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ORIGINAL ARTICLE

Biologic Evaluation of Diabetes and Local Recurrence in Non-Small Cell Lung Cancer

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Abstract A recent multicenter study led by our institution demonstrated that local recurrence of non-small cell lung cancer (NSCLC) was significantly more frequent in patients with diabetes, raising the possibility of different tumor biology in diabetics. Epithelial-to-mesenchymal transition (EMT) plays a key role in local tumor recurrence and metastasis. In the present study, we investigated differences of tumor microenvironment between patients with and without diabetes by examining expression of EMT markers. Seventy-nine NSCLC patients were selected from the cohort of our early multicenter study. These patients were classified into 4 groups: 39 with adenocarcinoma with (n = 19) and without (n = 20) diabetes, and 40 with squamous cell carcinoma with (n = 20) and without (n = 20) diabetes. Immunohistochemical expression of eight EMT markers was analyzed, including transforming growth factor-beta (TGF-β), epidermal growth factor receptor (EGFR), insulin-like growth factor 1 receptor (IGF-1R), vimentin, E-cadherin, N-cadherin, HtrA1, and beta-catenin. Five markers (E-cadherin, HtrA1, TGF-β, IGF-1R and vimentin) demonstrated significantly higher expression in diabetics than in non-diabetics in both histology types. N-cadherin had higher expression in diabetics, though the difference did not reach statistical significance. EGFR showed a higher expression in diabetics in squamous cell carcinoma only. Beta-catenin was the only marker with no difference in expression between diabetics versus non-diabetics. Our findings suggest that diabetes is associated with enhanced EMT in NSCLC, which may contribute to growth and invasiveness of NSCLC.

Keywords Non-small cell lung cancer · Epithelial-to-mesenchymal transition · Diabetes

Introduction

Early-stage non-small cell lung cancers (NSCLCs) are primarily treated with surgery. Local tumor recurrence following surgical resection has a major impact on long-term patient survival [1]. More than half of all post-surgical deaths are attributed to tumor recurrence [2]. Therefore, better understanding of the role of potential biological factors involved in local recurrence would help identify specific group of patients who may benefit from more aggressive therapy. So far, well-defined biological factors and pathophysiologic mechanisms associated with local recurrence have not been precisely characterized.

A recent multicenter study led by our institution demonstrated that diabetes mellitus is an independent risk factor for local recurrence in patients with NSCLC undergoing surgical resection [3]. The finding was further confirmed in a separate cohort of patients [4]. The observation raises intriguing questions about whether diabetic status facilitates tumor progression and or recurrence in NSCLC and if so, by what mechanisms. Epithelial-to-mesenchymal transition (EMT) plays a key role in physiological processes of embryogenesis, tissue repair, and in tumor dissemination [5, 6]. In cancer, EMT is considered to be a critical process enabling invasion and dissemination. EMT has also been shown to play an important

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role in pathogenesis of diabetes complications such as diabetic nephropathy [7]. Given the central role of EMT in the biology of both cancer and diabetes, we hypothesized that the tumor environment in diabetes may favor EMT and contribute to cancer recurrence.

Taking advantage of a well-characterized cohort of NSCLC patients [3], we studied the difference of expression in EMT related factors in tumors of diabetics and non-diabetics. We evaluated growth factors with known role in both diabetes and lung cancer, including transforming growth factor beta (TGF-β), epidermal growth factor receptor (EGFR), and insulin-like growth factor 1 receptor (IGF-1R). In addition, we examined several well-established EMT markers, including glycogen adhesion protein E-cadherin, beta-catenin, mesenchymal marker neuronal cadherin (N-cadherin), and a protein marker vimentin. Moreover, we studied expression of the tumor suppressor HtrA1 which has recently been identified as a major regulator of EMT [8, 9].

Materials and Methods

Case Selection

Following approval by the local institutional review board, 79 cases of surgically resected primary NSCLC samples from patients with and without diabetes were selected from a well-characterized cohort of our early multicenter study [3]. There were a total of 39 cases of cancers in diabetics including 19 adenocarcinomas and 20 squamous cell carcinomas. We then selected 20 consecutive cases each of adenocarcinomas and squamous cell carcinomas among non-diabetics. We thus had 4 groups for analysis: 19 adenocarcinomas in diabetics, 20 adenocarcinomas in non-diabetes, 20 squamous cell carcinomas in diabetics, and 20 squamous cell carcinomas in non- diabetics. Due to pathogenetic and clinical difference between type 1 and 2 diabetes, we only included type 2 diabetes in this study to minimize potential bias in the results. Clinicopathological information for these patients is shown in Table 1.

