RESEARCH ARTICLE

Expression Characteristics of Proteins of the Insulin-like Growth Factor Axis in Non-small Cell Lung Cancer Patients with Preexisting Type 2 Diabetes Mellitus

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Abstract

Background: Preexisting type 2 diabetes mellitus (T2DM) affects the prognosis and mortality of patients with some cancers. Insulin like growth factor (IGF) and insulin receptor (IR) signaling axes play important roles in both cancer and diabetes development. We aimed to explore the expression characteristics of proteins in IGF/IR axis in non-small cell lung cancer (NSCLC) cases with preexisting T2DM. Methods: Fifty-five NSCLC patients with preexisting T2DM were retrospectively included and matched by 55 NSCLC without diabetes at a 1:1 ratio. The expression of proteins in IGF/IR axis was detected by immunohistochemical staining. Clinicopathological data were collected to analyze their relationship with the protein expression. Results: Both IGF 1 receptor (IGF-1R) and insulin receptor substrate 2 (IRS-2) showed higher expression in the NSCLC with T2DM group, compared with those without T2DM. The high expression of IGF-1R and IRS-2 were found to be negatively associated with lymph node metastases and T staging in the T2DM group, respectively, and IRS-2 expression was also found more in the subgroup whose T2DM duration was more than 4 years. No difference was detected in the expression of IRS-1, IGF-1, IGF-2, IGFBP3, IR and mTOR between groups with or without T2DM. Conclusion: Our study found higher expression of IGF-1R and IRS-2 proteins in NSCLC patients with preexisting T2DM, and that there was an association with early stage NSCLC, which suggested that IGF signaling may play an important early event in development of NSCLC associated with diabetes.

Keywords: Non-small cell lung cancer - diabetes mellitus - insulin-like growth factor - insulin receptor - early event

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Introduction

Both cancer and diabetes mellitus are multi-mechanism and systemic diseases which affect human health in the world. Accumulating epidemiologic evidence suggests that type 2 diabetes mellitus (T2DM) and/or certain diabetes treatments may be associated with prevalence, progression, or prognosis of some malignancies, but the risk may vary according to diabetes therapy and cancer site (Barone et al., 2008; Currie et al., 2012; Wang et al., 2013). For example, the cohorts of patients with T2DM were reported to have increased prevalence in several types of malignancies, such as liver, colorectal, pancreas, endometrial and breast cancers, etc (He et al., 2010; Wang et al., 2012; Elena et al., 2013; Zhang et al., 2013), and the all-cause mortality in patients with preexisting T2DM (preDM) was also increased in those with breast, prostate, pancreas and colorectal cancer, etc (Arif, et al., 2011; Currie et al., 2012; Hwang et al., 2013; Walker et al., 2013). Non-small-cell lung cancer (NSCLC) is the leading cause of cancer-related deaths worldwide (Siegel et al., 2013). Although no certainly increased, even decreased risks were observed in prevalence or mortality of NSCLC with preDM (Atchison et al., 2011; Kurishima et al., 2012; Lee et al., 2013), diabetes was confirmed to be an independent predictor of the risk of local recurrence following resection of NSCLC (Varlotto et al., 2012), and was reported to be associated with a lower risk of metastasis in people with NSCLC (Hanbali et al., 2007). Furthermore, our prior study showed that antidiabetic drug metformin may affect chemotherapy outcomes and survival for patients who have NSCLC with diabetes (Tan et al., 2011). Although the underlying mechanism of interaction of T2DM and NSCLC remains unclear, accumulating data showed that the preexisting diabetes actually affected the development of NSCLC as mentioned above. Therefore, we speculate that there may be distinctive pathogenesis of neoplasms in patients with preDM which is different from those without T2DM.

The insulin-like growth factor (IGF) system and

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insulin signaling pathway simultaneously play an important part in hyperinsulinemia, insulin resistance and tumor pathogenesis, which is more likely to be the potential mechanism. In this study, we performed an immunohistochemical analysis of molecules in IGF and insulin receptor (IR) axes in NSCLC with or without preDM, in order to investigate the potential molecules which play roles in mechanism that diabetes affects NSCLC.

