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Expression heterogeneity research of ITGB3 and BCL-2 in lung adenocarcinoma tissue and adenocarcinoma cell line

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ABSTRACT

Objective: To analyze expression heterogeneity of Integrin beta 3 (ITGB3) and B-cell lymphoma 2 (BCL-2) in lung adenocarcinoma tissue and adenocarcinoma cell line and further provide theoretical direction for molecular biological research of lung adenocarcinoma. **Methods:** Tissue microarray was used to observe relation among expression, heterogeneitpy and clinical characteristics of ITGB3 and BCL-2 in lung cancer. **Results:** ITGB3 and BCL-2 increased significantly in A549 cells in CAFs group with β -actin as control; the expression level of BCL-2 also increased in ITGB3 transfected cells with GFP plasmid transfected A549 cells as control; immunohistochemistry staining showed that positive rates of ITGB3, ITGB1 and BCL-2 in normal lung tissues were 0, the positive rates in lung adenocarcinoma were 7.04%, 84.51% and 4.23%, respectively; in the results of immunohistochemistry staining, the expression of Girdin protein in lung adenocarcinoma was homogeneous, however protein expression of ITGB3, ITGB1 and BCL-2 showed different patterns in the same location with significant heterogeneity; majority of ITGB3, ITGB1 or BCL-2 positive tissue showed heterogeneity that expression in trailing edge was higher than that of trailing edge in lung adenocarcinoma tissue, the patients with BCL-2 heterogeneity showed higher lymph node metastasis ratio and lower clinical stage ($P<0.05$); and the expression of ITGB3 and the clinical characteristics of patients were not significant related ($P>0.05$). **Conclusions:** Expression of ITGB3 and BCL-2 in lung adenocarcinoma and adenocarcinoma cell line showed heterogeneity that expression in trailing edge was higher than that of trailing edge, which may play an important role in promoting tumor lymph node metastasis and vascular invasion, and provides a new research direction for exploration of lung adenocarcinoma metastasis mechanism.

1. Introduction

Liver cancer is a malignant cancer that threatens human life and health with ascending trend of modality and mortality and it requires higher standard in clinical prevention and treatment of lung cancer[1,2]. Integrin beta 3 (TGB3) and B-cell lymphoma 2 (BCL-2) function in cell adhesion and apoptosis respectively. Recent research considered that ITGB3 and BCL-2 had significantly effects in occurrence and development of cancer[3,4]. To explore the exact influencing mechanism, we conducted the related research, the procedure and conclusion are reported as following.

2. Materials and methods

2.1. Experimental methods

Paraffin embedded samples from 576 lung cancer patients during January, 2005-January, 2013 in our hospital were selected, including 213 cases of adenocarcinoma, 222 cases of squamous carcinoma, 27 cases of large cell carcinoma, 45 cases of small cell carcinoma, 42 cases of carcinoid, 18 cases of bronchial adenoma, 9 cases of adenosquamous

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carcinoma, which were all diagnosed referring to Lung cancer clinical diagnostic criteria proposed by WHO in 1999[5,6]. In the same period, paraffin embedded samples of normal lung tissue from 17 normal patient were collected.

2.2. Experimental treatment

2.2.1. Production of tissue microarray

Production of tissue microarray was referred to experimental method in related references[7,8], mouse anti-human Skp2 monoclonal antibody and 0.1% citric acid buffer solution were used for 10 min microwave repair, and then primary antibody was added, DAB developing was used after being preserved at 4 ℃ for 12 h. PBS was used as negative control, and immunohistochemical labeling was used. Mouse anti-human Skp2 monoclonal antibody was purchased from OriGene Technologies, USA

2.2.2. HE staining

After dewaxing and hydration of tissue microarray, the tissues were put in xylene and immersed for 10 min, xylene was changed and immersed again for 10 min, repeated for 4 times; the processed tissue microarrays were put in absolute ethanol for 1 min and repeated for 3 times; and then put in either 95% ethanol and 75% ethanol for 5 min and washed under water flow. Hematoxylin stained for 5 min and washed in water flow for 15 min, and then stained by eosin for 5 s. At last, the tissues were dehydrated and observed under microscope after sealed.

