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Correlation of epithelial-mesenchymal transition markers with clinicopathologic parameters in adenocarcinomas and squamous cell carcinoma of the lung

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Summary. Epithelial-mesenchymal transition (EMT) is characterized by the loss of epithelial cell junction proteins and the gain of mesenchymal markers. The aim of this study was to analyze the associations between the EMT-related markers vimentin, E-cadherin, ß-catenin, slug, snail, and twist1 and clinicopathologic parameters as well as epidermal growth factor receptor (EGFR) gene copy number and protein expression in non-small cell lung carcinoma (NSCLC). Fifty-nine squamous cell carcinomas (SCC) and 43 adenocarcinomas (AD) were immunohistochemically examined for respective EMT markers and for EGFR, using the EGFR PharmDx kit (Dako) for protein expression and automated silver enhanced *in situ* hybridization (SISH) for copy number. Vimentin expression in tumor epithelia was significantly higher in AD samples than in SCC samples (P=0.015). Among AD samples, vimentin expression was positively correlated with histologic grade (2 vs. 3; P=0.021) and exhibited a tendency toward a positive correlation with pTNM stage (I vs. II–IV; P=0.052). EGFR gene copy number was positively correlated with EGFR protein expression among both AD samples (P=0.008) and SCC samples (P=0.042), with EGFR protein expression being significantly higher in SCC samples compared with AD (P=0.038). Among AD samples, EGFR protein expression was associated with higher cytoplasmic expression of ß-catenin (P=0.031). Among SCC samples, EGFR protein expression was negatively correlated with nuclear expression of ß-catenin (P=0.033) but positively with nuclear slug (P=0.021). The expression pattern of EMT markers in AD suggests that vimentin is a possible immunohistochemical predictor of tumor progression.

Key words: Epithelial–mesenchymal transition, EGFR, Non-small cell lung carcinoma, Adenocarcinoma, Squamous cell carcinoma

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Introduction

Epithelial–mesenchymal transition (EMT), a process by which epithelial cells exhibit reduced intercellular adhesion and acquire fibroblast-like properties, is common to both development and carcinogenesis (Xu et al., 2009) and is related to mechanisms inducing tumor invasion and metastasis (Thiery, 2002; Gupta and Massague, 2006). By this process epithelial cells are converted to mesenchymal phenotypes and towards dedifferentiated and more malignant states. Repression of E-cadherin and expression of vimentin are hallmarks of EMT (Gupta and Massague, 2006; Yauch et al., 2005; Hugo et al., 2007). Cancer cells can trigger EMT upon activation of transcription factors such as snail, twist,

and slug; these same factors are involved in EMT during embryogenesis (Gupta and Massague, 2006).

Lung carcinoma is the leading cause of cancer-related death in both men and women worldwide (Jemal et al., 2006). Pathologic EMT is important to the progression of colon, prostate, breast, and non-small cell lung carcinoma (NSCLC) (Yauch et al., 2005; Bremnes et al., 2002; Prudkin et al., 2009; Hugo et al., 2007). In NSCLC an EMT pattern of decreased E-cadherin and elevated vimentin is significantly associated with ZEB1 and ZEB2 expression, but not with the expression of snail, slug, twist1 or twist2, suggesting that ZEB1 and ZEB2 are key regulators of the EMT process (Gemmill et al., 2011). Upregulation of snail, a transcriptional repressor of E-cadherin, promoted angiogenesis and tumor progression via CXCR2 ligands in a murine lung cancer model (Yanagawa et al., 2009). Knockdown of LKB1 in lung cancer cells increases cell motility and invasiveness and induces the expression of several mesenchymal marker proteins accompanied by the expression of ZEB1, which strongly indicates that LKB1 inactivation triggers EMT via the induction of ZEB1 (Roy et al., 2010). The expression of SIP1 is associated with reduced E-cadherin expression and with advanced NSCLC stage, suggesting that EMT might be activated via SIP1 expression (Miura et al., 2009). Overexpression of the EMT regulators HIF-1alpha, twist1 or snail in primary NSCLCs is associated with shorter overall survival, and overexpression of HIF-1alpha is associated with a shorter recurrence-free survival (Hung et al., 2009). Overexpression of the EMT indicator periostin in stroma or tumor epithelia correlates with more advanced stage, larger tumor size, and in stroma, with tumor relapse (Soltermann et al., 2008).

Epidermal growth factor receptor (EGFR) is overexpressed in human cancers, including NSCLC (Sharma et al., 2007). EGFR protein expression does not correlate with patient survival. However, patients with adenocarcinoma (AD) and squamous cell carcinoma (SCC) of the lung who exhibited an E-cadherin(-)/EGFR(+) phenotype had lower survival rates than E-cadherin(+)/EGFR(-) patients (Deeb et al., 2004). An increase in EGFR gene copy number in NSCLC trends toward poorer prognoses (Hirsch et al., 2003), but few medical studies have tested for correlations between EMT and EGFR protein expression or gene copy number in NSCLC. An evaluation of the relationship between EGFR protein expression, EGFR gene copy number, and the expression of EMT-associated proteins may facilitate an interpretation of the contributions of EMT and EGFR to NSCLC.

We examined 59 SCC samples and 43 AD samples immunohistochemically for expression of the EMT markers and related proteins, vimentin, E-cadherin, ß-catenin, slug, snail and twist1. We determined an association of EMT expression with EGFR gene copy number, EGFR protein expression and clinico-pathological parameters.