Table 1 Demographic and pathologic staging data by histologic tumor type and diabetic status

Characteristics	Adenocarcinoma		Squamous cell carcinoma	
	Non-diabetics	Diabetics	Non-diabetics	Diabetics
Sex				
Female	16	9	5	6
Male	3	11	15	14
Age (yr, mean ± SD)	69.1 ± 10.1	66.7 ± 7.4	69.1 ± 8.1	67.2 ± 7.8
Pathologic staging				
Tumor size				
PT1a	8	7	7	3
PT1b	0	2	4	4
PT2a	8	8	4	9
PT2b	2	1	3	2
PT3	1	2	2	2
Lymph node				
PN0	16	19	15	18
PN1	2	0	4	0
PN2	1	1	0	1
NA	0	0	1	1

Immunohistochemical Staining and Assessment

Formalin-fixed, paraffin-embedded samples were sectioned (5 μm thick) and heated for 1 h at 60 °C. Immunohistochemistry was performed on a Ventana Discovery XT automated staining platform (Ventana Medical Systems, Tuscon, AZ). Antibodies for TGF-β (ab66043) and HtrA1 (ab38611) were purchased from Abcam (Cambridge, MA). Antibodies for EGFR (790–2988), IGF-1R (7,904,346), vimentin (790–2917), E-cadherin (790–4497) and beta-catenin (7,604,242) were obtained from Ventana (Tucson, AZ). N-cadherin (M3613) was purchased from Dako Cytomation (Carpinteria, CA). Immunohistochemical staining was visualized using the OmniMAP DAB anti-mouse or anti-rabbit detection kits appropriate to each antibody, according to manufacturer's recommendations (Ventana Medical Systems, Tuscon, AZ). Finally, sections were dehydrated, cleared and coverslipped prior to viewing.

The sections were evaluated on an Olympus BX53 light microscope at 100× magnification, and representative images were captured by an Olympus DP25 camera (Olympus America) and Olympus CellSens Dimension software version 1.11. Two anatomical pathologists (one of whom with fellowship training in pulmonary pathology and more than 5 years experience in this filed) performed scoring of the immunohistochemical studies independently, in a blinded fashion. Discrepancy in data interpretation was discussed until consensus agreement was reached between the two pathologists. For each marker, we used a control with diffuse and strong positive staining (designated as "3+" staining intensity). Based on comparison with the positive control, staining intensity of each case was graded as follows: "0" no staining, "1+" weak staining, "2+" intermediate staining, and "3+" strong staining. Next, the ratio of total number of immunoreactive cells for each staining intensity to the total tumor cells was estimated. The average percentage of positive cells was calculated and the following formula was applied to produce a semi-quantitative H-score = (% of cells stained at intensity category 1 × 1) + (% of cells stained at intensity category 2 × 2) + (% of

cells stained at intensity category 3 × 3). This yielded an H-score ranging from "0" to "300" for each case where 300 was equal to 100 % of the cells stained strongly (3+).

Statistical Analysis

Data were summarized using standard descriptive statistics and frequency tabulation. Associations between categorical variables were assessed via cross-tabulation, the chi-square test. Wilcoxon rank-sum test was performed to determine the difference in continuous variables by expression scores in different groups of patients based on diabetic status and histologic types. All computations were carried out using standard statistical software (SPSS V.20.0; SPSS, Chicago, Illinois, USA). *P* values less than 0.05 were considered statistically significant.

Results

The immunohistochemical expressions (H-scores) for the eight examined EMT markers are summarized in Table 2. Five of the eight markers (i.e., E-cadherin, HtrA1, vimentin, TGF-β, and IGF-1R) demonstrated significantly different EMT phenotypes between diabetic samples and non-diabetic samples for both adenocarcinoma and squamous cell carcinoma. In both histologic groups, diabetic patients had significantly reduced level of E-cadherin and HtrA 1 expression, as compared to non-diabetic patients. TGF-β, IGF-1R, and vimentin demonstrated significantly higher level of expression in diabetic samples than in non-diabetic samples. EGFR expression was significantly higher in diabetic samples than in non-diabetic samples in squamous cell carcinoma, but not in adenocarcinoma.

Although N-cadherin expression was higher in diabetics, its expression was minimal to weak in most cases, and differences were not significant. Beta-catenin expression was relatively robust, but similar in all four groups.

Discussion

While lung cancer recurrence has been well documented in the literature, the underlying physiopathologic mechanism has not

Table 2 Comparison of immunohistochemical expression (H-scores) of the epithelial-to-mesenchymal transition markers in non-diabetics versus diabetics in NSCLC specimens