2.3. Observational indexes

GFP-A549 cell line was established referring to related method in references[7,8], FACS was used to separate GFP marked A549, Western blot was used to detect expression difference between ITGB3 and BCL-2. ITGB3 plasmid was used to transfect A549 cells to observe the influence of ITGB3 overexpression on BCL-2 expression[9]. Positive rates of ITGB3, ITGB1 and BCL-2 expression in various types of lung cancer and normal lung tissue were observed, 213 samples of lung adenocarcinoma expression heterogeneity at leading edge and trailing edge of cancer tissue were specially analyzed. Girdin protein was used as negative control, immunohistochemical staining was applied in paraffin section of lung adenocarcinoma to observe expression of ITGB3 and BCL-2 at the same location. Relation between expression heterogeneity of lung adenocarcinoma tissue and adenocarcinoma cells and clinical indexes were analyzed[10].

2.4. Statistical analysis

All date in our study were analyzed by SPSS13.0. Enumeration data was analyzed by *Chi*-square test and measurement data was analyzed by *t* test. The test level was set as α =0.05. The difference was considered as statistically significant when $P<0.05$.

3. Results

3.1. Result of Western blot

ITGB3 and BCL-2 increased significantly in A549 cells in CAFs group with β -actin as control, as shown in Figure 1-2.

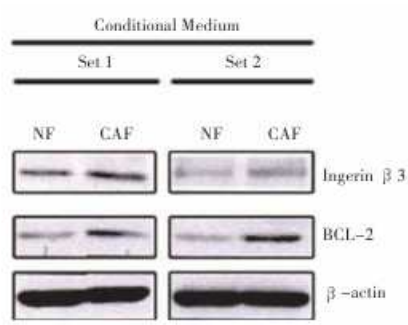

Figure 1. Expression of ITGB3 and BCL-2 in conditional medium culture.

Table 1
Positive rate of ITGB3, ITGB1 and BCL-2 expression in various lung cancers and normal lung tissue (%).

Sample type	Number of samples	ITGB3		ITGB1		BCL-2	
		Number of positive cases	Positive rate	Number of positive cases	Positive rate	Number of positive cases	Positive rate
Adenocarcinoma	213	15	7.04	180	84.51	9	4.23
Squamous carcinoma	222	93	41.89	183	82.43	51	22.97
Large cell carcinoma	27	3	11.11	21	77.78	0	0.00
Small cell carcinoma	45	33	73.33	9	20.00	3	6.67
Carcinoid	42	33	78.57	24	57.14	6	14.29
Bronchial adenoma	18	0	0.00	6	33.33	0	0.00
Adenosquamous carcinoma	9	9	100.00	9	100.00	6	66.67
Normal lung tissue	17	0	0.00	0	0.00	0	0.00

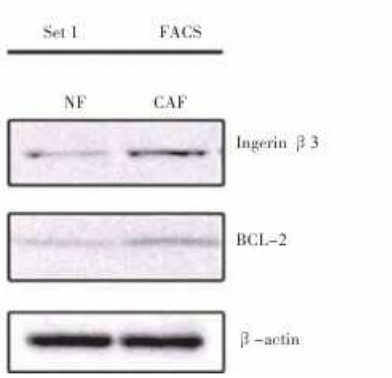

Figure 2. Expression of ITGB3 and BCL-2 in co-culture system. Note: Set 1:A549 cells cultured in NF medium/CAF medium; Set 2:A549 cells cultured in NHLF medium/LCAF medium.

3.2. Result of ITGB3 plasmid transfection

The expression level of BCL-2 was also increased in ITGB3 transfected cells with GFP plasmid transfected A549 cells as control, as shown in Figure 3.