Materials and methods

Tissue samples

We obtained 102 surgically resected NSCLC samples (43 ADs and 59 SCCs) from the 2000-2008 surgical pathology files at the Department of Pathology, Chungnam National University Hospital and at Eulji University Hospital. A medical record review was conducted for all cases. None of the patients from whom samples were obtained had undergone preoperative chemotherapy or radiotherapy. All samples were derived from lobectomy or pneumonectomy specimens. This study was approved by the Institutional Review Board of Chungnam National University Hospital.

All cases were histologically reviewed by two pathologists (K.H.K and S.H.K), and the two most representative areas of viable carcinoma tissue were selected and marked on H&E slides. To create a tissue microarray, we punched tissue columns (3.0 mm in diameter) from the original paraffin blocks and inserted them into new paraffin blocks (each containing 30 holes to accept the tissue columns). These arrays were constructed using two 3-mm diameter cores per tumor.

Histological grading

SCCs were separated into three subgroups according to the degree of histological differentiation as follows: grade 1, well-differentiated; grade 2, moderately differentiated; and grade 3, poorly differentiated (Greene, 2002). Grade 1 cases were characterized by similarity to the stratified squamous epithelium of the airway and by clear keratinization. Grade 3 cases were difficult to distinguish from other lung cancer subtypes.

ADs were graded according to histological staining, as previously described (Sica et al., 2010). Histological subtypes were assigned grades as follows: grade I, bronchioloalveolar carcinoma subtype; grade II, acinar and papillary subtypes; and grade III, micropapillary and solid subtypes. Subtypes were identified and graded based on the predominant staining patterns found in microarrays of the AD cases.

Immunohistochemistry

Tissue sections on microslides were deparaffinized using xylene, rehydrated in serial dilutions of alcohol, and heated in a pressure cooker at full power for 3 min in 10 mM sodium citrate (pH 6.0) for antigen retrieval. Peroxide blocking was conducted with 3% H_2O_2 in methanol at ambient temperature for 15 min. Sections were then incubated with primary antibodies at ambient temperature for 60 min. The following primary mouse monoclonal antibodies were used: anti-vimentin (1:500; V9; Zymed Laboratories, Inc., San Francisco, CA, USA), anti-E-cadherin (1:100; NCL-E-Cad; Novocastra, Bannockburn, IL, USA), anti-ß-catenin (1:200; BD-

610154; BD Transduction Laboratories, BD Biosciences), anti-twist1 (1:50; Abcam 50887; Abcam, Cambridge, MA, USA), anti-slug (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), and anti-snail (1:100; Santa Cruz Biotechnology). After rinsing, sections were incubated with REAL EnVision/HRP Rabbit/Mouse detection reagent (Dako, Glostrup, Denmark) for 20 min at ambient temperature. After rinsing, the chromogen was developed for 2 min. Slides were counterstained with Mayer's hematoxylin, dehydrated, and mounted using Canada balsam. Immunohistochemical staining for EGFR was performed using the Food and Drug Administration-approved Dako EGFR PharmDx kit (Clone 2-18C9, predilution kit, Dako Cutomation; Dako) according to the manufacturer's instructions.

All immunostained slides were digitally scanned using a ScanScope CS system (Aperio, Vista, CA, USA). The central areas of each core were selected for analysis; these areas contained the most preserved and representative tumor cells and were less susceptible to the staining artifacts and edge effects characteristic of peripheral areas. Cytoplasmic membrane staining of E-cadherin, cytoplasmic/membranous staining of ß-catenin, cytoplasmic staining of vimentin, and nuclear staining of ß-catenin, slug, snail and twist1 were scored using digitally scanned files and light microscopy. A proportion score (range, 0-5) and an intensity score (range, 0-3) were multiplied to obtain a total score (range, 0-15) (Sinicrope et al., 1995; Allred et al., 1998). For the immunohistochemical staining of vimentin, the immunoreactivity in tumor cells was graded as low (total score <3) or high (total score ≥3) (Fig. 1). Staining with EGFR PharmDx was scored as 0 when there was no specific membrane staining within the tumor and as a positive integer when staining was observed above background level. Positive cases were classified as 1, 2, or 3 based on the staining intensity (Fig. 1) (Shia et al., 2005). Each sample was examined individually and classified by two pathologists (K.H.K. and S.H.K.).

Silver enhanced in situ hybridization for EGFR

Automated silver enhanced in situ hybridization (SISH) analysis of consecutive slides was performed using EGFR dual SISH-2p4 according to the manufacturer's protocol (Roche, Tucson, AZ, USA). The protocol was optimized for use with XT Dual uView SISH RedISH v1 on a Benchmark XT IHC/ISH Staining Module (Roche). The EGFR DNA probe (SISH V-probe) was denatured at 95°C for 12 min, and hybridization was performed at 52°C for 6 h. Following hybridization, appropriate stringency washes (3 times at 72°C) were performed. The chromosome 7 probe (Red ISH V-probe) was denatured at 95°C for 12 min, and hybridization was performed at 44°C for 2 h. After hybridization, appropriate stringency washes (3 times at 59°C) were performed. The EGFR DNA probe was visualized using a rabbit anti-DNP primary antibody with an ultraView SISH Detection kit. The chromosome 7 probe was visualized using rabbit anti-DNP primary antibody and an ultraView ISH AP Detection kit. Specimens were counterstained with Ventana Hematoxylin II. Nuclear precipitation of silver allowed EGFR gene signals to be visualized as black dots. The chromosome 7 centromere was visualized as a red spot.

