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From Cell Biology to Tissue Engineering

Expression of EMT inducers integrin-linked kinase (ILK) and ZEB1 in phyllodes breast tumors is associated with aggressive phenotype

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Summary. Phyllodes tumors (PTs) of the breast constitute an uncommon group of mammary fibroepithelial lesions with ambiguous biologic behavior. Recent evidence suggests that epithelial mesenchymal transition (EMT), a driving force of cancer progression is implicated in PTs pathogenesis. Integrin-linked kinase (ILK), a focal adhesion kinase, has been implicated in cancer and EMT and represents a novel cancer therapeutic target. In this study, we aimed to investigate ILK and EMT markers expression in phyllodes breast tumors in relation to tumor grade. Expression of ILK and EMT markers E-cadherin, β-catenin, N-cadherin, vimentin, Snail, ZEB1 and Twist was evaluated by immunohistochemistry in paraffin-embedded tissue sections from 96 human phyllodes breast tumors (48 benign, 27 borderline, 21 malignant). Cytoplasmic and nuclear immunopositivity of ILK were observed in both the epithelial and the stromal component of phyllodes breast tumors and were significantly higher with increasing tumor grade. An EMT-related expression profile consisting of decreased membranous and increased nuclear/cytoplasmic immunoreactivity of E-cadherin and β-catenin and increased expression of N-cadherin, vimentin, Snail, ZEB1 and Twist was observed in tumor epithelial and stromal component and was significantly associated with malignant phyllodes breast tumor histopathology. Interestingly, there was a significant correlation of ILK expression with all of the EMT markers examined. Our results suggest that EMT significantly contributes to phyllodes tumor pathogenesis and originally implicate ILK and ZEB1 in phyllodes tumors malignant phenotype.

Key words: Phyllodes tumors (PTs), EMT, ILK, ZEB

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Introduction

Phyllodes breast tumors (PTs) are uncommon but clinically important fibroepithelial neoplasms (Parker and Harries, 2001; Cheng et al., 2006; Jacklin et al., 2006; Tse et al., 2010; Hanby et al., 2017). They consist of epithelial and mesenchymal components and represent part of the spectrum of fibroepithelial breast neoplasms rather than a single disease entity (Parker and Harries, 2001; Hanby et al., 2017). PTs tend to recur locally and may have metastatic potential (Parker and Harries, 2001). They are currently classified as benign, borderline and malignant but unfortunately, there are no reliable markers that can predict their biological behavior and help identify patients that may benefit from more aggressive treatments (Cheng et al., 2006; Tse et al., 2010).

Epithelial to mesenchymal transition (EMT), the process by which epithelial cells acquire mesenchymal phenotype, plays a fundamental role in embryogenesis, wound healing, fibrosis and carcinogenesis (Kalluri and Weinberg, 2009; Thiery et al., 2009). EMT is implicated

not only in tumor progression but also in cell plasticity and in the acquisition of stem cell-like traits (Turley et al., 2008; Iwatsuki et al., 2010; Fabregat et al., 2016). Cells that undergo EMT are characterized by altered cell-cell and cell-extracellular matrix (ECM) interactions, cytoskeleton remodeling and a switch from epithelial to mesenchymal gene expression (Kalluri and Weinberg, 2009; Thiery et al., 2009). A hallmark of EMT is the loss of epithelial cell-cell junctions due to downregulation of the epithelial marker E-cadherin, while mesenchymal N-cadherin is upregulated, a phenomenon called cadherin switching (Kalluri and Weinberg, 2009; Thiery et al., 2009). Other important characteristics of cells undergoing EMT are the nuclear translocation of β-catenin, an effector of the Wnt pathway and the increased expression of the intermediate filament protein vimentin, reflecting cytoskeleton remodeling (Kalluri and Weinberg, 2009; Thiery et al., 2009; Zeisberg and Neilson, 2009). This molecular reprogramming that takes place in EMT is regulated mainly by three major groups of transcription factors, Snail, ZEB, and Twist families (Peinado et al., 2007; Kalluri and Weinberg, 2009; Thiery et al., 2009; Zeisberg and Neilson, 2009; Liu et al., 2016).

Integrin-linked kinase (ILK) is an evolutionally conserved component of cell-extracellular (ECM) adhesions linking integrins to the actin cytoskeleton (Wu and Dedhar, 2001; Hannigan et al. 2005; McDonald et al., 2008). ILK, through its scaffolding and signaling functions, has been shown to regulate cell adhesion, survival, proliferation, mitosis, migration, invasion and angiogenesis (Wu and Dedhar, 2001; Hannigan et al. 2005; McDonald et al., 2008). Aberrant expression of ILK has been reported in several epithelial cancers and sarcomas and several lines of evidence suggest that ILK represents a promising therapeutic target in cancer (Hannigan et al. 2005; Bravou et al., 2006; McDonald et al., 2008; Papachristou et al., 2008; McDonald et al., 2009; Papanikolaou et al., 2010; Rhee et al., 2013). Previous studies have also shown that ILK contributes to tumor progression partly due to induction of EMT and mechanisms involved in ILK mediated EMT include activation of β-catenin, induction of Snail and downregulation of E-cadherin (Novak et al., 1998; Oloumi et al., 2004; Bravou et al., 2006; Papanikolaou et al., 2010; Gil et al., 2011; Serrano et al., 2013).

PTs are true biphasic neoplasms with unclear cell of origin. Epithelial-mesenchymal interactions have been shown to play a crucial role in the pathogenesis of PTs and evidence suggests that the epithelial component should not be considered an innocent bystander (Sawyer et al., 2003; Karim et al., 2009a,b). Recently, aberrant expression of EMT markers has been reported in PTs suggesting that an EMT-like process may be implicated in PTs pathogenesis (Huang et al., 2010; Kwon et al., 2012; Do et al., 2013; Lim et al., 2015). However, a detailed analysis of an EMT-related expression profile and furthermore the implication of ILK and ZEB1 in PTs pathology has not been previously addressed. We therefore examined by immunohistochemistry the expression of the EMT related molecules ILK, E-cadherin, β-catenin, N-cadherin, vimentin, Snail, ZEB1 and Twist in a series of 96 PTs and evaluated the correlations between these markers and tumor grade.

Materials and methods

Tissue speciments

The study was performed in accordance with the Helsinki declaration and the institutional ethical guidelines and has been approved by the Committee on Research and Ethics of the University Hospital of Patras, Greece. Formalin-fixed paraffin-embedded tissue samples from 96 PTs were obtained from the Departments of Pathology of University Hospital of Patras, Greece, General Hospital "Elena Venizelou", Athens, Greece and General Hospital "Alexandra", Athens, Greece. According to the WHO classification of tumors 48/96 (50%) tumors were classified as benign PTs, 27/96 (28.1%) as borderline and 21/96 (21.9%) as malignant PTs.