Materials and Methods

Patients and tissue sample

Data on patients with NSCLC who admitted in West China Hospital of Sichuan University during December 2008 to March 2012 were retrospectively searched. Inclusion criteria were: histologic or cytologic confirmation of NSCLC; patients who had undergone radical surgical removal of primary lesions; newly diagnosed NSCLC without radiotherapy or chemotherapy before surgery; pre-existing T2DM before the diagnosis of NSCLC. Patients were excluded for the following reasons: insufficient clinical or pathological data; concurrent other malignancies or NSCLC mixed with small cell lung cancer.

For comparison with NSCLC with preDM, we matched NSCLC without the diagnosis of diabetes from the database at a 1:1 ratio on gender, age (± 3 years), pathological type and the stage of cancers. All matched NSCLC without diabetes should be verified for the same inclusion and exclusion criteria mentioned above except for the diagnosis of T2DM, and the matched NSCLC patients should have their morning fasting blood glucose lower than 5.9 mmol/L. Tumor tissues were acquired from formalin fixed pathological samples taken from the resected NSCLC specimens. Clinicopathological characteristics of patients with preDM and paired patients were collected. The study was approved by the Biomedical Ethics Committee of West China Hospital, Sichuan University, China.

Immunohistochemical staining

The tissue samples of the tumor were cut into sections of 4 μm which were mounted on silanized slides. The sections were deparaffinized in a xylene bath, rehydrated using a graded alcohol series, and retrieved in pH 6.0 sodium citrate buffer via microwave heating for 7 min at 95°C. Subsequently, the sections were incubated with the primary antibody at 4°C overnight. All primary antibodies were purchased from Abcam, Hong Kong, China and belonged to rabbit polyclonal: IGF 1 receptor (IGF-1R, clone ab131476) at 1:200; IGF-1 (clone ab9572), IGF-2 (clone ab9574), insulin receptor substrate 1 (IRS-1, clone ab52167) and mTOR (clone ab2732) at 1:100; IR (clone ab5500) and IRS-2 (clone ab46811) at 1:50; and IGFBP3 (clone ab76001) at 1:40, respectively. The sections were then incubated by PV6001 Two-Step immunohistochemistry Detection Reagent (ZSJQ-BIO, Bei Jing, China) for one hour at 37°C. 3, 3'-diaminobenzidine was used as a chromogen, and commercial hematoxylin as a counterstaining agent.

Evaluation of Immunohistochemical staining results

Expression of all antigens was examined by an investigator who was blinded to clinical data of the patients. Each sample was examined in a high power field at 200 times magnification. The evaluation of staining was referred to previous report with some modifications (Herberger et al., 2007), as following: Immunostaining was classified based on staining intensity and percentage of positive tumor cells. Staining intensity was determined as 0 (absent), 1 (weak), 2 (moderate), 3 (strong). For comparison with the positive degree of antigen expression between two groups, expression levels of the antigens were semi-quantified using an immunohistochemistry score (range, 0-300) calculated by multiplying staining intensity with the percentage of positive tumor cells. The immunoreactivity was classified: 0, score ≤ 60; 1+, 60 < score ≤ 140; 2+, 140 < score ≤ 220; 3+, 220 < score ≤ 300. Patients with an immunohistochemistry score of ≤ 140 were considered as negative to weak immunoreactivity and those with a score of > 140 as moderate to strong immunoreactivity, which will be used for the subsequent multivariate analysis.

Statistical analysis

Chi-square test was used for comparison between clinicopathological characteristics of two groups. Wilcoxon's rank sum test was used to compare differences of antigens expression between two groups. For multivariate analysis, binary logistic regression analysis was used to determine whether the clinicopathological variables, including gender, age, smoke history, hypertension history, T staging, lymph node metastasis, tumor differentiation, blood gluscose, diabetes duration, were jointly associated with expression of the antigens. A p-value of < 0.05 was considered statistically significant. Statistical analysis was performed using SPSS 17.0.

Results

Clincopathological characteristics

Totally, the pathological reports and clinical data of 1371 lung cancer during December 2008 to March 2012 in our hospital were reviewed, 78 newly diagnosed, resectable NSCLC patients with pre-existing T2DM met the inclusion criteria, while 23 patients were excluded for the data deficiency. Finally, 55 cases with T2DM were included. The clincopathological characteristics of the included and paired patients are shown in Table 1. The gender, age, and pathological type were paired in all patients between two groups, and the stages were matched at 81.8%, those who didn't match the same stages were paired with the nearest stages of cancers.