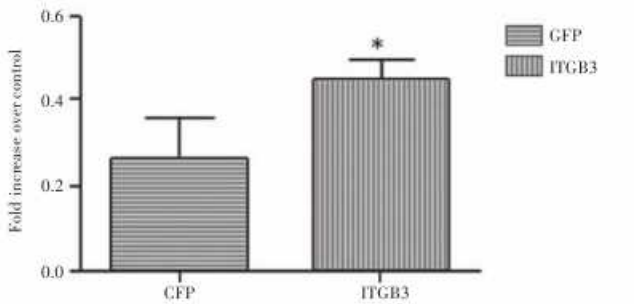

Figure 3. Influence of ITGB3 plasmid transfection on BCL-2 expression of A549 cells.

3.3. Positive rates of ITGB3 and BCL-2 in various lung cancers and normal lung tissue

Immunohistochemistry staining showed that positive rates of ITGB3, ITGB1 and BCL-2 in normal lung tissues were 0, the positive rates in lung adenocarcinoma were 7.04%, 84.51% and 4.23% respectively, as shown in Table 1.

3.4. Results of immunohistochemical staining

The expression of Girdin protein in lung adenocarcinoma was homogeneous, however protein expression of ITGB3, ITGB1 and BCL-2 showed different patterns in the same location with significant heterogeneity, as shown in Figure 4.

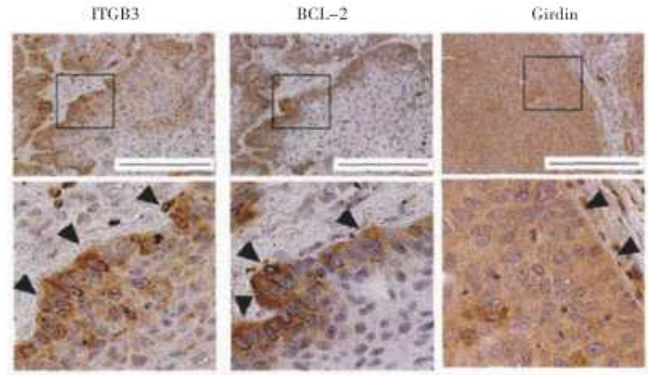

Figure 4. Results of immunohistochemical staining.

3.5. Heterogeneity analysis

Majority of ITGB3, ITGB1 or BCL-2 positive tissue showed heterogeneity that expression in trailing edge was higher than that of trailing edge in lung adenocarcinoma tissue, as shown in Table 2 and Figure 5.

Table 2
Expression heterogeneity of ITGB3, ITGB1 and BCL-2 in leading edge and trailing edge of lung adenocarcinoma tissue (*n*).

Heterogeneity	ITGB3	ITGB1	BCL-2
Leading edge<trailing edge	0	16	1
Leading edge = trailing edge	6	83	2
Leading edge>trailing edge	9	81	6
In total	15	180	9

Table 3
Relation between heterogeneity of ITGB3 and BCL-2 and clinical characteristics of patients (%).

Clinical characteristics		ITGB3(*n*=15)			BCL-2(*n*=9)		
		Leading edge≤trailing edge	Leading edge> trailing edge	*P* value	Leading edge≤trailing edge	Leading edge> trailing edge	*P* value
Age(year)	≥60	7(46.7)	2(13.3)	0.535	3(33.3)	1(11.1)	1.000
	<60	5(33.3)	1(6.7)		4(44.4)	1(11.1)	
Gender	Male	6(40.0)	1(6.7)	1	2(22.2)	0	1.000
	Female	8(53.3)	0		3(33.3)	4(44.4)	
Clinical stage	Stage Ⅰ	3(20.0)	2(13.3)	0.704	1(11.1)	1(11.1)	0.019△
	Stage Ⅱ	5(33.3)	0		3(33.3)	2(22.2)	
	Stage Ⅲ	4(26.7)	1(6.7)		1(11.1)	1(11.1)	
Clinical grade	Grade 1-2	3(20.0)	3(20.0)	0.263	3(33.3)	1(11.1)	0.588
	Grade 3-4	5(33.3)	4(26.7)		3(33.3)	2(22.2)	
Lymph node metastasis	Yes	4(26.7)	3(20.0)	0.296	3(33.3)	1(11.1)	0.039▲
	No	7(46.7)	1(6.7)		5(55.6)	0	

Note: △: χ^2=8.067, *OR*=7.138, *P*<0.05; ▲: χ^2=6.255,OR=8.209, *P*<0.05.