		Adenocarcinoma			Squamous cell carcinoma		
		Non-diabetics	Diabetics	*P* value	Non-diabetics	Diabetics	*P* value
Beta-catenin	Mean (SD) Median	273.6 (33.2) 290	266.2 (26.7) 267.5	0.207	265.2 (36.2) 280	263 (27.6) 265	0.398
	(Range)	(195–300)	(230–300)		(170–300)	(190–300)	
E-cadherin	Mean (SD) Median	282.3 (24.4) 295	236.5 (28.7) 240	**<0.0001**	273.2 (27.5) 287	239.7 (36.5) 240	**0.001**
	(Range)	(225–300)	(200–290)		(230–300)	(175–290)	
EGFR	Mean (SD) Median	90.5 (55.0) 95	77.7 (78.5) 37.5	0.422	133 (61.6) 150	201 (89.5) 230	**0.003**
	(Range)	(0–195)	(0–210)		(0–200)	(10–290)	
HtrA1	Mean (SD) Median	182.1 (61.4) 180	69.7 (66.3) 40	**<0.0001**	79.2 (72.4) 50	12.2 (19.4) 5	**<0.0001**
	(Range)	(80–290)	(0–220)		(0–250)	(0–80)	
IGF-1R	Mean (SD) Median	140.5 (83.6) 160	242 (45.3) 250	**0.001**	173.5 (72.8) 170	275 (47.3) 300	**<0.001**
	(Range)	(20–300)	(160–300)		(40–300)	(120–300)	
N-cadherin	Mean (SD) Median	4.7 (16.4) 0	28.2 (59.3) 0	0.060	4 (11.8) 0	29 (60.3) 0	0.097
	(Range)	(0–70)	(0–180)		(0–50)	(0–220)	
TGFB	Mean (SD) Median	180 (62.1) 180	212 (57.6) 230	**0.047**	151 (58.9) 140	211 (60.1) 210	**0.002**
	(Range)	(50–270)	(80–280)		(60–260)	(50–290)	
Vimentin	Mean (SD) Median	3.1 (9.4) 0	39.2 (72.9) 0	**0.012**	2.5 (5.5) 0	61.8 (95.4) 17.5	**0.0005**
	(Range)	(0–40)	(0–300)		(0–20)	(0–300)	

P values less than 0.05 are in bold

been precisely characterized [10]. Local recurrence may occur even following complete surgical resection, suggesting persistent exposure to independent etiologic risk factors [2]. The TNM staging is commonly used as a predictor factor for risk of post-surgery recurrence [11]; however, wide variation in incidence of recurrence occurs in patients with similar TNM stage. Therefore, the current TNM staging system, which is based on clinical and pathological findings, has limits of its usefulness [12]. Other possible biomarkers including circulating tumor cells and proteins, RNA, and circulating tumor DNA (ctDNA) [13], have been studied and hold promise as early predictors of disease recurrence. However, at this stage their use is compromised by limited sensitivity, specificity, and relatively high costs [13].

The association of diabetes with and its effect on human cancer have been extensively investigated. Microangiopathy and metabolic modification are common complications of diabetes and may play a role in tumorigenesis. Studies have shown angiogenesis is an early- to mid-stage event in many human cancers and a crucial step for the transition of a small avascular tumor into a large vascularized cancerous mass [14], implying the potential connection of diabetes with tumorigenesis. In addition, chronic hyperinsulinemia may contribute to cancer initiation and/or progression in diabetic patients due to the mitogenic effect of insulin [15].

In our study, tumors of diabetics demonstrated a significantly higher expression of IGF-1R and TGF-β. Growth factors play key roles in development of both diabetes and lung cancer. Mice receiving injected human lung adenocarcinoma cells had elevated and specific expression of IGF-1R in hypoxic tumor areas [16]. TGF-β is dysregulated in diabetes and modulates cell to cell communication in early EMT [17]. Chronic TGF-β overexpression may exert an altered cell-matrix interaction in diabetes, implying its role in both diabetes and carcinogenesis [18]. TGF-β is also a major mediator of EMT [19, 20], a process that is important in both diabetes and cancer [21]. Our results showed EGFR expression was significantly higher only in squamous cell carcinomas in diabetics. This, at first, seems curious since EGFR pathways are prognostically and therapeutically important in adenocarcinomas, with inhibition of EGFR tyrosine kinase being now a standard of care in selected patients. However, EGFR protein expression has been an important target in the treatment of squamous cell carcinomas of various sites, using the monoclonal antibody Cetuximab. Higher EGFR expression in squamous carcinoma of diabetics raises the question of whether Cetuximab could be routinely used in these patients, a subject requiring further investigation.

HtrA1 is a heat shock-induced envelop-associated serine protease [22]. Over-expression of HtrA1 in human cancer cells inhibits cell growth and proliferation in vitro and in vivo [8, 9]. The lower expression of HtrA1, along with significant reduction in E-cadherin expression in diabetics, suggests that the microenvironment in diabetes facilitates loss of tumor cell cohesion and therefore migration. Interestingly, a study on breast cancer demonstrated that reduction of HtrA1 levels resulted in EMT with acquisition of mesenchymal phenotypic characteristics, including increased growth rate, migration, and invasion, as well as expression of mesenchymal biomarkers [23]. Mounting evidence suggested multiple reciprocal interactions of E-cadherin and beta-catenin with EMT-inducing transcriptional repressors to stabilize an invasive mesenchymal phenotype of epithelial tumor cells [24]. In this study, we did not observe difference in beta-catenin expression or nuclear localization between diabetics and non-diabetics, which seems unexpected as E-cadherin expression level was reduced significantly in diabetics. One possible explanation is that interaction between E-cadherin and β-catenin is complex and loss of E-cadherin is not necessarily linked to nuclear localization of β-catenin and transcriptional activation of responsive target genes.

In summary, we found significantly elevated expression of the EMT phenotype for EMT markers in surgically-resected NSCLC specimens in diabetic versus nondiabetic patients. Our findings suggest that the tumor microenvironment in diabetic patients, in concert with an exaggerated EMT, conspire to increase the growth of NSCLC.

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