Immunohistochemistry

Immunohistochemistry was performed using secondary antibodies conjugated to peroxidase- labeled polymer (Envsion detection kit, DAKO, Hamburg, Germany) and DAB as the chromogen as previously described (Bravou et al., 2006; Papanikolaou et al., 2010). Primary antibodies used were anti ILK (Santa Cruz Biotechnology, CA, USA, 1:100), anti ZEB1 (Sigma-Aldrich, St. Luis, USA, 1:200), anti Snail1 (Acris, Herford, Germany, 1:80) anti Twist1 (EMD Millipore, USA, 1:400), anti E-cadherin (BD Biosciences, CA, USA, 1:1000), anti β-catenin (BD Biosciences, CA, USA, 1:1000), anti vimentin (Novocastra, UK, 1:800) and anti N-cadherin (Acris, Herford, Germany, 1:300). Negative controls (by omitting the primary antibody) and positive controls (basal cell carcinoma and colorectal carcinoma tissue samples) were performed in all cases.

Immunohistochemical evaluation

All slides were assessed by two pathologists (HP, VB) and two investigators (IA, SN) independently and blinded to the case. Cytoplasmic, membranous and nuclear immunoreactivity was evaluated for each protein in the epithelial and the stromal tumor component separately. Immunoreactivity was scored on a scale of 0-3 according to the intensity of immunoreactivity and the percentage of positive cells. Intensity of immunoreactivity was scored as 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). Percentage of positive cells was scored as 0 (<10%), 1 (10-25%), 2 (25-50%), 3 (50-75%) and 4 (75-100%). The two scores were multiplied and the immunoreactivity score (values from 0 to 12)

was determined: score 0 as negative; score 1 (multiplication values 1,2) as weakly positive; score 2 (multiplication values 3,4,6) as moderately positive; score 3 (multiplication values 8,9,12) as strongly positive.

Statistical analysis

Statistical analysis was performed with the SPSS for Windows, release 22.0 (SPSS Inc., Chicago, IL, USA). To test the significance of differences in protein expression among benign, borderline and malignant PTs, ordinal data were analyzed with the non-parametric Kruskal Wallis test, whereas correlations between expression of proteins (immunoreactivity scores) were evaluated by the Spearman rank order correlation coefficient. The significance level was defined as $p<0.05$.

Results

ILK is overexpressed in phyllodes breast tumors and is associated with tumor grade

Adjacent non-neoplastic breast epithelium showed no expression for ILK, while cytoplasmic and nuclear ILK immunoreactivity was observed in the epithelial component of the tumors in 94/96 (97.9%) and 93/96 (96.9%) cases, as well as in the stromal tumor cells in 49/96 (51%) and 50/96 (52.1%) of the tumors examined, respectively (Fig. 1, Table 1). There was a statistically significant difference of ILK immunohistochemical expression among benign, borderline and malignant PTs with higher ILK immunoreactivity scores in both the epithelial (Kruskal Wallis, $p=0.013$ for cytoplasmic and $p=0.017$ for nuclear ILK expression) and the stromal tumor cells ($p<0.001$ for cytoplasmic and $p<0.001$ for nuclear ILK expression) in malignant and borderline compared to benign phyllodes tumors.

Decreased membranous and increased nuclear/cytoplasmic immunoreactivity of E-cadherin and β-catenin in PTs are associated with tumor grade

Adjacent non-neoplastic breast epithelium showed strong membranous immunoreactivity for E-cadherin and β-catenin, while in the epithelial component of the tumors there was decreased membranous and increased cytoplasmic and nuclear expression of both proteins (Fig. 2). Specifically, we observed membranous immunopositivity for E-cadherin and β-catenin in 73/96 (76%) and 59/96 (61.5%) cases respectively, cytoplasmic expression in 91/96 (94.8%) and 86/96 (87.5%) cases respectively and nuclear positivity in 87/96 (90.6%) and 59/96 (61.5%) of cases respectively. In the stromal tumor cells nuclear reactivity for E-cadherin was found in 40/96 (41.7%) cases and nuclear and cytoplasmic immunoreactivity for β-catenin in 51/96 (53.1%) and 21/96 (21.9%) cases of PTs (Table 2).

There was a statistically significant difference in the immunohistochemical expression of E-cadherin in both the epithelial and stromal component among benign, borderline and malignant phyllodes tumors. Significantly decreased membranous ($p<0.001$), as well as significantly higher cytoplasmic ($p=0.033$) and nuclear ($p=0.005$) E-cadherin immunopositivity in epithelial cells were observed in malignant and borderline phyllodes tumors. Stromal nuclear positivity of E-cadherin and stromal nuclear/cytoplasmic positivity for β-catenin also differed among PTs with stronger expression observed in the malignant tumors ($p=0.017$ for E-cadherin and $p=0.004$ and $p<0.001$ for nuclear and cytoplasmic β-catenin respectively), while epithelial

Table 1. ILK expression in epithelial and stromal components of phyllodes tumors: Correlation with tumor type.

	Expression (IR score)		Phyllodes tumors total (N=96)	Benign (N=48)	Borderline (N=27)	Malignant (N=21)	p value *
Stromal cytoplasmic ILK	0	n (%)	47 (49)	36 (75)	9 (33.3)	2 (9.5)	<0.001
	1	n (%)	19 (19.8)	7 (14.6)	10 (37)	2 (9.5)	
	2	n (%)	21 (21.9)	5 (10.4)	7 (26)	9 (42.9)	
	3	n (%)	9 (9.4)	0 (0)	1 (3.7)	8 (38.1)	
Stromal nuclear ILK	0	n (%)	46 (47.9)	35 (72.9)	9 (33.3)	2 (9.5)	<0.001
	1	n (%)	25 (26)	7 (14.6)	13 (48.2)	5 (23.8)	
	2	n (%)	18 (18.8)	6 (12.5)	4 (14.8)	8 (38.1)	
	3	n (%)	7 (7.3)	0 (0)	1 (3.7)	6 (28.6)	
Epithelial cytoplasmic ILK	0	n (%)	2 (2.1)	0 (0)	0 (0)	2 (9.5)	0.013
	1	n (%)	30 (31.3)	18 (37.5)	10 (37)	2 (9.5)	
	2	n (%)	48 (50)	28 (58.3)	13 (48.2)	7 (33.3)	
	3	n (%)	16 (16.7)	2 (4.2)	4 (14.8)	10 (47.7)	
Epithelial nuclear ILK	0	n (%)	3 (3.1)	1 (2.1)	0 (0)	2 (9.5)	0.017
	1	n (%)	32 (33.3)	18 (37.5)	11 (40.7)	3 (14.3)	
	2	n (%)	40 (41.7)	25 (52.1)	11 (40.7)	4 (19)	
	3	n (%)	21 (21.9)	4 (8.3)	5 (18.6)	12 (57.2)	

*Non-parametric Kruskal Wallis test. The significance level is 0.05.

expression of β-catenin did not differ significantly among tumors.