Expression characteristics of the proteins in the IGF/IR system in NSCLC

The expressions of IGF-1 and IGF-2 were observed in 80.0% and 91.8% of samples and mainly located in the cytoplasm of malignant cells in NSCLC tissues. IGFBP3 and mTOR expression were found in 53.6% and 94.5% of samples mostly in the cytoplasm and a small part of mTOR expression in the nucleus. Most of the NSCLC

Table 1. Clinicopathological Characteristics of the NSLCC with DM and Paired Patients Without DM (n = 55)

	With DM	Without DM	*P*-value
Male: Female	32:23 (55)	32:23 (55)	1.000
Median age, years (range)	63.6 (47-81)	63.4 (47-81)	1.000
Smoking status			0.849
Never smoker	28 (50.9%)	29 (52.7%)	
Former/current smoker	27 (49.1%)	26 (47.3%)	
Pathological type			1.000
Adenocarcinoma	32 (58.2%)	32 (58.2%)	
Squamous carcinoma	19 (34.5%)	19 (34.5%)	
Adenosquamous carcinoma	4 (7.3%)	4 (7.3%)	
Tumor differentiation			0.823
Well	5 (9.1%)	4 (7.3%)	
Moderate	20 (36.4%)	23 (41.8%)	
Poor	30 (54.5%)	28 (50.9%)	
T staging			0.496
T1	6 (10.9%)	2 (3.6%)	
T2	38 (69.1%)	42 (76.4%)	
T3	5 (9.1%)	4 (7.3%)	
T4	6 (10.9%)	7 (12.7%)	
Lymph node metastasis			0.295
N0	32 (58.2%)	33 (60.0%)	
N1	9 (16.4%)	4 (7.3%)	
N2	14 (25.4%)	18 (32.7%)	
Distant metastasis			0.495
M0	53 (96.4%)	55 (100.0%)	
M1	2 (3.6%)	0 (0.0%)	
Stage			0.120
I	18 (32.7%)	21 (38.2%)	
II	17 (31.0%)	9 (16.4%)	
III	18 (32.7%)	25 (45.5%)	
IV	2 (3.6%)	0 (0.0%)	

tissues were found to overexpress IGF1R and IR at 87.3% and 79.1% in the cell membrane and partly in the cytoplasm. IRS-1 and IRS-2 expressed prominently in the cytoplasm, and a few of them was still observed in the cell membrane and nucleus of malignant cells with positive rates of 80.0% and 90.9%. The representative pictures of immunohistochemistry of these molecules were shown in Figure 1.

Expression differences of the proteins in the IGF/IR system between groups with or without T2DM

IGF-1R staining in the group with preDM was observed to be obviously stronger than that in the group without diabetes (mean rank 59.25 vs. 51.75, P = 0.004), IRS-2 staining was also stronger in the preDM group than group without diabetes (mean rank 61.03 vs. 49.97, *P* = 0.048). IRS-1 was expressed with a stronger tendency

Table 2. The Expression Differences of Proteins in IGF/IR Signaling Pathway Between NSCLC with and Without Diabetes

	DM	Negative	Positive			Mean rank	*P*-value
		0-	1+	2+	3+		
IGF-1	+	8	16	27	4	57.42	0.502
	-	14	13	22	6	53.58	
IGF-2	+	4	20	28	3	54.46	0.714
	-	5	20	21	9	56.54	
IGFBP3	+	27	20	7	1	52.50	0.287
	-	24	16	14	1	58.50	
IGF-1R	+	7	8	28	12	59.25	0.004
	-	9	20	23	3	51.75	
IR	+	11	11	25	8	59.25	0.196
	-	12	19	18	6	51.75	
IRS-1	+	8	20	23	4	59.69	0.063
	-	14	21	16	2	49.11	
IRS-2	+	4	14	29	8	61.03	0.048
	-	6	21	25	3	49.97	
mTOR	+	3	15	26	11	58.71	0.256
	-	3	20	25	7	52.29	

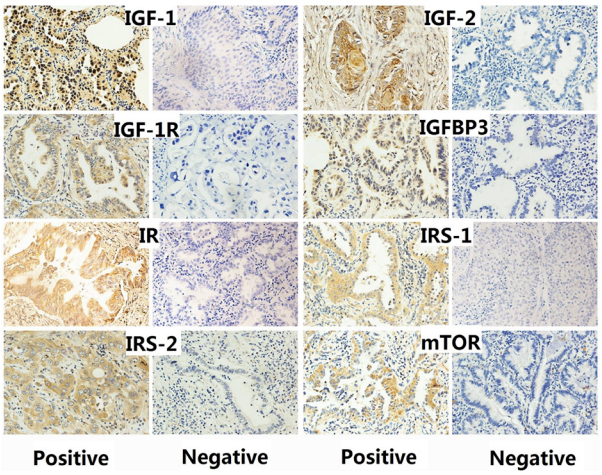

Figure 1. The Representative Figures of Expression of the Proteins in NSCLC (× 200)

in the group with T2DM, but the difference didn't reach statistical significance (*P* = 0.063). No statistically significant differences were found in the expression of IGF-1, IGF-2, IGFBP3, IR and mTOR between NSCLC with or without preDM, as shown in Table 2.

