although Gab2 is implicated in breast cancer development, the only studies that have analyzed Gab2 protein expression in primary breast cancers[4,9] have lacked the power to accurately determine the frequency of Gab2 upregulation and its association with clinicopathological characteristics and patient outcome. In addition, its expression during early breast cancer evolution and disease progression is uncharacterized. To address these issues, we characterized Gab2 expression in a large and well-defined cohort of primary breast cancer specimens, as well as a separate cohort that included premalignant and preinvasive breast lesions as well as regional lymph node metastases.

Key words: tyrosine kinase, signal transduction, ductal carcinoma *in situ*

Abbreviations: AML: acute myeloid leukemia; Cdk: cyclin-dependent kinase; DCIS: ductal carcinoma *in situ*; ER: estrogen receptor; Erk: extracellular signal regulated kinase; Gab2: Grb2-associated binder 2; HER2: human epidermal growth factor receptor 2; IDCa: invasive ductal carcinoma; IHC: immunohistochemistry; MDS: myelodysplastic syndrome; MEK: mitogen activated protein kinase/Erk kinase; PI3K: phosphatidyinositol 3-kinase; Shp2: src homology 2-containing phosphatase 2; UDH: usual ductal hyperplasia

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Material and Methods

Generation of anti-Gab2 polyclonal antibody, Western blotting and immunohistochemistry

An affinity-purified sheep polyclonal antibody was generated against the C-terminal Gab2 peptide sequence WTDV RQSSEPSKGAKL in association with Symansis (Auckland, New Zealand). Although the peptide sequence exhibits similarity to the Gab1 C-terminus, lack of crossreactivity of the antibody against Gab1 was confirmed by Western blot and immunohistochemistry (IHC; see Results). The peptide antigen sequence is poorly conserved in Gab3. Western blot analysis was undertaken as previously described.[4,15] The anti-β-actin antibody (clone AC-15) was from Sigma (Castle Hill, Australia). Before immunohistochemical staining, formalin-fixed, paraffin-embedded breast cancer cell line pellets and human or mouse tissue sections were dewaxed in xylene and subsequently rehydrated. Antigen retrieval was performed in a low pH target retrieval solution (S1699, DAKO Corporation, Carpinteria, CA) in a pressure cooker for 10 sec at target pressure. IHC was performed using a Dako autostainer (DAKO Corporation). Nonspecific background staining was prevented by blocking with hydrogen peroxide (3% v/v). Sections were incubated with the anti-Gab2 antibody (1:100) for 60 min at room temperature. Substitution of the primary antibody by normal sheep serum or diluent served as an additional negative control. Sections were then incubated with rabbit-anti-sheep antibody (Vector Laboratories, Burlingame, CA); (1:200 in diluent) for 15 min, followed by the addition of Envision+ Rabbit (DAKO Corporation) for 15 min. Gab2 staining was then visualized by incubation of the slides with DAKO DAB+ chromogen (DAKO Corporation) for 10 min. Slides were counterstained with haematoxylin, dehydrated and coverslipped. Slides were coscored for Gab2 expression by 2 independent observers that included a breast pathologist (S.O'T.). For each tissue microarray section, the level of Gab2 staining was assessed with respect to both the percentage of cells expressing the protein (0–100%) and the intensity of staining (0–3). The scoring system for the staining intensity was as follows: 0, no cells staining positive; 1, weak staining intensity; 2, moderate staining intensity; 3, high staining intensity. Finally, an *H*-score was designated to each core, which was generated by multiplying the percentage of cells stained by the predominant intensity score. All the scores were based on cytoplasmic staining. The *H*-score was used for further analysis.

Patient characteristics

The cohort of primary human breast cancers of known outcome has been described previously.[16] Briefly, 40% of tumors were >20 mm with a mean size of 19.6 mm, 45% were grade >2, 43% were lymph node positive, 68% were estrogen receptor (ER) positive, 57% were progesterone receptor (PR) positive and 18% were *HER2* FISH positive (>2.2 ratio of HER2:chr.17 centrosome). Median age was 54 years and patients were treated with endocrine therapy (49%), chemotherapy (38%) or both (24%). Cases were prospectively followed-up for a median of 64 months and outcome events measured were: recurrence (local or distant) (25%), metastasis (23%) and all deaths, but only breast cancer-related deaths (18%) were considered for survival analyses.