The EGFR copy number was classified into two strata based on the frequency of cells exhibiting increased EGFR gene copies per cell: (1) SISH-negative, with no or low genomic gain (≥4 EGFR copies in <40% of cells); or (2) SISH-positive, characterized by the following: (a) a high level of polysomy (≥4 EGFR

Table 1. Clinicopathological characteristics of a cohort of 59 SCC samples and 43 AD samples.

Characteristic	Adenocarcinoma (No.)	Squamous cell carcinoma (No.)	P
Age	43	59	0.393*
Mean±SD (Year)	61.05±10.319	63.07±8.859	
Sex	43 (100%)	59 (100%)	<0.001†
Male	26 (60.5%)	56 (94.9%)	
Female	17 (39.5%)	3 (5.1%)	
EGFR PharmDx	42 (100.0%)	57 (100.0%)	0.038†
Negative	28 (66.7%)	26 (45.6%)	
Positive	14 (33.3%)	31 (54.4%)	
EGFR SISH	42 (100%)	59 (100%)	0.202†
Negative	24 (57.1%)	41 (69.5%)	
Positive	18 (42.9%)	18 (30.5%)	
Vimentin	43	59	0.015*
Mean±SD	2.37±4.467	0.63±1.809	
E-cadherin	42	59	0.550*
Mean±SD	2.36±2.739	2.49±2.706	
ß-catenin(CM)	43	59	0.246*
Mean±SD	7.86±4.491	9.02±3.985	
ß-catenin(Nu)	43	59	0.184*
Mean±SD	0.07±0.457	0.73±2.632	
Slug	43	58	0.140*
Mean±SD	2.56±3.556	1.64±2.982	
Snail	43	58	0.809*
Mean±SD	13.95±3.000	14.05±2.724	
Twist	43	58	0.755*
Mean±SD	0.81±1.868	1.29±3.044	
EGFR mutation	8 (100%)	12 (100%)	0.034‡
Negative	4 (50.0%)	11(91.7%)	
Positive	4 (50.0%)	1 (8.3%)	
Grade	42 (100.0%)	58 (100.0%)	
1	2 (4.8%)	43 (74.1%)	
2	28 (66.7%)	10 (17.2%)	
3	12 (28.6%)	5 (8.6%)	
TNM-Stage	43 (100.0%)	58 (100.0%)	
I	21 (48.8%)	26 (44.8%)	
II	6 (14.0%)	17 (29.0%)	
III	14 (32.6%)	15 (25.9%)	
IV	2 (4.7%)	0 (0.0%)	

SCC, squamous cell carcinoma; AD, adenocarcinoma; *, Mann Whitney U-test; †, Pearson's chi-square test; ‡, likelihood-ratio test; No., total number of cases investigated; SD, standard deviation; CM, cytoplasmic/membranous expression; Nu, Nuclear expression

Table 2. Characteristics of the EGFR mutation cases.

	EGFRmutation	Type	Grade	Sex	Age	Stage	EGFR IHC	EGFR SISH	E-cadherin	ß-catenin (CM)	ß-catenin (Nu)	vimentin	slug	snail	twist1
1	Exon 19 15bp del (2237-2251)	AD	3	M	63	IIIa	2	6	0	1	0	0	0	15	0
2	Exon 19 12bp del (2240-2251)	AD	2	F	70	Ia	NA	NA	NA	10	0	1	0	15	0
3	Exon 20 codon 787 (CAG>CAA)/ Exon 19 18bp del (2253-2270)	AD	2	F	67	Ib	0	1	0	12	0	0	0	10	5
4	Exon 18 codon 695(AGT>AAT)	SCC	2	M	58	IIa	0	4	5	15	0	0	0	15	5
5	Exon 19 15bp del (2235-2249)	AD	2	F	62	Ia	0	4	0	1	0	0	0	15	0

AD, adenocarcinoma; SCC, squamous cell carcinoma; Grade, histologic grade; NA, not available; M. male; F, female; IHC, immunohistochemical staining; CM, cytoplasmic/membranous; Nu, nucleus

Table 3. Analysis of EGFR SISH status; expression of EGFR, E-cadherin, ß-catenin, slug, snail, and twist1; and clinicopathologic features with vimentin expression in AD and SCC.