The Correlation of clinicopathological factors with the expression of IGF-1R in NSCLC with preDM

The T staging, tumor differentiation, lymph node metastasis, blood glucose, and diabetes duration were included as main covariances for multivariate analysis

Table 3. Correlation of the IGF-1R Expression[#] and Clinicopathological Factors in NSCLC with DM

	DM group				Non-DM group			
	N	Odds ratio	95%CI	*P*-Value	N	Odds ratio	95%CI	*P*-Value
T staging (T3-4 vs.T1-2)	11 vs.44	0.24	0.03-1.93	0.179	11 vs.44	1.77	0.39-8.02	0.460
Tumor differentiation (Moderate/well vs.poor)	25 vs.30	4.06	0.73-22.72	0.111	27 vs.28	1.87	0.45-7.68	0.388
Lymph node metastasis (Positive vs. negative)	23 vs.32	0.08	0.01-0.55	0.009	22 vs.33	1.33	0.33-5.34	0.685
Bloodglucose (mmol/L) (>5.9 vs.≤5.9)	33 vs.22	1.68	0.30-9.42	0.551				
DM duration (≥4y vs.<4y)	29 vs.26	0.45	0.07-2.99	0.410				

[#]moderate to strong expression vs. negative to weak expression

Table 4. Correlation of the IRS-2 Expression[#] and Clinicopathological Factors in NSCLC with DM

	DM group				Non-DM group			
	N	Odds ratio	95%CI	*P*-Value	N	Odds ratio	95%CI	*P*-Value
T staging (T3-4 vs.T1-2)	11 vs.44	0.13	0.02-0.69	0.016	11 vs.44	3.47	0.69-17.46	0.131
Tumor differentiation (Moderate/well vs.poor)	25 vs.30	0.91	0.18-4.65	0.831	27 vs.28	0.65	0.15-2.81	0.563
Lymph node metastasis (Positive vs. negative)	23 vs.32	0.69	0.13-3.79	0.673	22 vs.33	0.32	0.09-1.13	0.077
Bloodglucose (mmol/L) (>5.9 vs.≤5.9)	33 vs.22	0.63	0.11-3.45	0.590				
DM duration (≥4y vs.<4y)	29 vs.26	4.45	1.07-18.49	0.040				

[#]moderate to strong expression vs. negative to weak expression

to identify the potential association factors with IGF-1R or IRS-2 expression in preDM group. Lymph node metastasis was negatively associated with strong IGF-1R expression. No relationship was found between the other clinicopathological factors and IGF-1R expression in the group with preDM. Otherwise, none of clinicopathological factors were found to be related with IGF-1R expression in the group without preDM (Table 3).

The Correlation of clinicopathological factors with the expression of IRS-2 in NSCLC with preDM

IRS-2 expression was significantly associated with diabetes duration in the NSCLC with preDM, and was higher when diabetes duration was over four years. Interestingly, T staging (T3-4 vs. T1-2) was negatively associated with IRS-2 expression. Other factors like lymph node metastasis, tumor differentiation and blood glucose level was not found to be significantly associated with IRS-2 expression. Correspondingly, no association between the clinicopathological factors and IRS-2 expression was found in the group without preDM (Table 4).

Discussion

In our study, we found that the proteins in IGF-1R/IR signaling pathway were highly expressed in all NSCLC tissues, except for IGFBP3 with a relatively negative expression, which is constant with previous reports and suggests that IGF-1R/IR signaling plays an important role in NSCLC development (Pavelic et al., 2005; Fidler et al., 2012). Of those, two of the proteins, namely IGF-1R and IRS-2, were found differences in their expression between NSCLC groups with or without preDM. The higher expression of IGF-1R and IRS-2 in preDM group suggested that there might be potential difference in mechanism of NSCLC development in patients with preDM.