To complement the data on expression of Gab2 from the outcome series, a "progression series" was assembled consisting of tissue microarrays (TMAs) containing usual ductal hyperplasia (UDH; $n = 39$), ductal carcinoma *in situ* (DCIS) nuclear grades 1 ($n = 12$), 2 ($n = 54$), and 3 ($n = 34$) and regional lymph node metastases ($n = 80$), all derived from randomly selected primary breast cancer resections of patients with either pure DCIS ($n = 8$), DCIS with invasive ductal carcinoma (IDC; $n = 37$) or IDC alone ($n = 49$). Because 1 patient can have several different types of lesion (*e.g.*, ductal hyperplasia adjacent to DCIS, or multiple lymph node metastases), cores were scored per lesion and not per patient. Normal ducts ($n = 43$) derived from patients who had undergone reduction mammoplasty were also included in this series, none of which contained premalignant breast lesions or breast cancer within the surgical specimen. This "progression series" (excluding the IDC cases for which the clinicopathological features have already been described) had a mean age of 54.3 years and a mean tumor size of 25.4 mm. Twelve percent of the invasive carcinomas were grade 1, 36% were grade 2 and 52% were grade 3. Hormone and HER2 receptor status or additional clinical information was not available on these cases.

Prior approval for this study was obtained from the Human Research Ethics Committee of St. Vincent's Hospital, Sydney (HREC SVH H94/080, HREC 06338 SVH H00 036).

Statistical analyses

Statistical evaluation was performed using Statview 5.0 Software (Abacus Systems, Berkeley, CA). A p-value of <0.05 was accepted as statistically significant. Baseline characteristics of the cohort were defined using simple frequency distributions. Patient outcomes were analyzed using Kaplan-Meier analysis, and a one way analysis of variance (ANOVA) with Fisher's least significant difference post-hoc test was used to determine the relationship between Gab2 expression levels and clinicopathological and molecular markers.

Results

Our initial study on Gab2 in breast cancer used a commercially available polyclonal Gab2 antibody for IHC,[4] however, subsequent batches of this antibody failed to exhibit appropriate selectivity for this application. Therefore, a panel of polyclonal antisera against Gab2 was generated in sheep and tested for their sensitivity and specificity in detecting Gab2. A C-terminal antibody detected marked Gab2 expression in MDA-MB-134, −175 and −468, and low levels in HMEC-219-4 and BT-549 cells, as previously reported (Fig. 1*a*).[4] Prolonged exposure of the blot indicated that the antibody was highly selective for Gab2. In particular, it did not detect