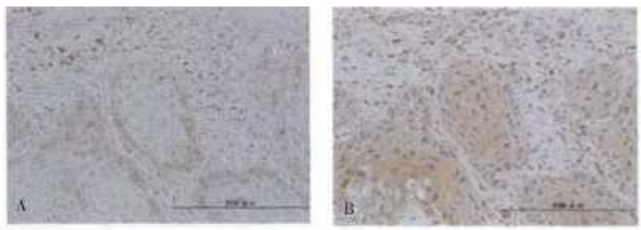

Figure 5. Expression difference between cancer cells of leading edge and trailing edge.
A: Leading edge<trailing edge; b: Leading edge<trailing edge.

3.6. Relation between heterogeneity and clinical characteristics

The patients with BCL-2 heterogeneity showed higher lymph node metastasis ratio and lower clinical stage ($P<0.05$); and the expression of ITGB3 and the clinical characteristics of patients were not significant related ($P>0.05$), as shown in Table 3.

4. Discussion

Adenocarcinoma is a histological type of lung cancer, deep research in molecular biology can possibly direct the exploration of lung cancer mechanism and affect significantly in prevention and treatment of lung cancer. Meanwhile, invasion and metastasis of cancer are considered as the reasons that shorten patients' survival time[11,12]. Thus, research of tumor microenvironment in metastasis of lung adenocarcinoma is significant. Researches reported that ITGB3 and BCL-2 could function in cell adhesion and cell apoptosis, which were highly expressed in various cancer tissues and considered as critical genes in occurrence and metastasis of cancer[13-15].

In our research were selected ITGB3 and BCL-2 as our chief objectives to explore the expression heterogeneity in lung adenocarcinoma and adenocarcinoma cells. We found that ITGB3 and BCL-2 both significantly increased in A549 cells in CAFs group with β-actin as control, indicating that CAFs may be the mediated factor in increase of ITGB3 and BCL-2[16,17], and in the result of ITGB3 plasmid transfection, we found that BCL-2 expression also increased when transfected by ITGB3 with GFP plasmid transfected A549 as control, indicating that CAFs may induce overexpression of BCL-2 by up-regulating ITGB3 expression. Although positive rates of ITGB3 and BCL-2 were low in lung adenocarcinoma tissue microarray, we still selected part of positive samples for further research. Immunohistochemical staining result showed that the expression of Girdin protein in lung adenocarcinoma is homogeneous, however protein expression of ITGB3, ITGB1 and BCL-2 showed different patterns in the same location with significant heterogeneity; majority of ITGB3, ITGB1 or BCL-2 positive tissue showed heterogeneity that expression in trailing edge was higher than that of trailing edge. The characteristics of cancer are closely related to its metastasis and reproduction, and the expression heterogeneity of leading edge cells and trailing edge cells it the important factor that affects budding, tissue type and blood vessel invasion in cancer metastasis[18-21]. It is reasonable to consider that the effects of ITGB3 and BCL-2 in occurrence and development of lung adenocarcinoma may affect heterogeneity of leading edged cells and trailing edge cells, and induce metastasis and invasion of cancer cells. The patients with BCL-2 expression heterogeneity in lung adnocarcinoma tissue had higher lymph node metastatic rate and lower clinical stage ($P<0.05$); and the expression of ITGB3 and the clinical characteristics of patients were not significant related ($P>0.05$). In research of non-small cell lung cancer research by Felicetti et al[22-26], patients with high BCL-2 expression showed poor prognosis, and in our research BCL-2 expression in leading edge cells of lung adenocarcinoma was high, indicating that BCL-2 expressed in leading edge cells of lung adenocarcinoma possibly affects significantly in metastatic invasion of cancer. Besides, many researches also found that transforming growth factor family is also significantly in promoting ITGB3 expression[27,28], the main mechanism are as following: cancer fibroblast first promote epithelium-mesenchyme neoplasis of cancer invasive edge, and fibroblast secrete a lot of transforming growth factor to cause phosphorylation of locally adhered kinase, causing the up-regulation of ITGB3 with main function of cell adhesion[29]. Thus, we consider that in the beginning of cancer occurrence, ITGB3 is activated and expression of ITGB3 is up-regulated to further induce BCL-2 overexpression, which is the significant influencing factor causing occurrence and development of lung adenocarcinoma.