Characteristics	Vimentin in AD.			Vimentin in SCC		
	low	high	P	low	high	P
EGFR SISH	30 (100.0%)	12 (100.0%)	0.554†	53 (100.0%)	6 (100.0%)	0.656‡
Negative	18 (60.0%)	6 (50.0%)		36 (67.9%)	5 (83.3%)	
Positive	12 (40.0%)	6 (50.0%)		17 (32.1%)	1 (16.7%)	
EGFR PharmDx	30 (100.0%)	12 (100.0%)	0.169‡	51 (100.0%)	6 (100.0%)	1.000‡
Negative	22 (73.3%)	6 (50.0%)		23 (45.1%)	3 (50.0%)	
Positive	8 (26.7%)	6 (50.0%)		28 (54.9%)	3 (50.0%)	
E-cadherin			0.874*			0.154*
No.	30	12		53	6	
Mean±SD	2.10±2.249	3.00±3.741		2.64±2.781	1.17±1.472	
ß-catenin (CM)			0.469*			0.066*
No.	31	12		53	6	
Mean±SD	7.52±4.396	8.75±4.808		9.32±4.042	6.33±2.160	
ß-catenin (Nu)			0.534*			0.018*
No.	31	12		53	6	
Mean±SD	0.10±0.539	0.00±0.000		0.40±1.864	3.67±5.715	
Slug			0.018*			0.433*
No.	31	12		52	6	
Mean±SD	1.61±2.704	5.00±4.395		1.73±3.073	0.83±2.041	
Snail			0.486*			0.786*
No.	31	12		52	6	
Mean±SD	13.71±3.408	14.58±1.443		14.04±2.807	14.17±2.041	
Twist1			0.966*			<0.001*
No.	31	12		52	6	
Mean±SD	0.81±1.869	0.83±1.946		0.67±1.987	6.67±5.164	
Tumor diameter	31 (100.0%)	12 (100.0%)	0.481†	52 (100.0%)	6 (100.0%)	0.166‡
≤3.0cm	14 (45.2%)	4 (33.3%)		17 (32.7%)	0 (0.0%)	
>3.0cm	17 (54.8%)	8 (66.7%)		35 (67.3%)	6 (100.0%)	
Grade	30 (100.0%)	12 (100.0%)	0.021‡	52 (100.0%)	6 (100.0%)	1.000‡
1	2 (6.7%)	0 (0.0%)	(^{A} vs.^{B})	39 (75.0%)	4 (66.7%)	(^{A} vs.^{B})
2^{A}	23 (76.7%)	5 (41.7%)		9 (17.3%)	1 (16.7%)	
3^{B}	5(16.7%)	7 (58.3%)		4 (7.7%)	1 (16.7%)	
TNM-Stage	31 (100.0%)	12 (100.0%)	0.052†	52 (100.0%)	6 (100.0%)	0.209‡
I	18 (58.1%)	3 (25.0%)		25 (48.1%)	1 (16.7%)	
II-IV	13 (41.9%)	9 (75.0%)		27 (51.9%)	11 (83.3%)	
TNM-Stage	31 (100.0%)	12 (100.0%)	0.092‡	52 (100.0%)	6 (100.0%)	0.643‡
I-II	22 (71.0%)	5 (41.7%)		39 (75.0%)	4 (66.7%)	
III-IV	9 (29.0%)	7 (58.3%)		13 (25.0%)	2 (33.3%)	

*, Mann Whitney U-test; †, Pearson's chi-square test; ‡, Fisher exact test; No., total number of cases investigated; SD, standard deviation.; AD, adenocarcinoma; SCC, squamous cell carcinoma; CM, cytoplasmic/membranous expression; Nu, Nuclear expression

copies in ≥40% of cells); or (b) gene amplification, defined by the presence of tight EGFR gene clusters, a ratio of the EGFR gene to chromosomes of ≥2, or ≥15 copies of EGFR per cell in ≥10% of analyzed cells (Fig. 1) (Cappuzzo et al., 2005; Hirsch et al., 2005).

EGFR mutation analysis

Twenty cases were examined in total, including 8 AD cases and 12 SCC cases.

DNA was extracted from 5 paraffin sections of 10 μm thickness each containing a representative portion of tumor tissue. DNA extraction from formalin-fixed paraffin-embedded (FFPE) tissue was conducted using the QIAamp DNA FFPE Tissue kit (Qiagen, Hilden, Germany) per the manufacturer's protocol. Fifty nanograms of DNA were amplified in a 20 μl reaction solution containing 10 μl of 2X concentrated HotStarTaq Master Mix (Qiagen) composed of polymerase chain reaction (PCR) buffer with 3 mM MgCl2, 400 μM each dNTP, and 0.3 μM each primer pair (Exon 18F: 5'-cca tgt ctg gca ctg ctt t-3', 18R: 5'-cag ctt gca agg act ctg g-3', Exon 19F: 5'- tgt ggc acc atc tca caa ttg-3', 19R: 5'-gga ccc cca cac agc aa-3', Exon 20F: 5'-ggt cca tgt gcc cct cct-3', 20R: 5'-tgg ctc ctt atc tcc cct cc-3', Exon 21F: 5'-cca tga tga tct gtc cct ca-3', 21R: 5'-aat gct ggc tga

Table 4. Analysis of EGFR, E-cadherin, ß-catenin, vimentin, slug, snail, and twist1 expression, and clinicopathologic features with EGFR SISH status in AD and SCC.