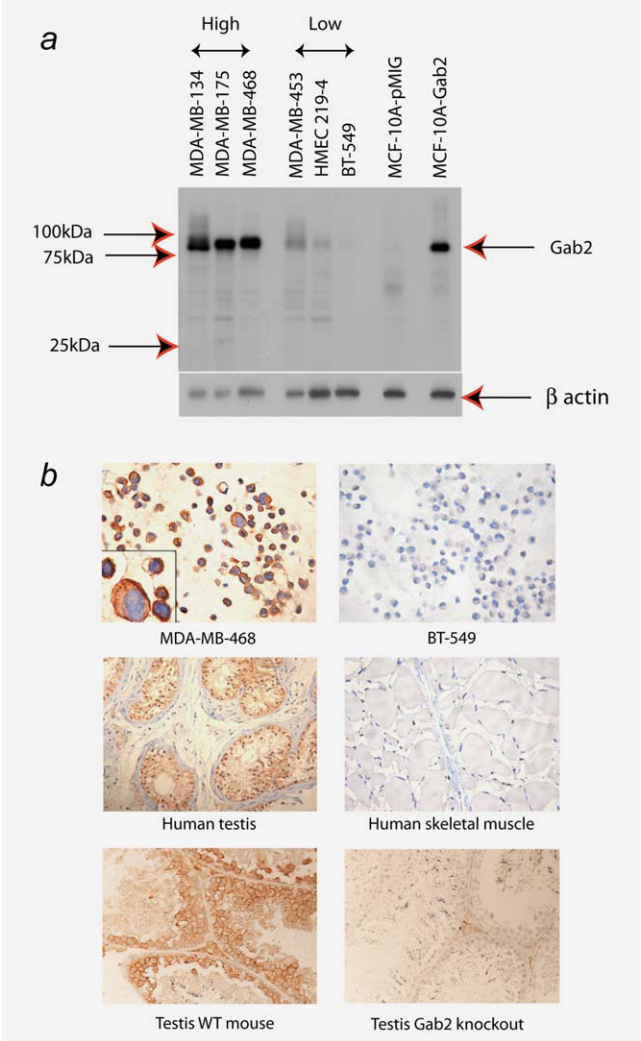

Figure 1. Characterization of anti-Gab2 polyclonal antibody. (*a*) Testing of antibody by Western blot. Lysates from the normal human mammary epithelial cell strain HMEC-219-4, the indicated breast cancer cell lines, and control or Gab2-overexpressing MCF-10A cells[19] were subjected to Western blot analysis as indicated. (*b*) Validation of the antibody for immunohistochemistry. Immunohistochemistry for Gab2 was performed on formalin-fixed, paraffin-embedded breast cancer cell line pellets, human testis and skeletal muscle and wild-type mouse testis and Gab2 gene knockout testis. Cell pellet images are ×400 magnification, all tissues are ×200 magnification. The higher power image in the inset highlights localization of Gab2 at the plasma membrane and in the cytoplasm.

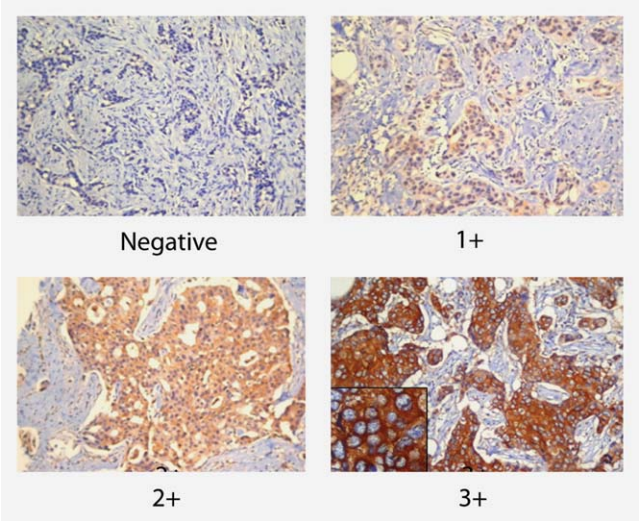

Figure 2. Immunohistochemistry of human breast cancer specimens. Representative specimens demonstrating negative, weak, moderate and high staining intensity are shown. All images are ×200 magnification, haematoxylin counterstain. The higher power inset highlights cytoplasmic localization of Gab2.

Gab1, which has a lower gel mobility and is expressed in all of these cell lines.[4]

All Gab2-positive cell lines demonstrated cytoplasmic expression by IHC and a higher staining intensity at the plasma membrane (Fig. 1*b* and data not shown). Notably, the intensity of expression corresponded with the Western Blot data, with little or no staining observed in the low-expressing cell lines (data for BT-549 is shown in Fig. 1*b*). Normal human tissue controls were also utilized, with testis as a positive control and skeletal muscle as a negative control, based on Northern Blot results.[17] Gab2 staining was strong in the seminiferous tubules, whereas no Gab2 immunopositivity was detected in skeletal muscle (Fig. 1*b*). In addition, we utilized specific tissues from wild-type and *Gab2* gene knock-out mice[18] to test the specificity of our antibody. As with human testis tissue, wild-type mouse testis exhibited strong staining in the seminiferous tubules, but this was absent in the gene knock-out, confirming its dependency on Gab2 expression (Fig. 1*b*). Wild-type mammary gland exhibited weak staining that was reduced to background in corresponding knock-out tissue (data not shown).