Increased expression of EMT markers N-cadherin and vimentin in PTs is associated with tumor grade

While no expression of N-cadherin and vimentin was noted in adjacent non-neoplastic epithelium, cytoplasmic/membranous immunoreactivity for N-cadherin and cytoplasmic immunoreactivity for vimentin in the epithelial component of the PTs was observed in 93/96 (96.9%) and in 66/96 (68.7%) of the tumors examined. Immunopositivity of both markers was also detected in stromal tumors cells in 64/96 (66.7%) and 83/96 (86.5%) cases respectively (Fig. 3, Table 3). Stromal expression of N-cadherin and both epithelial and stromal expression of vimentin in PTs differ significantly among benign, borderline and malignant tumors with the higher expression levels observed in tumors with malignant histology (Kruskal Wallis, $p<0.001$ for stromal N-cadherin, $p=0.011$ for epithelial vimentin and $p<0.001$ for stromal vimentin).

EMT regulators Snail, ZEB1 and TWIST are overexpressed in PTs and associate with tumor grade

Increased nuclear immunoreactivity of Snail, ZEB1 and Twist was observed in the epithelial component in 90/96 (93.8%), 93/96 (97%) and 87/96 (91%) of the tumors respectively and in the stromal component in 49/96 (51%), 87/96 (91%) and 67/96 (70%) of the phyllodes tumors respectively (Fig. 4, Table 4). Immunohistochemical expression of all EMT-related transcription factors significantly differ among benign,

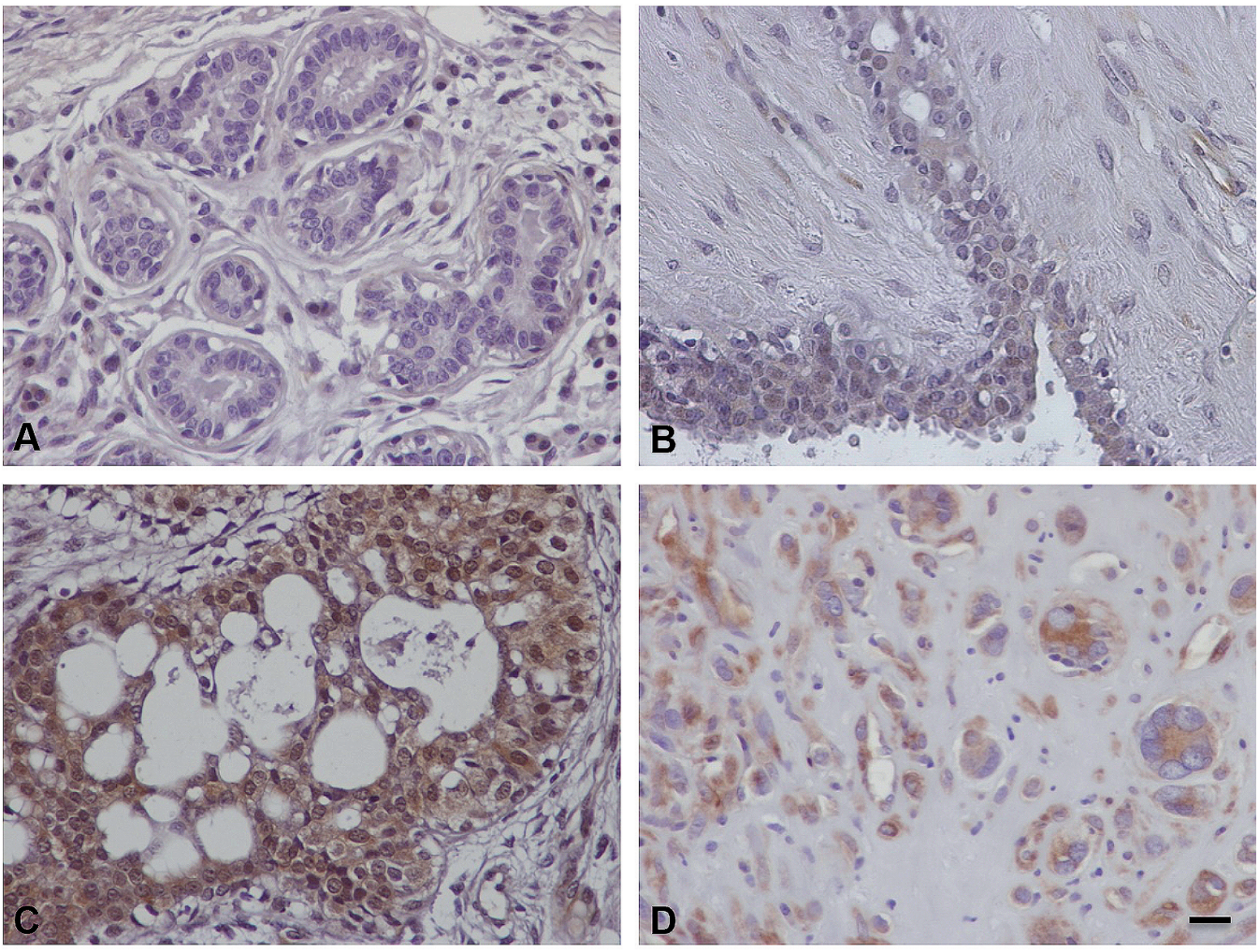

Fig. 1. ILK is overexpressesed in phyllodes breast tumors. **A.** Negative staining for ILK in adjacent non neoplastic breast. **B.** Negative to weak ILK expression in a case of benign PT. **C, D.** Representative cases of malignant PT with strong nuclear and/or cytoplasmic expression of ILK in the epithelial **(C)** and stromal component **(D)** of the tumor. Scale bars: 50 μm.

borderline and malignant tumors. Specifically, higher nuclear expression of Snail and ZEB1 in both the epithelial (Kruskal Wallis, p<0.001 for both) and stromal (Kruskal Wallis, p<0.001 for both) component of the tumors correlated with malignant histology. Stromal expression but not epithelial expression of Twist was also significantly higher with advanced tumor histology (Kruskal Wallis, p<0.001).

ILK expression in PTs strongly correlates with EMT markers expression

In the epithelial component of the PTs examined ILK expression significantly correlated with decreased membranous E-cadherin expression (p=0.031, r=-0.221 for cytoplasmic and p=0.021, r=-0.236 for nuclear ILK expression), nuclear accumulation of β-catenin (p=0.018, r=0.242 for nuclear and p=0.003, r=0.301 for cytoplasmic ILK expression) and expression of N-cadherin (p<0.001, r=0.350 for nuclear and p=0.023, r=0.232 for cytoplasmic ILK expression).