Characteristics	EGFR-SISH in AD			EGFR-SISH in SCC		
	Negative	Positive	P	Negative	Positive	P
EGFR PharmDx	24 (100.0%)	18 (100.0%)	0.008*	40 (100.0%)	17 (100.0%)	0.042‡
Negative	20 (83.3%)	8 (44.4%)		22 (55.0%)	4 (23.5%)	
Positive	4 (16.7%)	10 (55.6%)		18 (45.0%)	13 (76.5%)	
E-cadherin			0.470*			0.762*
No.	24	18		41	18	
Mean±SD	1.83±1.903	3.06±3.506		2.54±2.785	2.39±2.593	
ß-catenin (CM)			0.654*			0.796*
No.	24	18		41	18	
Mean±SD	7.42±4.106	8.33±5.122		9.17±3.748	8.67±4.576	
ß-catenin (Nu)			0.386*			0.125*
No.	24	18		41	18	
Mean±SD	0.13±0.612	0.00±0.000		1.05±3.114	0.00±0.000	
Vimentin			0.619*			0.719*
No.	24	18		41	18	
Mean±SD	2.04±4.418	2.89±4.727		0.66±1.783	0.56±1.917	
Slug			0.740*			0.821*
No.	24	18		41	17	
Mean±SD	2.71±3.529	2.50±3.738		1.65±3.093	1.62±2.787	
Snail			0.722*			0.072*
No.	24	18		41	17	
Mean±SD	13.96±3.290	13.89±2.742		13.66±3.167	15.00±0.000	
Twist1			0.098*			0.406*
No.	24	18		41	17	
Mean±SD	1.25±2.212	0.28±1.179		1.59±3.435	0.59±1.661	
Tumor diameter	24 (100.0%)	18 (100.0%)	0.856†	40 (100.0%)	18 (100.0%)	0.540‡
≤3.0cm	10 (41.7%)	7 (38.9%)		13 (32.5%)	4 (22.2%)	
>3.0cm	14 (58.3%)	11 (61.1%)		27 (67.5%)	14 (77.8%)	
Grade	23 (100.0%)	18 (100.0%)	0.748†	41 (100.0%)	17 (100.0%)	0.699†
1	2 (8.7 %)	0 (0.0 %)	(^{A} vs.^{B})	31 (75.6%)	12 (70.6%)	(^{A} vs.^{B})
2^A	15 (65.2 %)	12 (66.7 %)		7 (17.1%)	3 (17.6%)	
3^B	6 (26.1 %)	6 (33.3%)		3 (7.3%)	2 (11.8%)	
TNM-Stage	24 (100.0%)	18 (100.0%)	0.721†	41 (100.0%)	17 (100.0%)	0.347†
I	12 (50.0%)	8 (44.4%)		20 (48.8%)	6 (35.3%)	
II-IV	12 (50.0%)	10 (55.6%)		21 (51.2%)	11 (64.7%)	
TNM-Stage	24 (100.0%)	18 (100.0%)	0.927†	41 (100.0%)	17 (100.0%)	0.291†
I-II	15 (62.5%)	11 (61.1%)		32 (78.0%)	11 (64.7%)	
III-IV	9 (37.5%)	7 (38.9%)		9 (22.0%)	6 (35.3%)	

*, Mann Whitney U-test; †, Pearson's chi-square test; ‡, Fisher exact test; No., total number of cases investigated; SD, standard deviation.; AD, adenocarcinoma; SCC, squamous cell carcinoma; CM, cytoplasmic/membranous expression; Nu, Nuclear expression