The growth factors in IGF axis play a critical role in the growth and development of malignant cells and the regulation of cells growth and metabolism in normal conditions (Arcaro, 2013). The IGF-1R is commonly overexpressed in many cancers, including NSCLC, and IGF-1 and IGF-2 link to IGF-1R leading the consequent activation of intracellular signaling pathways, then affecting cancer cell proliferation, adhesion, migration and are critical for cancer cell survival and metastases (LeRoith et al., 2003). What's more, the insulin-like effects of IGF-1 via IGF-1R played a crucial part in the maintenance of normal glucose homeostasis and may contribute to the etiopathogenesis of type 2 diabetes (Rajpathak et al., 2009). In our study, higher IGF-1R expression was found in NSCLC with preDM, which is supported by previous study reported by Rosalyn Ferguson whose study found that, in conditions of hyperinsulinemia, there was the higher phosphorylation of IR/IGF-1R which contributed to the increased growth of mammary tumors (Ferguson et al., 2013). Consequently, we speculated that the strengthened IGF/IGFR signaling is one of possible mechanisms of NSCLC development in patients with preDM. In our study, we also found in NSCLC with preDM that IGF-1R expression was relatively higher in those without lymph nodes metastasis, which suggested that the strengthened IGF/IGFR signaling may be an early event and exert a certain effect in early stage of NSCLC development. Similarly, IGF-1R was reported to be lost in metastatic prostate cancer and the loss of the IGF-1R contributes to prostate cancer progression in Chott's experiment (Chott et al., 1999). And, Satyamoorthy's study in melanoma cells also found that IGF-1 inducing downstream signaling, such as MAP kinase pathway and PI3 kinase pathway, promoted malignant development at early stages while during later stage IGF-1 signaling was not required for further progression (Satyamoorthy et al., 2001). These results from other malignancies may help us explain the phenomenon that higher IGF-1R expression was found in relative early stages of NSCLC in preDM in our study.

The IRS proteins which are substrates of the IGF-IR and IR, function as docking proteins that link these receptors to various downstream signaling pathways (LeRoith et al., 2003; Shaw, 2011). IGF-1R or hybrid IGF-1R/IR signaling may play a more active role than IR signaling through IRS-2, because the unique KRLB domain of IRS-2 limited IRS-2 tyrosine phosphorylation in IR signaling but not in IGF-1R or hybrid IGF-1R/IR signaling (Wu et al., 2008). IRS-2 branch of IGF-1R signaling plays different parts from IRS-1 branch which was involved in cancer initiation and primary cancer growth, while IRS-2 mainly promoted cancer cell mobility, invasion and metastasis, as well as glycolysis (Shaw, 2011), as studied in pancreatic cancer (Kornmann et al., 1998), breast cancer (Jackson et al., 2001; Gibson et al., 2007), etc. The strengthened IGF-1R signaling may enhance the activity of IRS-2, which may support our discovery that IRS-2 expression was higher in NSCLC with preDM. In our research, we found the relatively high expression of IRS-2 in NSCLC with the lower T staging, along with our finding that IGF-1R was higher expressed in non-lymph node metastasis NSCLC, suggesting that

IGF/IGFR signaling may be an important signaling in early development of NSCLC patients with preDM. In our study, we also found IRS-2 expressed relatively higher when diabetes duration was over four years, which logically supports that long term strengthened IGF/IGFR signaling might be one of possible mechanisms of NSCLC development.

In our study, only diabetic patients who had undergone radical surgical removal of primary NSCLC lesions were included, which could represent the relatively early stage of NSCLC patients. We employed immunohistochemistry to analyze the proteins expression in IGF-1R/IR axis, which is a classical technology with a qualitative, quantitative and positioning detection of antigen or antibody expression, with high sensitivity and specificity, while it is the drawback of a semi-quantitative approach with the possibility of false positive and negative staining. Our research is just a preliminary exploration of IGF-1R/IR signaling pathway in cancer development with diabetes, meanwhile, we are carrying on series of studies in other malignancies and conducting prospective experiments for further confirmation. Hence, our research provided some important information to illuminate the mechanism of NSCLC development in patients with preexisting diabetes.

In conclusion, our study found that the higher expression of IGF-1R and IRS-2 proteins was in NSCLC patients with preDM, and was associated with early stage of NSCLC, which suggested that IGF signaling may play an important role in early development of NSCLC in patients with diabetes.

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