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# 14-3-3ζ Cooperates with ErbB2 to Promote Ductal Carcinoma In Situ Progression to Invasive Breast Cancer by Inducing Epithelial-Mesenchymal Transition

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## SUMMARY

**ErbB2, a metastasis-promoting oncoprotein, is overexpressed in ~25% of invasive/metastatic breast cancers, but in 50%–60% of noninvasive ductal carcinomas in situ (DCIS). It has been puzzling how a subset of ErbB2-overexpressing DCIS develops into invasive breast cancer (IBC). We found that co-overexpression of 14-3-3ζ in ErbB2-overexpressing DCIS conferred a higher risk of progression to IBC. ErbB2 and 14-3-3ζ overexpression, respectively, increased cell migration and decreased cell adhesion, two prerequisites of tumor cell invasion. 14-3-3ζ overexpression reduced cell adhesion by activating the TGF-β/Smads pathway that led to ZFHX1B/SIP-1 upregulation, E-cadherin loss, and epithelial-mesenchymal transition. Importantly, patients whose breast tumors overexpressed both ErbB2 and 14-3-3ζ had higher rates of metastatic recurrence and death than those whose tumors overexpressed only one.**

## INTRODUCTION

ErbB2 overexpression is found in approximately 25% of invasive breast cancers (IBC) and is strongly associated with poor patient survival (Slamon et al., 1989). Overexpression of ErbB2 has been demonstrated to promote breast cancer invasion and metastasis (Yu and Hung, 2000). However, ErbB2 is overexpressed in 50%–60% of ductal carcinomas in situ (DCIS) in general and 60%–70% of high-grade DCIS (Nofech-Mozes et al., 2005). DCIS, a precursor of IBC, consists of clonal proliferation of malignant cells within the lumen of mammary ducts, with no evidence of invasion through the basement membrane into the surrounding stroma (Burstein et al., 2004). The apparent paradox that ErbB2, the well-known metastasis-promoting oncoprotein, is more frequently overexpressed in noninvasive DCIS than in IBC has been puzzling.

This stimulated debate about whether ErbB2 overexpression alone is sufficient to promote progression from noninvasive DCIS to IBC. The limited number of studies that have used patient follow-up data on invasive recurrence of primary DCIS have yielded ambiguous results. Some studies indicated that ErbB2-overexpressing DCIS had an increased risk of invasive recurrence (Provenzano et al., 2003), while others suggested the opposite (Perin et al., 1996; Ringberg et al., 2001). Interestingly,

## SIGNIFICANCE

More than 90% of breast cancer-related deaths are caused by metastasis not primary tumor. For effective reduction of cancer mortality, it is extremely important to predict the risk of, and to intervene in, the critical transition from noninvasive ductal carcinomas in situ (DCIS) to life-threatening invasive breast cancer (IBC). Here, we discovered that 14-3-3ζ overexpression is a "second hit" or "risk factor" facilitating a subset of ErbB2-overexpressing DCIS transition into IBC and identified molecular mechanisms/pathways through which ErbB2 and 14-3-3ζ co-overexpression promotes invasion. This study identified biomarkers that allow selection of high-risk DCIS patients for more aggressive treatments at early stages of cancer development, while saving low-risk patients from ablative clinical procedures. Moreover, it provided promising targets for future intervention strategies to prevent DCIS progression to IBC.

**Table 1. Expression of ErbB2 and 14-3-3ζ in 25 DCIS Cases with up to 7 Years of Follow-Up Data and the Incidence of Metastatic Recurrence**

| | Number of Cases (Number of Metastatic Recurrence) | | |
|---|---|---|---|
| | 14-3-3ζ (<3+) | 14-3-3ζ (3+) | Total |
| ErbB2 (<3+) | 5 (0) | 6 (0) | 11(0) |
| ErbB2 (3+) | 6 (0) | 8 (4) | 14 (4) |
| Total | 11(0) | 14 (4) | 25 (4) |

ErbB2 and 14-3-3ζ expression levels were examined by IHC staining in 25 DCIS patients' samples. Fisher's exact test showed that ErbB2 and 14-3-3ζ co-overexpression (3+/3+) in this cohort significantly ($p < 0.05$) correlated with distant site metastasis.

studies using 3D culture of mammary epithelial cells (MECs) showed that ErbB2 activation in preformed, growth-arrested mammary acini led to disruption of the well-organized acinar structure that shared several properties with DCIS in vivo, including uncontrolled cell proliferation, luminal filling, and no invasion (Muthuswamy et al., 2001). Moreover, transgenic mice expressing *neu* (rat homolog of human ErbB2) under its endogenous promoter developed DCIS-like mammary tumors after a long latency with rare metastasis (Andrechek et al., 2003).

These indicate that ErbB2 activation/overexpression may be involved in DCIS formation and that ErbB2 overexpression alone is not sufficient to drive invasion/metastasis. It was suggested that greater ErbB2 activity or additional genetic/epigenetic events ("second hits") are needed for MECs to gain invasive capability and for a subset of ErbB2-overexpressing DCIS to transition into IBC (Muthuswamy et al., 2001). However, it remained unclear as to what the second hits are.