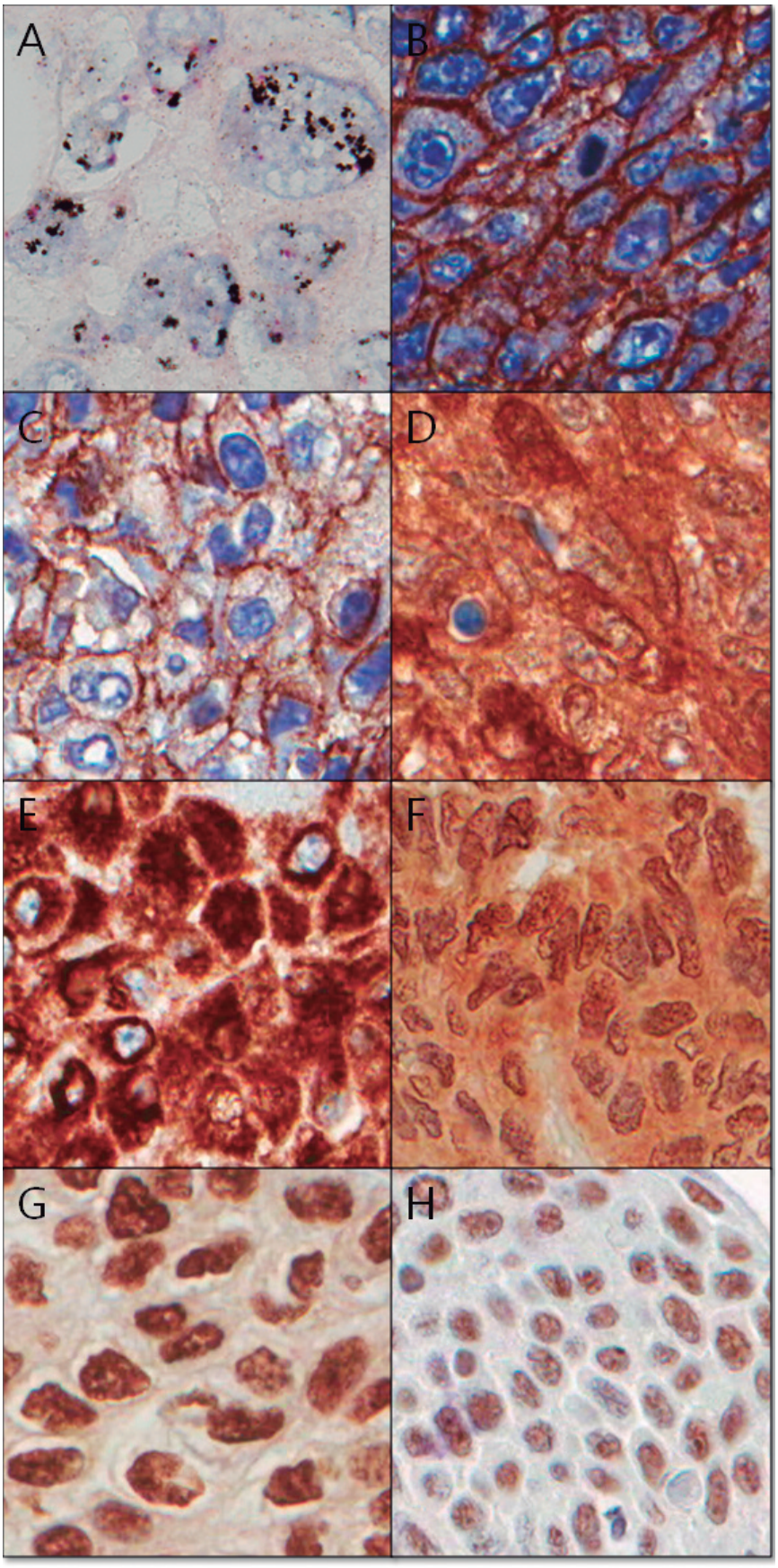

Fig. 1. Representative micrographs of tumor cells showing EGFR gene amplification by SISH, EGFR immunohistochemical expression (Dako PharmDx), and expression of EMT-related proteins (E-cadherin, ß-catenin, vimentin, slug, snail, and twist1) (Light microscope). **A.** Positive-EGFR SISH: The red signals represent the centromere of chromosome 7; the black signals represent the EGFR gene locus on chromosome 7p12. **B.** Positive EGFR PharmDx result, scored as 3. **C.** Cytoplasmic/membranous expression of E-cadherin. **D.** Cytoplasmic/membranous and nuclear expression of ß-catenin. **E.** Cytoplasmic expression of vimentin. **F.** Nuclear and cytoplasmic expression of slug. **G-H.** Nuclear expression of snail and twist1. x 400

cct aaa gc-3'). EGFR (exons 18–21) was amplified via a 15-min initial denaturation at 95°C; 35 cycles of 30 s at 94°C, 30 s at 59°C, and 30 s at 72°C and a 10-min final extension at 72°C. PCR products then were 2% gel-purified using a HiYield Gel/PCR DNA Extraction Kit (Real Biotech Corporation, Banqiao, Taipei, Taiwan). DNA templates were processed for DNA sequencing using the ABI-PRISM BigDye Terminator v3.1Cycle Sequencing kit (Applied Biosystems, Foster, CA, USA) with forward and reverse sequence-specific primers. Twenty nanograms of purified PCR products were used in a 10 μl sequencing reaction solution containing 1 μl of BigDye Terminator v3.1 and 0.1 μM of PCR primer. Sequencing reactions were performed using 25 cycles of 10 s at 96°C, 5 s at 50°C, and 4 min at 60°C. Sequence data were generated on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Sequences were examined for variations using Sequencing Analysis 5.1.1. software (Applied Biosystems).

Statistical analysis

Statistical analyses for continuous variables were performed using the Mann Whitney U-Test. Associations between categorical variables were assessed via cross-tabulation, the Pearson's chi-square test, the likelihood-ratio test, and Fisher's exact test. Statistical significance was assigned at $P<0.05$. All statistical analyses were performed using PASW Statistics 18.0 (SPSS Inc., Chicago, IL, USA).

Results

Clinicopathological features

The average patient age at the time of surgery was

Table 5. Analysis of E-cadherin, ß-catenin, vimentin, slug, snail, and twist1 expression, and clinicopathologic features with EGFR expression (assessed using PharmDx) in AD and SCC.

Characteristics	EGFR PharmDx in AD			EGFR PharmDx in SCC		
	Negative	Positive	P	Negative	Positive	P
E-cadherin			0.508*			0.325*
No.	28	14		26	31	
Mean±SD	1.96±2.063	3.14±3.718		2.77±2.388	2.39±3.008	
ß-catenin (CM)			0.031*			0.375*
No.	28	14		26	31	
Mean±SD	6.57±4.068	10.29±4.531		9.46±4.042	8.87±3.956	
ß-catenin (Nu)			0.355*			0.033*
No.	28	14		26	31	
Mean±SD	0.00±0.000	0.21±0.802		1.65±3.805	0.00±0.000	
Vimentin			0.260*			0.823*
No.	28	14		26	31	
Mean±SD	1.64±4.011	3.93±5.210		0.73±1.991	0.58±1.728	
Slug			0.655*			0.021*
No.	28	14		26	30	
Mean±SD	2.23±2.992	3.39±4.558		0.77±2.094	2.08±3.222	
Snail			0.574*			0.355*
No.	28	14		26	30	
Mean±SD	13.57±3.563	14.64±1.336		13.65±3.019	14.33±2.537	
Twist1			0.459*			0.772*
No.	28	14		26	30	
Mean±SD	1.07±2.089	0.36±1.336		1.54±3.397	1.17±2.842	
Tumor diameter	28 (100.0%)	14 (100.0%)	0.657†	26 (100.0%)	30 (100.0%)	0.603†
≤3.0cm	12 (42.9%)	5 (35.7%)		7 (26.9%)	10 (33.3%)	
>3.0cm	16 (57.1%)	11 (64.3%)		19 (73.1%)	20 (66.7%)	
Grade	27 (100.0%)	14 (100.0%)	0.221†	26 (100.0%)	30 (100.0%)	1.000‡
1	2 (7.4 %)	0 (0.0 %)	(A vs.B)	18 (69.2%)	23 (76.7%)	(A vs.B)
2A	19 (70.4 %)	8 (57.1 %)		5 (19.2%)	3 (16.7%)	
3B	6 (22.2 %)	6 (42.9%)		3 (11.5%)	2 (6.7%)	
TNM-Stage	28 (100.0%)	14 (100.0%)	0.081†	26 (100.0%)	30 (100.0%)	0.743†
I	16 (57.1%)	4 (28.6%)		11 (42.3%)	14 (46.7%)	
II-IV	12 (42.9%)	10 (71.4%)		15 (57.7%)	16 (53.3%)	
TNM-Stage	28 (100.0%)	14 (100.0%)	0.261†	26 (100.0%)	30 (100.0%)	0.560†
I-II	19 (67.9%)	7 (50.0%)		20 (76.9%)	21 (70.0%)	
III-IV	9 (32.1%)	7 (50.0%)		6 (23.1%)	9 (30.0%)	