In the stromal component of the PTs, ILK expression significantly correlated with both cytoplasmic (p<0.001, r=0.460 and p<0.001, r=0.368 for cytoplasmic and nuclear ILK expression respectively) and nuclear (p=0.003, r=0.297 and p=0.007, r=0.275 for cytoplasmic and nuclear ILK expression respectively) β-catenin expression, expression of N-cadherin (p<0.001, r=0.484 for nuclear ILK and p<0.001, r=0.497 for cytoplasmic ILK expression) and expression of vimentin (p<0.001, r=0.466 for cytoplasmic ILK and p<0.001, r=0.499 for nuclear ILK)

There was also a significant positive correlation between ILK expression and expression of Snail, ZEB1 and Twist in both the epithelial and the stromal tumor cells (Table 5).

Table 2. E-cadherin and β-catenin expression in epithelial and stromal components of phyllodes tumors: Correlation with tumor type.

	Expression (IR score)		Phyllodes tumors total (N=96)	Benign (N=48)	Borderline (N=27)	Malignant (N=21)	p value*
Epithelial membranous E-cadherin	0	n (%)	23 (24)	2 (4.2)	3 (11.1)	18 (85.6)	<0.001
	1	n (%)	15 (15.6)	3 (6.2)	11 (40.7)	1 (4.8)	
	2	n (%)	32 (33.3)	18 (37.5)	13 (48.2)	1 (4.8)	
	3	n (%)	26 (27.1)	25 (52.1)	0 (0)	1 (4.8)	
Epithelial cytoplasmic E-cadherin	0	n (%)	5 (5.2)	0 (0)	0 (0)	5 (23.8)	0.033
	1	n (%)	7 (7.3)	7 (14.6)	0 (0)	0 (0)	
	2	n (%)	63 (65.6)	36 (75)	18 (66.7)	9 (42.9)	
	3	n (%)	21 (21.9)	5 (10.4)	9 (33.3)	7 (33.3)	
Epithelial nuclear E-cadherin	0	n (%)	9 (9.4)	4 (8.3)	0 (0)	5 (23.8)	0.005
	1	n (%)	21 (21.9)	19 (39.6)	1 (3.7)	1 (4.8)	
	2	n (%)	48 (50)	20 (41.7)	21 (77.8)	7 (33.3)	
	3	n (%)	18 (18.7)	5 (10.4)	5 (18.5)	8 (38.1)	
Stromal nuclear E-cadherin	0	n (%)	56 (58.3)	35 (72.9)	10 (37)	11 (52.4)	0.017
	1	n (%)	26 (27.1)	8 (16.7)	11 (40.7)	7 (33.3)	
	2	n (%)	10 (10.4)	2 (4.2)	5 (18.6)	3 (14.3)	
	3	n (%)	4 (4.2)	3 (6.2)	1 (3.7)	0 (0)	
Epithelial membranous β-catenin	0	n (%)	37 (38.5)	16 (33.3)	11 (40.8)	10 (47.6)	0.612
	1	n (%)	32 (33.3)	12 (25)	3 (11.1)	3 (14.3)	
	2	n (%)	18 (18.8)	13 (27.1)	10 (37)	7 (33.3)	
	3	n (%)	9 (9.4)	7 (14.6)	3 (11.1)	1 (4.8)	
Epithelial cytoplasmic β-catenin	0	n (%)	12 (12.5)	5 (10.4)	2 (7.4)	5 (23.8)	0.581
	1	n (%)	14 (14.6)	6 (12.5)	7 (25.9)	1 (4.8)	
	2	n (%)	45 (46.9)	23 (47.9)	13 (48.2)	9 (42.8)	
	3	n (%)	25 (26)	14 (29.2)	5 (18.5)	6 (28.6)	
Epithelial nuclear β-catenin	0	n (%)	37 (38.5)	20 (41.7)	9 (33.3)	8 (38.1)	0.333
	1	n (%)	32 (33.3)	18 (37.5)	12 (44.5)	2 (9.5)	
	2	n (%)	18 (18.8)	7 (14.6)	3 (11.1)	8 (38.1)	
	3	n (%)	9 (9.4)	3 (6.2)	3 (11.1)	3 (14.3)	
Stromal cytoplasmic β-catenin	0	n (%)	75 (78.1)	45 (93.7)	21 (77.8)	9 (42.9)	<0.001
	1	n (%)	3 (3.1)	2 (4.2)	1 (3.7)	0 (0)	
	2	n (%)	10 (10.4)	1 (2.1)	5 (18.5)	4 (19)	
	3	n (%)	8 (8.4)	0 (0)	0 (0)	8 (38.1)	
Stromal nuclear β-catenin	0	n (%)	45 (46.9)	29 (60.5)	8 (29.6)	8 (38.1)	0.004
	1	n (%)	19 (19.8)	11 (22.9)	7 (25.9)	1 (4.8)	
	2	n (%)	17 (17.7)	4 (8.3)	11 (40.8)	2 (9.5)	
	3	n (%)	15 (15.6)	4 (8.3)	1 (3.7)	10 (47.6)	

*Non-parametric Kruskal Wallis test. The significance level is 0.05.