We then analyzed Gab2 protein expression in a previously characterized breast cancer cohort.[16] Gab2 immunopositivity (defined as *H*-score > 0, see Fig. 2) was observed in 94% ($n = 236$) of 251 patients and the average *H*-score of the whole cohort was 136. The distribution of staining intensity over the cohort was: Gab2 low (*H*-score ≤ 100), $n = 140$; medium (*H*-score 101–200), $n = 78$; and high (*H*-score > 200), $n = 33$. Examples of breast cancer specimens negative for Gab2, or exhibiting low, medium and high expression, are shown in Figure 2. When present, Gab2 expression was cytoplasmic, as expected (Fig. 2). As reported previously,[4] normal breast epithelium was either negative for Gab2 or exhibited weak staining. In a recent study, *GAB2* gene amplification at 11q14.1 was detected in 8–15% of primary breast cancers.[11] However, our data indicate that Gab2 overexpression is a common event in this disease. Consequently, while gene amplification represents 1 mechanism leading to deregulated Gab2

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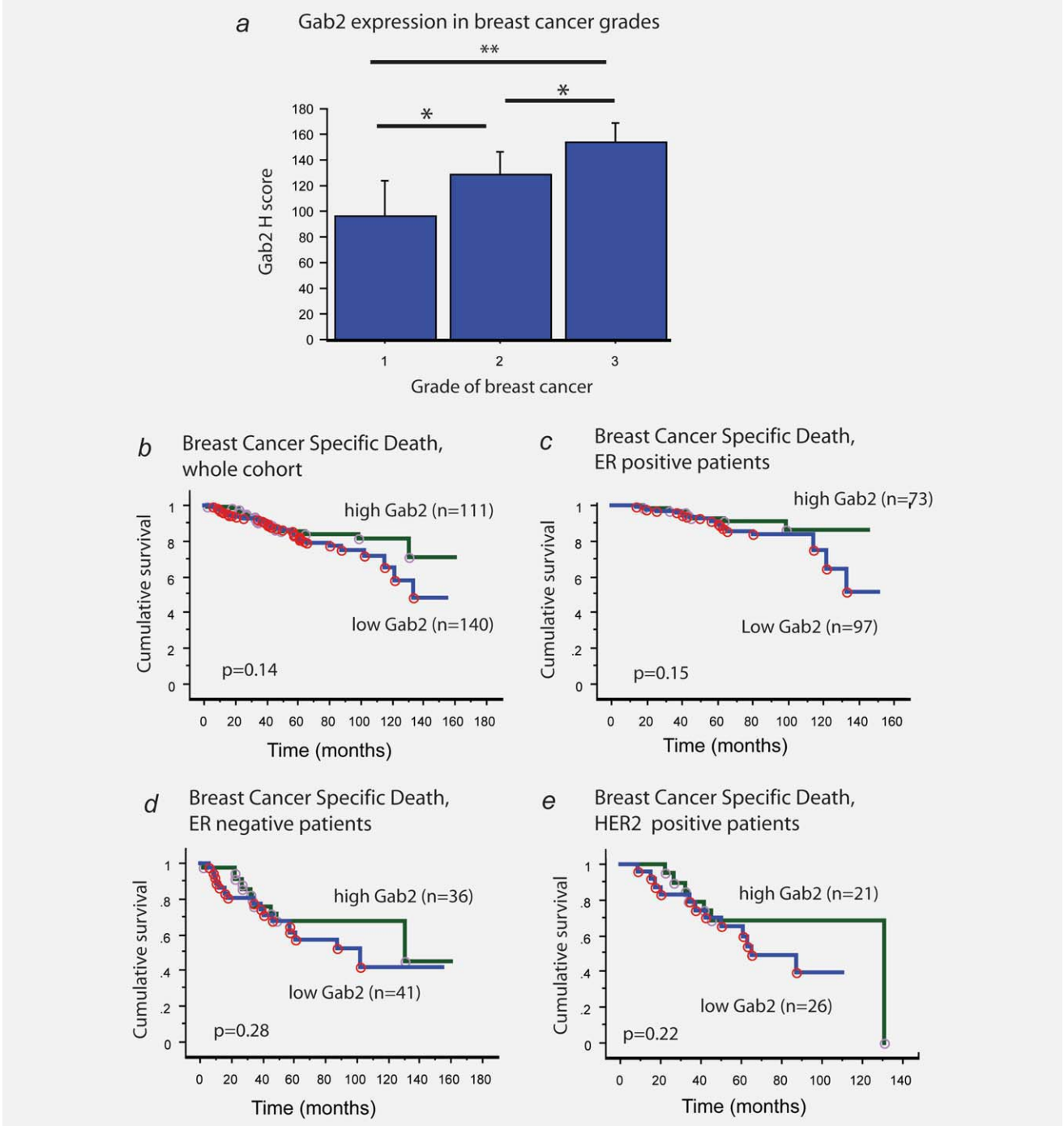