The transition from a normal cell to a malignant cell is a multistep process, and at least six hallmark alterations in cell physiology collectively drive the malignant progression (Hanahan and Weinberg, 2000). 14-3-3 is a family of evolutionally conserved proteins that can bind to many target proteins involved in each of these cancer hallmark alterations (Tzivion et al., 2006; Wilker and Yaffe, 2004). It is conceivable that deregulation of 14-3-3 may contribute to cancer development. Generally, 14-3-3 proteins are divided into two subgroups: 14-3-3σ is a tumor suppressor, whereas the other 14-3-3 isoforms may have oncogenic functions. Increased 14-3-3ζ expression was observed in several tumor types and in the early stages of breast diseases such as DCIS (Danes et al., 2008). This raised the interesting possibility that 14-3-3ζ overexpression might contribute to DCIS progression to IBC.

The epithelial-mesenchymal transition (EMT) is a process during which epithelial cells convert to a mesenchymal cell phenotype after losing cell polarity, disassembling cell-cell adhesion machinery, and subsequently acquiring cell motility (Guarino, 2007). EMT promotes tumor invasion and metastasis by facilitating escape of tumor cells from the original rigid constraints of the surrounding tissue architecture (Guarino, 2007). The EMT-mediated increase in invasion/metastasis is largely contributed by loss of E-cadherin function because E-cadherin is essential for the maintenance of adherent junctions between neighboring cells and thus confers physical integrity on epithelial cells (Beavon, 2000; Guarino, 2007). E-cadherin loss has been shown to increase cell invasion in multiple in vitro models and has been correlated with increased metastasis in several epithelial tumor types (Strathdee, 2002). Therefore, E-cadherin is considered a suppressor of tumor invasion.

Given that ErbB2 overexpression alone in DCIS is not sufficient for progression to IBC, we explored whether 14-3-3ζ overexpression in DCIS may serve as a second hit that cooperates with ErbB2 to drive a subset of ErbB2-overexpressing DCIS progression into IBC.

## RESULTS

### ErbB2 and 14-3-3ζ Co-overexpression in DCIS Is Associated with Increased Invasion Potential

To investigate whether 14-3-3ζ overexpression cooperates with ErbB2 to drive a subset of ErbB2-overexpressing DCIS progression to IBC, we initially examined DCIS samples from 25 patients for whom up to 7 years of follow-up data was available. We analyzed the expression of ErbB2 and 14-3-3ζ by immunohistochemistry (IHC) staining. Fourteen of the 25 cases (56%) showed a high level of ErbB2 expression (Table 1), which is consistent with previous reports of ErbB2 overexpression in 50%–60% of DCIS cases (Nofech-Mozes et al., 2005). Eight of the 25 cases (32%) exhibited high levels of both ErbB2 and 14-3-3ζ (Table 1 and see Figure S1 available online). Strikingly, four of these eight patients had disease recurrence with distant site metastasis, whereas none of the 17 DCIS patients whose tumors did not overexpress both proteins developed distant metastasis (Table 1). Thus, ErbB2 and 14-3-3ζ co-overexpression in this small cohort significantly ($p < 0.05$) correlated with distant site metastasis, suggesting that 14-3-3ζ cooperates with ErbB2 to promote the progression from DCIS to IBC and metastasis.

MCF10A, a nontransformed human MEC line, is an excellent in vitro model in 3D culture for studying breast cancer progression as it forms well-organized acinar structures that mimic the normal mammary end bud in vivo (Debnath et al., 2003). Here, we used the MCF10A 3D culture model system to study whether and how 14-3-3ζ cooperates with ErbB2 to gain invasiveness. We established multiple stable MCF10A sublines overexpressing ErbB2 (10A.ErbB2), HA-tagged 14-3-3ζ (10A.14-3-3ζ), or both ErbB2 and HA-tagged 14-3-3ζ (10A.ErbB2.ζ), with 10A.Vec as the control (Figure 1A). We found that only the 10A.ErbB2.ζ cells formed soft agar colonies, whereas 10A.ErbB2, 10A.14-3-3ζ, and 10A.Vec MECs did not (Figure 1B). The data indicated that ErbB2 or 14-3-3ζ overexpression alone was not sufficient to induce a full transformation in MCF10A MECs, but ErbB2 and 14-3-3ζ co-overexpression could cooperatively induce full transformation, an important step for cancer invasion/metastasis.

Strikingly, the four sublines showed distinct acinar structures when grown in 3D matrigel (Figures 1C and 1D and Figure S2). 10A.ErbB2 cells formed highly proliferative, but noninvasive, DCIS-like structures characterized by impaired proliferation suppression and luminal cell apoptosis resistance, similar to a previous report (Muthuswamy et al., 2001). 10A.14-3-3ζ cells developed into abnormal acinar structures with no lumen formation, but no growth advantage, as we recently reported (Danes et al., 2008). 10A.ErbB2.ζ cells, however, demonstrated severe disruption of the acinar architecture, characterized by increased acinar size and no lumen formation (Figure 1C and 1D). The most distinct feature of the 10A.ErbB2.ζ acini was the gain of invasive