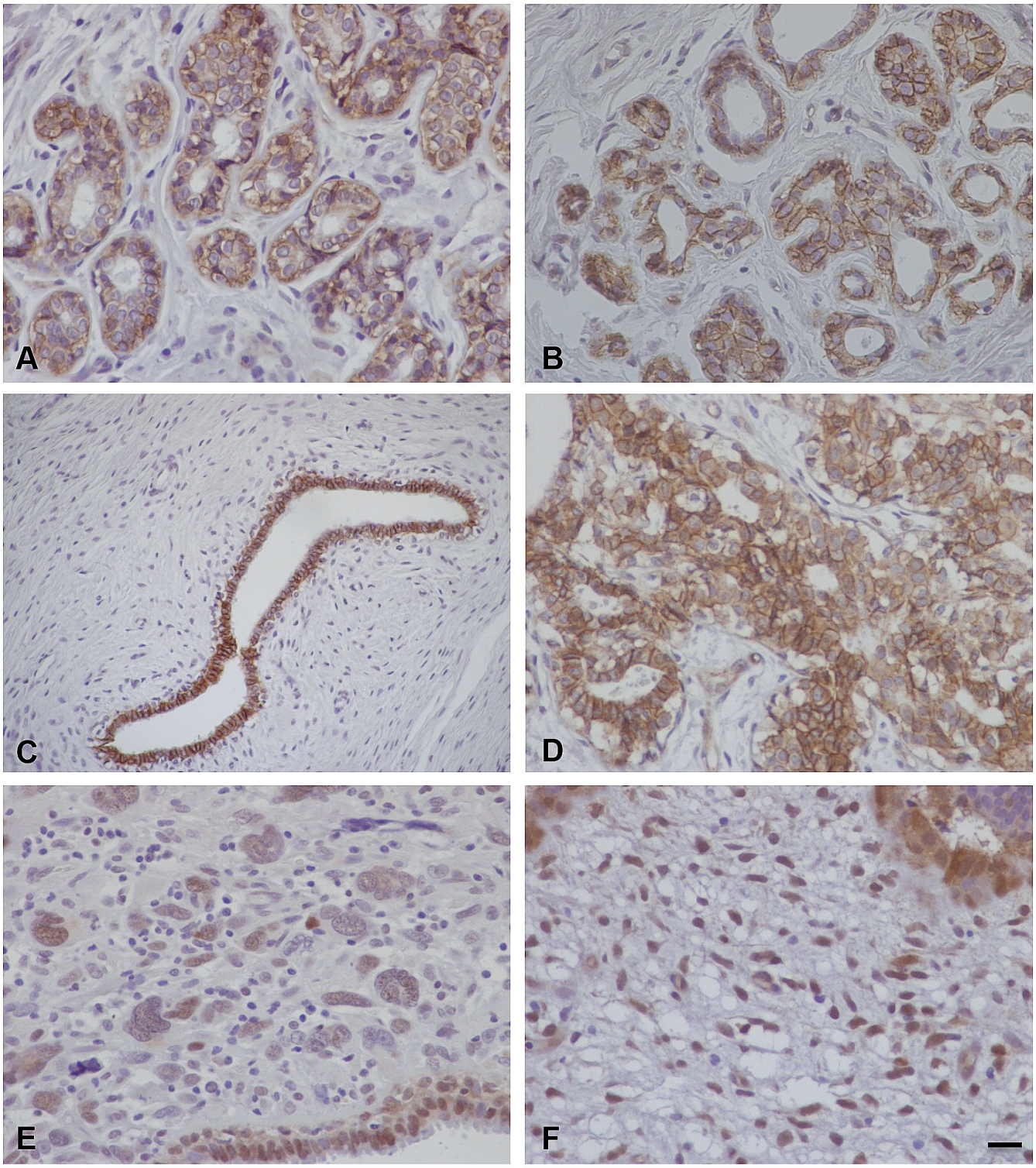

Fig. 2. Immunohistochemical staining of E-cadherin and β-catenin in phyllodes breast tumors. **A, B.** Adjacent non neoplastic breast showing membranous staining of E-cadherin **(A)** and β-catenin **(B)** in epithelial cells. **C, D.** Cases of benign PTs with membranous staining for E-cadherin **(C)** and β-catenin **(D)** in epithelial cells as in normal breast. **E, F.** Representative cases of malignant PT with strong nuclear staining for E-cadherin **(E)** and β-catenin **(F)** in both the epithelial and the stromal component of the tumor. Scale bars: 50 μm.

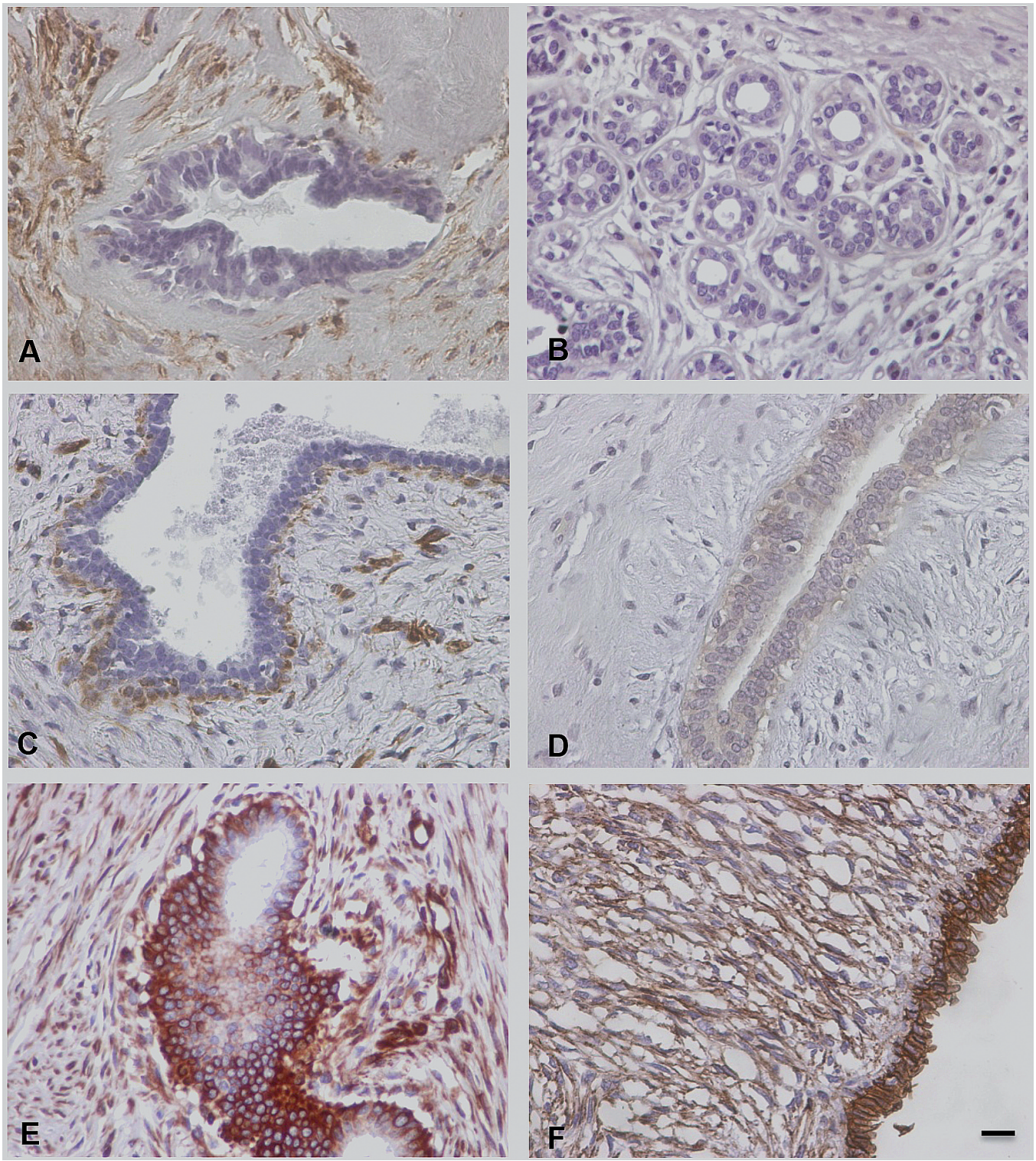

Fig. 3. Increased immunohistochemical expression of vimentin and N-cadherin in phyllodes breast tumors. **A, B.** Adjacent non neoplastic breast showing negative epithelial staining of vimentin **(A)** and N-cadherin **(B)**. **C, D.** Cases of benign PTs with negative to weak staining of vimentin **(C)** and N-cadherin **(D)** in epithelial cells. **E, F.** Representative cases of malignant PT with strong staining for vimentin **(E)** and N-cadherin **(F)** in both the epithelial and the stromal component of the tumor. Scale bars: 50 μm.