*, Mann Whitney U-test; †, Pearson's chi-square test; ‡, Fisher exact test; No., total number of cases investigated; SD, standard deviation.; AD, adenocarcinoma; SCC, squamous cell carcinoma; CM, cytoplasmic/membranous expression; Nu, Nuclear expression

61.05 y in AD cases and 63.07 y in SCC cases (Table 1). The ratio of males to females was 26:17 (60.5%:39.5%) among AD cases and 56:3 (94.9%:5.1%) among SCC cases (P<0.001). EGFR expression was higher in SCC (54.4%) than in AD (33.3%) (P=0.038). Vimentin expression was significantly higher in AD than in SCC (P=0.015). EGFR mutation was detected in 5 of 20 total cases, and specifically in 4 of 8 AD cases and 1 of 12 SCC cases (P=0.034, likelihood-ratio test; P=0.109, Fisher exact test) (Tables 1 and 2).

Correlation of vimentin expression with clinicopathological parameters

Among AD samples, elevated vimentin expression was associated with higher histologic grade (2 vs. 3; P=0.021) and showed a tendency toward a positive correlation with more advanced pathologic tumor, node and metastasis (TNM) stage (I vs. II–IV; P=0.052) (Table 3). Vimentin and slug expression were positively correlated among 42 AD samples (P=0.018), whereas vimentin and twist1 expression were positively correlated among 58 SCC samples (P<0.001).

Correlation of EGFR copy number status with clinicopathological parameters

EGFR SISH positivity was associated with immunohistochemically assessed EGFR protein expression in AD samples (P=0.008) and SCC samples (P=0.042) (Table 4). No significant correlation was found between EGFR SISH positivity and any other clinicopathological parameters examined.

Correlation of EGFR protein expression and EGFR mutation with clinicopathological parameters

Among AD samples, EGFR protein expression was associated with higher cytoplasmic/membranous expression of ß-catenin (P=0.031) (Table 5). Among SCC samples, EGFR protein expression was negatively correlated with nuclear expression of ß-catenin (P=0.033) and positively correlated with slug expression (P=0.021).

Table 6. Review of published reports examining EMT in NSCLC.

Reference	Genes/proteins	Histology	Results associated with EMT
Thomson et al (2005)	erlotinib	NSCLC lines undergone EMT	↓ erlotinib effect
Shin et al (2005)	slug	AD lines	↑Xenograft tumor growth, angiogenesis, invasive ability, MMP-2 activity ↓E-cadherin
Yauch et al (2005)	E-cadherin Vimentin E-cadherin	Erlotinib sensitive line NSCLC Erlotinib insensitive lines NSCLC	↑E-cadherin (+) vimentin ↑ time to progression
Witta et al (2006)	E-cadherin	NSCLC lines	↑ sensitivity to gefitinib
Soltermann et al (2008)	periostin vimentin	NSCLC NSCLC	↑ stage, size, Tumor relapse, versican, vimentin ↑ grade, pT, periostin
Prudkin et al (2009)	E-cadherin integrin-αvß6 N-cadherin	SCC AD and SCC AD and SCC	↑on brain metastasis, ?? than AD ↓on brain metastasis ↓on brain metastasis
Miura et al (2009)	sip-1	NSCLC	↓E-cadherin ↑N-cadherin ↑tumor growth &poor prognosis
Hung et al (2009)	HIF-1α, twist1, snail	NSCLC	↓survival
Deng et al (2009)	EGFR mutation	NSCLC	↑E-cadherin
Yanagawa et al (2009)	snail	NSCLC	↑angiogenesis (MECA-32, CXCL8,CXCL5) ↓survival
Chang et al (2010)	slug slug	NSCLC lines AD	↑ tumor growth ↑ resistant to EGFR TKIs
Gemmill et al	ZEB1	NSCLC lines	↑vimentin ↓E-cadherin
Roy et al	LKB1	AD with LKB1 knockdown	LKB1 knockdown triggers EMT by ZEB1
Li et al	Subcellular proteomics (106 proteins)	AD line (A549), Bronchial epithelia (HBE) line	↑EMT in A 549 cells (carcinogenesis)

MMP-2, matrix metalloproteinase-2; AD, adenocarcinoma; sip-1, smad interacting protein 1; A549, human lung adenocarcinoma cell; HBE, human bronchial epithelial cell

Discussion

The EMT process has prognostic and therapeutic implications for cancer patients. Pathologic EMT in cancer is related to tumor invasion and metastasis (Gupta and Massague, 2006; Thiery, 2002). In the classic model of human cancer development, metastasis is consistent with the final step of the tumorigenic cascade. For certain cancers, however, EMT may promote malignant conversion concomitantly with metastatic dissemination, suggesting that the dissemination of primary tumor cells may occur at any time during cancer development (Sanchez-Garcia, 2009). The inactivation of EMT-related proteins might be associated with resistance to carcinogenesis, invasiveness and metastatic dissemination (Sanchez-Garcia, 2009). A recent subcellular proteomics study demonstrated that the EMT phenotype was altered in A549 human lung AD cells compared with human bronchial epithelial (HBE) cells, implicating EMT in lung carcinogenesis (Li et al., 2011). As more effective and targeted treatments for NSCLC have become available, prognoses have been improved by early diagnosis and treatment. The EMT phenotype has been frequently expressed in primary AD and SCC of the lung (Prudkin et al., 2009), and EMT might act as a determinant of EGFR activity in NSCLC tumor cells (Yauch et al., 2005; Deng et al., 2009). Following EMT, NSCLC lines became insensitive to the growth-inhibitory effects of EGFR kinase inhibition in vitro and in xenografts (Thomson et al., 2005). Witta et al. reported that transfection of a gefitinib-resistant NSCLC line with E-cadherin increased cell sensitivity to gefitinib, suggesting that E-cadherin augments EGFR activity and restores the growth-inhibitory and apoptotic effects of gefitinib (Witta et al., 2006). Hirsh et al. reported that the majority of NSCLC tumors exhibit either intermediate or high levels of EGFR protein expression, and that EGFR protein overexpression correlates with increased EGFR gene copy number (Hirsch et al., 2003).