In conclusion, ITGB3 and BCL-2 both showed heterogeneity that expression in trailing edge was higher than that of trailing edge, function significantly in lymph node metastasis and blood vessel metastasis, providing a new direction in exploration of lung adenocarcinoma metastasis mechanism.

Conflict of interest statement

We declare that we have no conflict of interest.

References

[1] Kapoor S. Attenuating effect of gossypol on tumor growth in systemic malignancies. *Cell Biochem Biophys* 2013: 1–2.

[2] Shih YW, Chien ST, Chen PS, Lee JH, Wu SH, Yin LT. α-mangostin suppresses phorbol 12-myristate 13-acetate-induced MMP-2/MMP-9 expressions via αvβ3 Integrin/FAK/ERK and NF-κB signaling pathway in human lung adenocarcinoma A549 Cells. *Cell Biochem Biophys* 2010; 58(1): 31–44.

[3] Singh R, Mo YY. Role of microRNAs in breast cancer. *Cancer Biol & Ther* 2013; 14(3): 0–1.

[4] Gemei M, Corbo C, D'Alessio F, Di Noto R, Vento R, Del Vecchio L. Surface proteomic analysis of differentiated versus stem-like osteosarcoma human cells. *Proteomics* 2013; 13(22): 3293–3297.

[5] Jadhav V, Hackl M, Druz A, Shridhar S, Chung CY, Heffner KM, et al. CHO microRNA engineering is growing up: Recent successes and future challenges. *Biotechnol Adv* 2013; 31(8): 1501–1513.

[6] Sigalotti L, Covre A, Fratta E, Parisi G, Colizzi F, Rizzo A, et al. Epigenetics of human cutaneous melanoma: setting the stage for new therapeutic strategies. *J Translational Med* 2010; 8(1): 1–22.

[7] Hegde S, Raghavan S. A skin-depth analysis of integrins: role of the integrin network in health and disease. *Cell Comm Adhesion* 2013: 1–15.

[8] Segura MF, Greenwald HS, Hanniford D, Osman I, Hernando E. MicroRNA and cutaneous melanoma: from discovery to prognosis and therapy. *Carcinogenesis* 2012; 33(10): 1823–1832.

[9] Gnatenko DV, Zhu W, Xu X, Samuel ET, Monaghan M, Zarrabi MH, et al. Class prediction models of thrombocytosis using genetic biomarkers. *Blood* 2010; 115(1): 7–14.

[10]Tharaux PL, Huber TB. How many ways can a podocyte die? Seminars in nephrology. *WB Saunders* 2012; 32(4): 394–404.

[11]Watson PM, Watson DK. Alternative splicing in prostate and breast cancer. *Open Cancer J* 2010; 3: 62–76.

[12]Cherfils-Vicini J, Platonova S, Gillard M, Laurans L, Validire P, Caliandro R, et al. Triggering of TLR7 and TLR8 expressed by human lung cancer cells induces cell survival and chemoresistance. *J Clin Inv* 2010; 120(4): 1285.

[13]Desgrosellier JS, Cheresh DA. Integrins in cancer: biological implications and therapeutic opportunities. *Nature Rev Cancer* 2010; 10(1): 9–22.