Table 3. Vimentin and N-cadherin expression in the epithelial and the stromal components of phyllodes tumors: Correlation with tumor type.

	Expression (IR score)		Phyllodes tumors total (N=96)	Benign (N=48)	Borderline (N=27)	Malignant (N=21)	p value *
Epithelial vimentin	0	n (%)	30 (31.2)	17 (35.4)	9 (33.3)	4 (19)	0.011
	1	n (%)	55 (57.3)	30 (62.5)	16 (59.3)	9 (42.9)	
	2	n (%)	9 (9.4)	1 (2.1)	2 (7.4)	6 (28.6)	
	3	n (%)	2 (2.1)	0 (0)	0 (0)	2 (9.5)	
Stromal vimentin	0	n (%)	13 (13.5)	11 (22.9)	1 (3.7)	1 (4.8)	<0.001
	1	n (%)	41 (42.7)	28 (58.3)	12 (44.5)	1 (4.8)	
	2	n (%)	23 (24)	7 (14.6)	11 (40.7)	5 (23.8)	
	3	n (%)	19 (19.8)	2 (4.2)	3 (11.1)	14 (66.6)	
Stromal Cytoplasmic N-cadherin	0	n (%)	32 (33.3)	24 (50)	4 (14.8)	4 (19)	<0.001
	1	n (%)	28 (29.2)	13 (27.1)	9 (33.3)	6 (28.6)	
	2	n (%)	24 (25)	10 (20.8)	11 (40.8)	3 (14.3)	
	3	n (%)	12 (12.5)	1 (2.1)	3 (11.1)	8 (38.1)	
Stromal membranous N-cadherin	0	n (%)	32 (33.3)	24 (50)	4 (14.8)	4 (19)	<0.001
	1	n (%)	29 (30.2)	13 (27.1)	10 (37)	6 (28.6)	
	2	n (%)	23 (24)	10 (20.8)	10 (37)	3 (14.3)	
	3	n (%)	12 (12.5)	1 (2.1)	3 (11.2)	8 (38.1)	
Epithelial cytoplasmic N-cadherin	0	n (%)	3 (3.1)	1 (2.1)	0 (0)	2 (9.5)	0.965
	1	n (%)	21 (21.9)	10 (20.8)	5 (18.5)	6 (28.6)	
	2	n (%)	37 (38.5)	21 (43.8)	13 (48.2)	3 (14.3)	
	3	n (%)	35 (36.5)	16 (33.3)	9 (33.3)	10 (47.6)	
Epithelial membranous N-cadherin	0	n (%)	3 (3.1)	1 (2.1)	0 (0)	2 (9.5)	0.965
	1	n (%)	21 (21.9)	10 (20.8)	5 (18.5)	6 (28.6)	
	2	n (%)	37 (38.5)	21 (43.8)	13 (48.2)	3 (14.3)	
	3	n (%)	35 (36.5)	16 (33.3)	9 (33.3)	10 (47.6)	

*Non-parametric Kruskal Wallis test. The significance level is 0.05.

Table 4. ZEB1, Snail and Twist expression in the epithelial and the stromal components of phyllodes tumors: Correlation with tumor type.

	Expression (IR score)		Phyllodes tumors total (N=96)	Benign (N=48)	Borderline (N=27)	Malignant (N=21)	p value*
Epithelial ZEB1	0	n (%)	3 (3.1)	2 (4.2)	0 (0)	1 (4.8)	<0.001
	1	n (%)	30 (31.3)	22 (45.8)	5 (18.5)	3 (14.3)	
	2	n (%)	46 (47.9)	23 (47.9)	15 (55.6)	8 (38.1)	
	3	n (%)	17 (17.7)	1 (2.1)	7 (25.9)	9 (42.8)	
Stromal ZEB1	0	n (%)	9 (9.4)	9 (18.8)	0 (0)	0 (0)	<0.001
	1	n (%)	36 (37.5)	34 (70.8)	1 (3.7)	1 (4.8)	
	2	n (%)	21 (21.9)	4 (8.3)	14 (51.9)	3 (14.3)	
	3	n (%)	30 (31.2)	1 (2.1)	12 (44.4)	17 (80.9)	
Epithelial Snail	0	n (%)	6 (6.3)	3 (6.3)	1 (3.7)	2 (9.5)	<0.001
	1	n (%)	23 (23.9)	17 (35.4)	5 (18.5)	1 (4.8)	
	2	n (%)	55 (57.3)	27 (56.2)	20 (74.1)	8 (38.1)	
	3	n (%)	12 (12.5)	1 (2.1)	1 (3.7)	10 (47.6)	
Stromal Snail	0	n (%)	47 (48.9)	31 (64.5)	13 (48.2)	3 (14.3)	<0.001
	1	n (%)	26 (27.1)	15 (31.3)	9 (33.3)	2 (9.5)	
	2	n (%)	14 (14.6)	1 (2.1)	5 (18.5)	8 (38.1)	
	3	n (%)	9 (9.4)	1 (2.1)	0 (0)	8 (38.1)	
Epithelial Twist	0	n (%)	9 (9.4)	4 (8.4)	1 (3.8)	4 (19)	0.227
	1	n (%)	40 (41.6)	23 (47.9)	12 (44.4)	5 (23.8)	
	2	n (%)	29 (30.2)	16 (33.3)	12 (44.4)	1 (4.8)	
	3	n (%)	18 (18.8)	5 (10.4)	2 (7.4)	11 (52.4)	
Stromal Twist	0	n (%)	29 (30.2)	20 (41.6)	5 (18.5)	4 (19)	<0.001
	1	n (%)	32 (33.3)	17 (35.4)	13 (48.2)	2 (9.6)	
	2	n (%)	22 (22.9)	9 (18.8)	9 (33.3)	4 (19)	
	3	n (%)	13 (13.6)	2 (4.2)	0 (0)	11 (52.4)	

*Non-parametric Kruskal Wallis test. The significance level is 0.05.

Table 5. Correlations between the expression of ILK and the EMT master regulators ZEB1, Snail and Twist in both the epithelial and the stromal neoplastic cells in phyllodes breast tumors.