Figure 3. Relationship between Gab2 expression, clinicopathological parameters and patient outcome. (*a*) Relationship between Gab2 and tumor grade. (*b–e*) Kaplan-Meier curves for breast cancer-specific death. There was a progressive increase in Gab2 *H*-score between grades 1 to 2 and 2 to 3 of breast cancer (*a*). The number of samples per group was: Grade 1, 38 cases; Grade 2, 92 cases; Grade 3, 121 cases. Error bars indicate 95% CI. *$p < 0.05$, **$p < 0.01$. There was no prognostic significance of high Gab2 expression in the cohort as a whole (*b*), or in ER positive (*c*), ER negative (*d*), or HER2 amplified (*e*) subgroups, with all analyses exhibiting $p > 0.05$.

expression in breast cancer,[9,11] aberrant transcriptional or post-transcriptional regulation must also contribute.

Although Gab2 enhances cell proliferation in *in vitro* models of mammary tumorigenesis,[9,19] Gab2 was not associated with tumor size in this cohort. In addition, although previous research demonstrated a potential link between Gab2 and metastasis,[14] and Gab2 is present in a gene expression signature associated with lymph node metastasis in

breast cancer patients,[20] no correlation was found with lymph node status. Previous research using breast cancer cell lines demonstrated a trend toward increased Gab2 expression in ER-positive versus ER-negative lines, although this did not reach significance.[4] Therefore, it was of interest to examine whether a similar correlation could be demonstrated in a large cohort of primary breast cancers. However, such a relationship was not found. Also, Gab2 expression did not correlate with progesterone receptor- or *HER2*-positivity. However, significantly higher Gab2 *H*-scores were associated with increases in tumor grade from 1 to 3 (Fig. 3*a*).

To identify whether there was an association between Gab2 protein expression levels and patient outcome for the whole cohort, Kaplan-Meier survival analysis (log-rank test) was performed. The frequency distribution for Gab2 was analyzed to determine if any biologically significant cut points were observed. None were seen and optimal cut point determination by serial survival analysis similarly revealed no cut point. Therefore, the median *H*-score for Gab2 expression ($H > 100$) within the IDC cohort was used to dichotomize the cohort into Gab2-high and -low groups. Kaplan-Meier analysis revealed no significant differences in disease recurrence (data not shown) or breast cancer-specific death (Fig. 3*b*). Subgroup analysis was then performed to determine if Gab2 was prognostic within specific patient groups. Although Gab2 is estrogen-regulated,[4] there was no association between Gab2 expression and outcome in ER-positive patients (Fig. 3*c*). Gab2 functions downstream of the epidermal growth factor receptor (EGFR) and erbB2,[9,19] and both of these receptors are expressed at higher levels in ER-negative versus ER-positive breast cancers.[21] Therefore, we investigated the relationship between Gab2 levels and patient outcome in the ER-negative subgroup. However, Gab2 expression was not related to patient outcome in these patients (Fig. 3*d*). Furthermore, although Gab2 enhances erbB2-induced tumorigenesis in a transgenic mouse model,[9] Gab2 was not associated with poor prognosis in *HER2*-positive breast cancer patients (Fig. 3*e*). Finally, no association with outcome was detected in the lymph node-negative or-positive subgroups (data not shown).