[14]Melaiu O, Cristaudo A, Melissari E, Di Russo M, Bonotti A, Bruno R, et al. A review of transcriptome studies combined with data mining reveals novel potential markers of malignant pleural mesothelioma. *Mutation Res/Rev Mutation Res* 2012; 750(2): 132–140.

[15]Johnson JL, Mejia EG. Flavonoid apigenin modified gene expression associated with inflammation and cancer and induced apoptosis in human pancreatic cancer cells through inhibition of GSK-3β/NF-κB signaling cascade. *Mol Nutrition & Food Res* 2013; 57(12): 2112–2127.

[16]Gupta SC, Hevia D, Patchva S, Park B, Koh W, Aggarwal BB. Upsides and downsides of reactive oxygen species for cancer: the roles of reactive oxygen species in tumorigenesis, prevention, and therapy. *Antioxidants & Redox Signaling* 2012; 16(11): 1295–1322.

[17]Neelakandan K, Babu P, Nair S. Emerging roles for modulation of microRNA signatures in cancer chemoprevention. *Curr Cancer Drug Targets* 2012; 12(6): 716–740.

[18]Llorente Izquierdo C, Mayoral R, Flores JM, Cucarella C, Gran C, Alvarez MS, et al. Transgenic mice expressing cyclooxygenase-2 in hepatocytes reveal a minor contribution of this enzyme to chemical hepatocarcinogenesis. *Am J Pathol* 2011; 178(3): 1361–1373.

[19]Lallemand B, Chaix F, Bury M, Bruyère C, Ghostin J, Becker JP, et al. N-(2-[3-[3, 5-bis (trifluoromethyl) phenyl] ureido} ethyl)-glycyrrhetinamide (6b): a novel anticancer glycyrrhetinic acid derivative that targets the proteasome and displays anti-kinase activity. *J Med Chem* 2011; 54(19): 6501–6513.

[20]Reufsteck C, Lifshitz-Shovali R, Zepp M, Baeuerle T, Kuebler D, Golomb G, et al. Silencing of skeletal metastasis-associated genes impairs migration of breast cancer cells and reduces osteolytic bone lesions. *Clin & Experimental Metastasis* 2012; 29(5): 441–456.

[21]Roy MK, Mine Y. Nutrigenomics and proteomics of tea polyphenols. green tea polyphenols. *Nutraceuticals Modern Life* 2013: 285.

[22]Felicetti F, Errico IVC. MicroRNAs in melanoma. *MicroRNAs Cancer* 2013: 252.

[23]Gemei M, Corbo C, D'Alessio F, Di Noto R, Vento R, Del Vecchio L. Surface proteomic analysis of differentiated versus stem-like osteosarcoma human cells. *Proteomics* 2013; 13(22): 3293–3297.

[24]Jadhav V, Hackl M, Druz A, Shridhar S, Chung CY, Heffner KM, et al. CHO microRNA engineering is growing up: Recent successes and future challenges. *Biotechnol Adv* 2013; 31(8): 1501–1513.

[25]Singh R, Mo YY. Role of microRNAs in breast cancer. *Cancer Biol & Ther* 2013; 14(3): 0–1.

[26]Broustas CG, Zhu A, Lieberman HB. Rad9 protein contributes to prostate tumor progression by promoting cell migration and Anoikis resistance. *J Biol Chem* 2012; 287(49): 41324–41333.

[27]Segura MF, Greenwald HS, Hanniford D, Osman I, Hernando E. MicroRNA and cutaneous melanoma: from discovery to prognosis and therapy. *Carcinogenesis* 2012; 33(10): 1823–1832.

[28]Hosseini Rad SMA, Shanaki Bavarsad M, Arefian E, Jaseb K, Shahjahani M, Saki N. The role of microRNAs in stemness of cancer stem cells. *Oncol Rev* 2013; 7(1): e8.

[29]Watson PM, Watson DK. Alternative splicing in prostate and breast cancer. *Open Cancer J* 2010; 3: 62–76.