Correlations Spearman's rho		Epithelial tumor cells ILK		Stromal tumor cells ILK	
		nuclear	cytoplasmic	nuclear	cytoplasmic
ZEB1	p	<0.001	<0.001	<0.001	<0.001
	r	0.500	0.450	0.617	0.634
Snail	p	<0.001	<0.001	<0.001	<0.001
	r	0.561	0.543	0.585	0.574
Twist	p	<0.001	<0.001	<0.001	0.001
	r	0.389	0.360	0.384	0.339

Discussion

Understanding pathogenesis and mechanisms of tumor progression of PTs remains a challenge. There is evidence that EMT regulators and ILK are implicated in cancer progression and represent promising tumor biomarkers and therapeutic targets (Hannigan et al., 2005; McDonald et al., 2008; Turley et al., 2008; Kalluri and Weinberg, 2009; Thiery et al., 2009; Iwatsuki et al., 2010; Fabregat et al., 2016). In this study we provide novel evidence that ILK and a set of proteins involved in EMT are overexpressed in phyllodes breast tumors and are associated with aggressive tumor phenotype.

ILK was overexpressed in our series of phyllodes breast tumors in agreement with previous findings in several human cancers (Hannigan et al. 2005; Bravou et

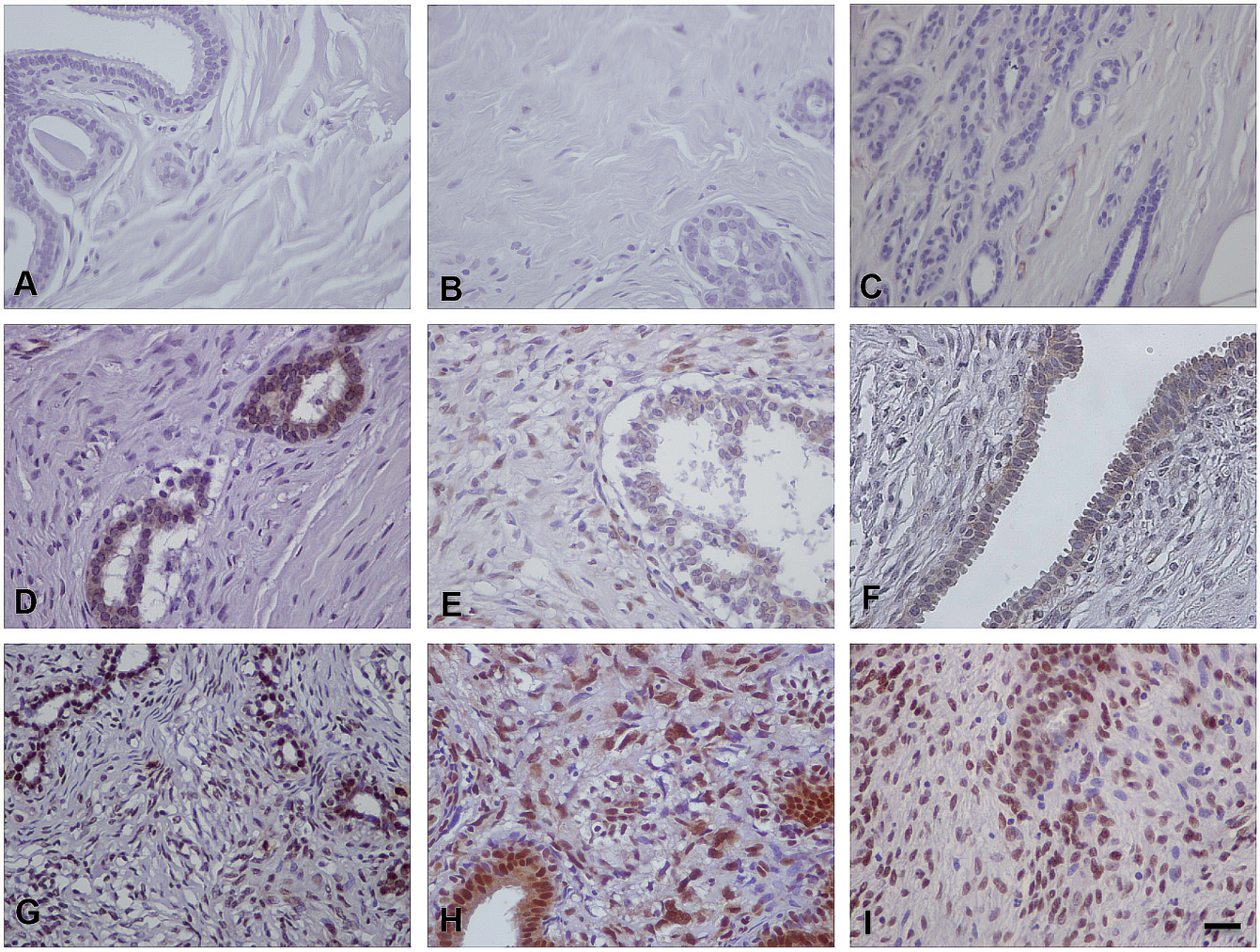

Fig. 4. EMT regulators Snail, ZEB1 and Twist are overexpressed in malignant phyllodes breast tumors. **A, B, C.** Adjacent non neoplastic breast showing negative staining of Snail **(A)**, ZEB1 **(B)** and Twist **(C)**. **D, E, F.** Cases of benign PTs with weak staining of Snail **(D)**, ZEB1 **(E)** and Twist **(F)** in epithelial and stromal tumor cells **G, H, I.** Representative cases of malignant PT with strong nuclear staining for Snail **(G)**, ZEB1 **(H)** and Twist **(I)** in both the epithelial in and the stromal component of the tumor. Scale bars: 50 μm.

al., 2006; Goulioumis et al., 2008; McDonald et al., 2008, 2009; Papachristou et al., 2008; Papanikolaou et al., 2010; Yu et al., 2011; Rhee et al., 2013). Interestingly specific overexpression of ILK in mouse mammary epithelium resulted in mammary hyperplasias and breast tumors (White et al., 2001). While the implication of ILK overexpression in breast cancer has been previously reported herein we provide novel evidence linking ILK to PTs pathology (White et al., 2001; Troussard et al., 2006; Hinton et al., 2008). We also demonstrated that ILK expression in both the epithelial and the stromal tumor cells was higher in malignant PTs, suggesting that overexpression of ILK may contribute to an aggressive tumor phenotype. This is supported by similar findings in other neoplasms, such as colon cancer and lung cancer where ILK expression correlated with adverse histological features and worse clinical outcome (Bravou et al., 2006; Papachristou et al., 2008; Papanikolaou et al., 2010; Yu et al., 2011; Rhee et al., 2013). In line with this, ILK has been shown to control processes that are fundamental to tumor progression such as cell adhesion, cytoskeletal dymamics, migration, invasion and epithelial to mesenchymal transition (Novak et al., 1998; Wu and Dedhar, 2001; Oloumi et al., 2004; Hannigan et al., 2005; Bravou et al., 2006; McDonald et al., 2008; Papanikolaou et al., 2010; Gil et al., 2011; Serrano et al., 2013). Interestingly several studies have demonstrated that pharmacologic inhibition of ILK shows significant anti-tumor effects and importantly ILK inhibition showed synergy with chemotherapy in breast cancer models rendering ILK a promising therapeutic target (Kalra et al., 2009; Lee et al., 2011; de la Puente et al., 2015). In this context, our finding of ILK overexpression in malignant PTs may be of clinical relevance.