The present study investigated samples of AD and SCC of the lung for correlations between clinico-pathologic parameters and EMT-related proteins, EGFR protein expression using PharmDx (Dako), EGFR gene copy number using SISH, and EGFR mutation. We observed significantly greater vimentin expression in AD samples compared with SCC samples (P=0.015), and in AD samples vimentin expression was associated with higher histologic grade (2 vs. 3) (P=0.021) and more advanced pathologic TNM stage (I vs. II–IV) (P=0.052). Among AD samples, a positive correlation was detected between vimentin and slug expression (P=0.018), whereas a positive correlation between twist1 and vimentin expression was observed in SCC samples ($P < 0.001$). These results suggest that vimentin overexpression contributes to AD progression, and different correlations among EMT-related markers in AD and SCC may be associated with different EMT pathways. A summary of published studies for EMT in NSCLC is shown in Table 6.

EGFR protein expression was higher in SCC samples (54.4%) than in AD samples (33.3%) (P=0.038). In agreement with previous reports, we found that EGFR protein expression was more common in SCC than in AD (Hirsch et al., 2003; Jeon et al., 2006; Lee et al., 2010). EGFR mutation was detected in 5 of 20 total samples examined, and specifically in 4 of 8 AD samples and 1 of 12 SCC samples (P=0.034, likelihood-ratio test; P=0.109, Fisher exact test). Although EGFR gene copy number and EGFR mutation frequency did not show any correlation with EMT-related proteins, EGFR SISH positivity was associated with expression of EGFR using PharmDx (Dako) in AD (P=0.008) and SCC samples (P=0.042). This result supports that EGFR protein overexpression correlates with increased EGFR gene copy number, implicating EGFR gene amplification as one mechanism for EGFR protein overexpression (Hirsch et al., 2003). A recent report demonstrated that slug expression was significantly increased in a gefitinib-resistant cell line compared with a gefitinib-sensitive cell line (Chang et al., 2011). Our data indicated that, among SCC samples, expression of slug was increased in EGFR PharmDx-positive cases compared to EGFR-PharmDx negative cases. Further studies will be necessary to elucidate the correlation between slug, EGFR protein expression and resistance to gefitinib. In our results, EGFR protein expression was significantly associated with higher cytoplasmic/membranous expression of ß-catenin among AD samples (P=0.031). SCC samples exhibited a negative correlation between nuclear expression of ß-catenin and EGFR expression (P=0.033). Thomson et al. reported that an NSCLC line exhibiting E-cadherin expression was more sensitive to EGFR inhibition than other NSCLC lines having vimentin or fibronectin expression (Thomson et al., 2005). We found that membranous expression of E-cadherin among AD samples was increased in EGFR PharmDx-positive cases but was not statistically significant (P=0.508). These results may be interpreted as a role for EMT as a determinant of EGFR activity in NSCLC tumor cells (Yauch et al., 2005; Deng et al., 2009).

Snail and slug of the Snail family of genes encode transcription factors expressed at different stages of development in different tissues (Seki et al., 2003). In addition, snail and slug are involved in the progression of human tumors via downregulation of E-cadherin expression (Sanchez-Garcia, 2009), which involves the interaction of snail and slug with the proximal E-boxes of the E-cadherin promoter (Bolos et al., 2003). Slug expression can be used to predict clinical outcomes of lung AD patients, and slug has been suggested as a novel invasion-promoting gene in lung AD (Shih et al., 2005). In our results, a positive correlation between slug and vimentin expression in AD but not in SCC (P=0.018 and 0.433, respectively) supports this idea and indicates that slug expression may contribute to EMT in AD of the lung.

The twist gene was originally identified as having important regulatory functions during embryogenesis in Drosophila (Leptin and Grunewald, 1990). In mammals, the two twist-like proteins (twist1 and twist2) share high structural homology (Li et al., 1995; Wolf et al., 1991). Twist1 is overexpressed in various human solid tumors, including numerous cancers (Yang et al., 2004), is known to trigger EMT (Oda et al., 1998, Sanchez-Garcia, 2009) and may act as a positive regulator of tumor metastasis (Sanchez-Garcia, 2009). Our finding that high vimentin expression correlates with increased twist1 expression in SCC but not in AD supports the possibility that twist1 plays a critical role in one of the EMT steps in SCC.

In conclusion, we examined the expression of the EMT-related factors vimentin, E-cadherin, ß-catenin, slug, snail and twist1, as well as the expression, gene copy number and mutation of EGFR in AD and SCC of the lung. Vimentin expression was associated with AD progression in terms of histologic grade and pathologic tumor stage. EMT-related markers, especially ß-catenin and slug, might act as determinants of EGFR protein expression. The disparate expression of EMT-related markers we observed might be interpreted as tumor-specific progression pathways of EMT in AD versus SCC of the lung. Additional research is warranted to test this possibility.

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