Next, we determined at which stage of breast cancer development Gab2 overexpression is first observed, and whether further alterations in expression are associated with metastatic progression. To address this, a cohort that included premalignant lesions including UDH and DCIS (grades 1, 2, and 3), as well as regional lymph node metastases, was subjected to Gab2 IHC. Notably, a significant increase in Gab2 expression was detected in the earliest lesion examined, UDH, and all of the different types of lesions exhibited significantly higher Gab2 expression when compared with the normal samples (Figs. 4*a* and *b*). Also, although there was no difference in Gab2 expression between UDH and DCIS grade 1, there was a significant increase in Gab2 expression between UDH and DCIS grade 2, and between DCIS grade 1 and DCIS grade 3 ($p = 0.011$). Interestingly, there was a significant decrease in expression when

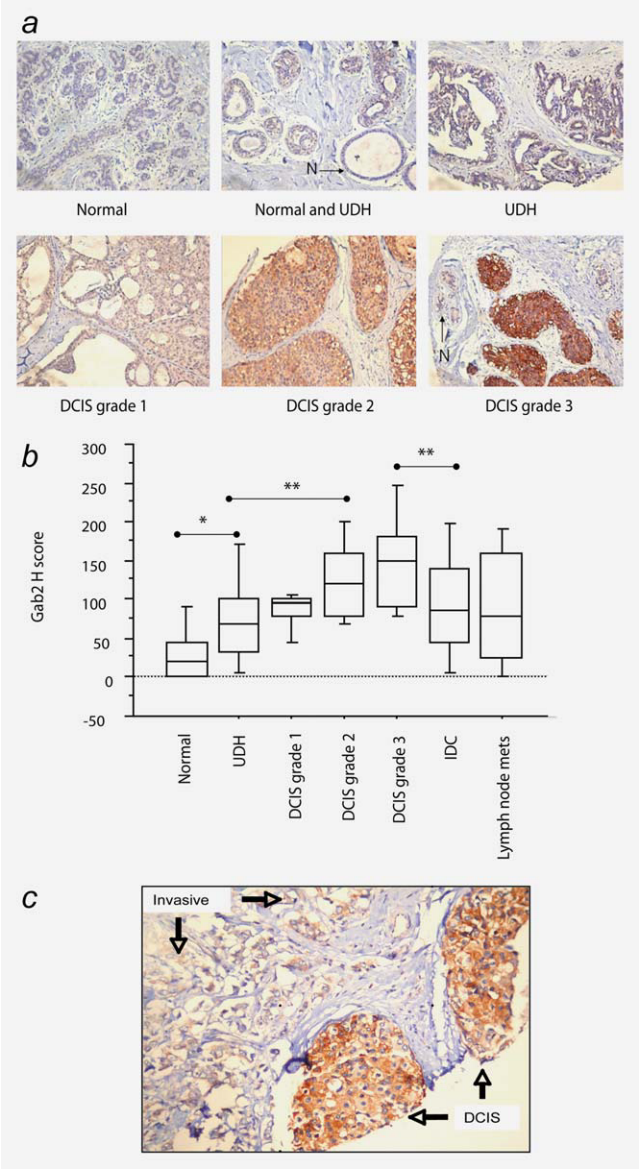

Figure 4. Gab2 expression during breast cancer evolution and progression. (*a*) Photomicrographs of Gab2 expression in a histological progression series. "N" denotes a normal duct. Note low levels of Gab2 in normal ducts and increasing expression in lesions with greater architectural and cytological atypia. All images are ×200 magnifications, haematoxylin counterstain. (*b*) Increased Gab2 expression occurs early in breast cancer evolution. Box plot of mean Gab2 *H*-score in each lesion. UDH, usual ductal hyperplasia; DCIS, ductal carcinoma *in situ*; IDC, invasive ductal carcinoma. $*p < 0.01$ and $**p < 0.005$. (*c*) Photomicrograph showing a decrease in Gab2 expression in invasive ductal carcinoma, compared to the adjacent DCIS.