We also showed that ILK expression in PTs is localized in the nucleus, a finding that may point to novel nuclear roles of this originally described focal adhesion kinase. Nuclear localization of ILK has been reported in a few other neoplasms, such as laryngeal cancer (Goulioumis et al., 2008). Previous published work provides evidence that ILK regulates the microtubule cytoskeleton and is involved in mitotic spindle organization and centrosome clustering indicating that apart from its role in cell matrix interactions ILK may be implicated in mitotic control in cancer (Fielding et al., 2008, 2011; Lim et al., 2013). In this context deregulation of ILK may lead to errors in cell division causing genomic instability, potentially further contributing to tumor progression and pharmacological inhibition of ILK may also exert anti-mitotic effects. Based on the above nuclear localization of ILK in PTs is an interesting finding that needs further investigation.

In support of our hypothesis that an EMT-like process is implicated in PTs biology, we found loss of membranous E-cadherin and β-catenin, and translocation to cytoplasm or the nucleus in the epithelial component of PTs, an expression profile that is consistent with the loss of intercellular junctions and activation of nuclear transcription leading to EMT (Chen et al., 2012; Valenta et al., 2012). In accordance with several studies in other tumors showing that loss of epithelial E-cadherin contributes to tumor progression and invasion we showed that loss of epithelial membranous E-cadherin increased with increasing tumor grade (Bravou et al., 2006; Onder et al., 2008; Chen et al., 2012; Valenta et al., 2012). In agreement with our results, it has been previously reported that epithelial E-cadherin associates with tumor grade and recurrence in PTs indicating the importance of the epithelial component in PT pathogenesis and prognosis (Tsang et al., 2012a,b; Feng et al., 2017). However, limited and contradictory results have been published regarding the epithelial expression of β-catenin in PTs (Sawyer et al., 2003; Karim et al., 2009).

In further support that the epithelial component of PTs is not an innocent bystander and may undergo an EMT process we provide evidence of increased expression of the mesenchymal markers vimentin and N-cadherin, as well as the EMT master regulators Snail, ZEB1 and Twist in the epithelial component of the phyllodes tumors examined. Importantly, expression of vimentin, Snail and ZEB1 in epithelial cells increased with tumor grade suggesting that an EMT-like process in the epithelial component may contribute to tumor progression of PTs. In agreement with our findings it was recently reported that epithelial expression of EMT markers Snail, Slug and Twist in PTs correlated with tumor grade (Feng et al., 2017).

Cytoplasmic/nuclear accumulation of E-cadherin and β-catenin and increased expression of mesenchymal markers vimentin and N-cadherin and EMT regulators ZEB1, Snail and Twist was also observed in the stromal component of the PTs examined and associated with increasing tumor grade. Our finding of nuclear E-cadherin expression in the stromal component (and in the epithelial tumor cells as well) is particularly interesting. Apart from its well-known role in intercellular junctions, nuclear localization of E-cadherin has been described and correlated with invasive properties in other human neoplasms, thus indicating a novel role in tumorigenesis (Chetty and Serra, 2008; Papanikolaou et al., 2010). Stromal nuclear β-catenin expression in PTs has also been reported by several other authors indicating the involvement of the Wnt pathway in the pathogenesis and progression of PTs (Sawyer et al., 2003; Karim et al., 2009a,b; Lacroix-Triki et al., 2010; Tsang et al., 2012a,b; Ho et al., 2013). In addition, consistent with our findings vimentin has been detected in malignant PTs and increased stromal Twist expression in PTs has been also reported and it correlated in some studies with worse patient outcome (Tsai et al., 2006; Kwon et al., 2012; Do et al., 2013; Lin et al., 2014; Lim et al., 2015; Feng et al., 2017). However, to the best of our knowledge, this is the first report of ZEB1 overexpression in phyllodes breast tumors. This finding is of importance as ZEB1 is a key promoting factor of

EMT, cancer progression and therapy resistance and a key determinant of mesenchymal phenotype of breast cancer (Peinado et al., 2007; Arima et al., 2012; Zhang et al., 2015). Moreover, pharmacologic inhibition of ZEB1 suppresses breast cancer growth and invasion suggesting that ZEB1 is a promising breast cancer therapeutic target (Arima et al., 2012).

Importantly, we also showed that ILK expression in PTs not only correlates with malignant histology, but is also associated with the expression of EMT-related markers. Overexpression of ILK has been associated with invasion and tumor progression in several cancers and some evidence indicates that induction of EMT may partially account for the oncogenic functions of ILK (Oloumi et al., 2004; Bravou et al., 2006; McDonald et al., 2008; Papanikolaou et al., 2010; Gil et al., 2011; Yu et al., 2011). Consistently, in human BCC, the correlation of ILK with EMT markers, invasion and infiltrative subtype suggests that ILK mediated EMT may significantly contribute to tumor progression in vivo (Papanikolaou et al., 2010). Interestingly, ILK overexpression has been shown to mesenchymally transform mammary epithelial cells in culture (Somarisi et al., 2001). In addition, transgenic mice overexpressing ILK in mammary epithelium developed breast tumors and some of these tumors showed a metaplastic-mesenchymal phenotype suggesting that an ILK-mediated epithelial-mesenchymal transition may occur during breast tumorigenesis (White et al., 2001). It is not unlikely therefore that ILK overexpression in PTs tumors may contribute to mesenchymal transformation of epithelium giving rise to the dual epithelial-stromal component of these tumors. However, this hypothesis needs further supporting evidence.

In conclusion, we confirm that EMT is implicated in phyllodes breast tumors and we show for the first time that EMT inducers ILK and ZEB1 are overexpressed in both the epithelial and the stromal component of these tumors and correlate with aggressive phenotype. ILK also correlates with all the EMT markers examined suggesting that ILK, probably through EMT, may contribute to the development and progression of phyllodes breast tumors. Since ILK and ZEB1 represent promising cancer biomarkers and therapeutic targets our results pave the way for further evaluation of the prognostic and therapeutic significance of these markers in phyllodes breast tumors.

Conflict of Interest. The authors declare that they have no conflict of interest.

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