DCIS grade 2 or 3 were compared to IDC or lymph node metastases, and expression in the latter 2 types of lesion was similar. Furthermore, for 12 patients, DCIS and IDC were present in the same core and 11/12 (92%) exhibited a reduction in Gab2 expression in the IDC compared to the

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associated DCIS (Fig. 4*c*). Therefore, Gab2 overexpression is an early event in breast cancer development, but it does not increase further in the progression to invasive or metastatic disease.

Discussion

In this manuscript, we report that Gab2 expression was not associated with outcome in a large breast cancer cohort or its constituent subgroups. This was a surprising result, given strong evidence from cell culture and mouse models linking Gab2 with mammary tumorigenesis and metastatic progression.[9,14,19,22] However, in a recently published study on breast cancer patients, amplification of the *GAB2* locus at 11q14.1 was not associated with overall survival,[11] supporting our findings. Consequently, while Gab2 possesses properties characteristic of a mammary oncogene, these data indicate that effects of increased Gab2 protein expression or gene copy number on disease course are not discernable for established breast cancers.

In addition, we detected a marked increase in Gab2 expression early in breast cancer evolution. The latter finding is potentially highly significant, as a functional role for Gab2 in this setting is supported by published *in vitro* studies. In 3D-culture, MCF-10A cells expressing Gab2 at levels comparable to those detected in breast cancer cell lines generate acini (spheroids) with enhanced diameter as a result of increased cellular proliferation, and further enhancement of Gab2 expression leads to defects in luminal clearance.[19] In addition, Gab2 cooperates with antiapoptotic proteins such as Bcl-X_L to induce luminal filling in this model.[9] Notably, such aberrant morphogenesis of MCF-10A cells is comparable to the histological phenotype of DCIS, which is also characterized by partial or complete luminal filling. A potential mechanism that may underpin the increase in Gab2 expression in early breast lesions is deregulation of cell cycle control mechanisms. The Retinoblastoma (Rb) tumor suppressor is a critical regulator of cell cycle entry *via* control of E2F family transcription factors, and Rb is itself regulated by specific cyclin/Cdk complexes, including cyclin D1/Cdk4.[23] Aberrations in the Rb pathway are known to occur early in breast cancer evolution, as cyclin D1 protein levels increase in the transition from normal mammary epithelium to UDH, and from the latter pathology to DCIS.[24] Furthermore, E2F-1 expression is enhanced in DCIS compared to the normal breast.[25] Importantly, Gab2 is a direct E2F target gene that mediates Akt activation, thereby attenuating the proapoptotic effects of the E2F-induced transcriptional program.[26] Indeed, MCF-10A cells overexpressing Gab2 exhibit enhanced Akt activity.[19] Therefore, we hypothesize that increased E2F activity early in breast cancer development leads to increased Gab2 expression, and Gab2 contributes to tumorigenesis by providing both proliferative and survival signals. Also, it remains possible that Gab2 promotes progression from *in situ* carcinoma to invasive cancer, although as expression of Gab2 is lower in invasive disease versus DCIS (but still higher than in normal tissue; Fig. 4), the effect must be mediated *via* cooperation with an additional oncogene. In support of the latter hypothesis, Gab2 cooperates with erbB2[9] and active Src[22] to promote invasive behavior in the MCF-10A 3D culture system. These potential roles of Gab2 early in breast cancer evolution warrant further investigation, particularly as targeting of its downstream effector pathways using Shp2-, MEK- or PI3K-directed small molecule inhibitors could provide a basis for breast cancer-preventative strategies.

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