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Higher Expression of Ubc9 in Human Lung Cancer Tissues and Its clinical Significance*

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ABSTRACT Objective: As Ubc9 may play a great role in tumor genesis and deterioration, the purpose of this study was to detect the expression of ubiquitin-conjugating enzyme (Ubc9) and assess the prognostic significance of Ubc9 in lung cancer. **Methods:** The expression of Ubc9 mRNA and Ubc9 protein in Non-small cell lung cancer and para-carcinoma tissue from 100 lung cancer patients were detected using Real-time, Immunohistochemistry and Western-blot. The correlation of Ubc9 expression with the clinical characteristics was analyzed by the single factor correlation analysis. **Results:** The Ubc9 protein stained brown granules in cytoplasm and nucleus of cancer cells. The higher expression of Ubc9 mRNA and Ubc9 protein were detected in cancer tissue than that in para-carcinoma tissue and correlated with TNM stage, lymph nodes metastases, differentiation and smoking. **Conclusions:** The expression level of Ubc9 mRNA or protein may independently predict postsurgical survival in lung cancer.

Key words: Lung cancer; Ubc9; Clinical characteristics

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Introduction

Lung cancer is one of the common malignant tumors and has become the leading cause of death in China [1]; late detection is a major contributor to this high mortality rates [2]. The lung cancer histologic subtypes (small cell and non-small cell) have different clinicopathological characteristics reflective of differences in carcinogenesis [3]. Undoubtedly, surgical resection remains the most effective therapeutic method for the lung cancer patients, but the survival rate remains at 20%-30% at 5 years. An important reason for poor outcomes of the patients is that lung cancer is often spread beyond the primary site at the time of diagnosis [4]. Therefore, effective measures such as prognostic factors on diagnosis, prediction and treatment of lung cancer at an earlier stage are extremely urgent[5].

Sumoylation, which includes small ubiquitin-like modifier (SUMO) protein addition or removal from other proteins, is a post-translational modification that plays an important role in diverse cellular processes, including transcriptional regulation, nuclear transport, cell cycle control and maintenance of genome in tegrity through modulating protein interactions of target proteins[6-8]. Ubiquitin-conjugating enzyme 9 (Ubc9), the sole E2-conjugating enzyme for sumoylation, plays an important role in tumorigenesis and progression[9]. Ubc9 has been reported to be expressed at high levels in advanced melanomas and was found to shield melanoma cells from apoptosis, and expressed at higher levels in head and neck tumor and lung tumor specimens than in the matched normal tissues [10]. However, whether Ubc9 expression is associated with the Lung cancer remains unknown. In this study, we were interested in analyzing the expression of Ubc9 in a series of 100 consecutive patients with lung cancer in order to better understand their roles in human lung cancer biology and their potential implications for prognostic significance .

1 Materials and methods

1.1 Patients

From July 2010 to April 2011, 100 Lung cancer patients treated by curative surgical resection at the Department of Thoracic Surgery in Affiliated Hospital of Medical College, Qingdao University were enrolled in our investigation. The patients' clinical and pathological data were recorded according to the World Health Organization (WHO) and the Union for International Cancer Control (UICC). Written and informed consent was obtained from all patients and the investigation was approved by ethical committee of our hospital. The clinico-pathological data analyzed include sex, age, smoking, histology, T-stage, N-stage, TNM-stage, lymph nodes metastasis and differentiation. Served as control group, the corresponding normal tissues from the same patients but different lung sites without tumor were also assessed for the expression of Ubc9 mRNA and protein.

1.2 Quantitative real–time PCR analysis

Each sample, including cancer tissue and normal tissue from the same persons, was frozen in liquid nitrogen immediately after surgical resection before extraction of RNA. Total RNA was isolated using Trizol reagent (Invitrogen, USA) according to the manufacture's protocol. The concentration and purity of the RNA sam-

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ples were determined by measuring the optical density in 260/280 nm ratios with a spectrophotometer. 10 ng of total RNA preparations from each sample was reverse transcribed to cDNA, stored at -80 ℃ until analysis. The primers, which were labeled with a 5'fluorescent reporter dye (6-carboxyfluorescein) and a 3' quenching dye (6-carboxytetramethylrhodamine), were synthesized by TaKaRa Biotechnology. The results were analyzed with 7500 Real Time PCR System software (ABI, Shanghai, China). For measurement of cDNA corresponding to human Ubc9 mRNA, the forward primer was 5'-TCTTCGCCACCAAAATGTAA-3', the reverse primer was 5'-CTCCAGTCCTTGTCCTCCTCTA-3'. For references, we quantified human GAPDH gene, the forward primer was 5'-TCAGCCGCATCTACTATIA G-3', the reverse primer was 5'- GATGGCATGGACTGTGGTC-3'.

1.3 Immunohistochemistry analysis

The paraffin section of all the samples, including cancer tissue and normal tissue from the same persons, was prepared. The goat antihuman polyclonal antibody Ubc9 was bought from Santa Cruz Biotechnology Company and the test was performed according to the manufacturer's instruction. IHC was performed as described in the literature. Briefly, after dewaxing and hydration, the slides were rinsed in phosphate-buffered saline (PBS) and blocked endogenous peroxidase activity with 3 % hydrogen peroxide for 15min. Antigen retrieval was achieved with preheated 10 mmol/L (pH 6) citrate buffer for 30 min to 95 ℃ in a microwave oven. Then the specimens were incubated with the goat anti-Ubc9 (diluted 1:50 in PBS) at 4 ℃ for 24h, PBS instead of antibody served as negative control and the secondary antibodies 90 min at 37 ℃ in a humid chamber. Finally, reaction products were visualized using 3, 3'-diaminobenzidine (DAB), and the sections were then counterstained with hematoxylin. The expression of Ubc9 protein was observed under light microscope and also analyzed using Image-Pro Plus.

1.4 Western blot analysis

Frozen tissue samples (100 mg) were homogenized in 400 μl of ice-cold RIPA buffer (PBS, pH 7.4, 0.5% sodium deoxycholate, 0.1% SDS with freshly added PMSF (100 μg/ml) (Sigma, USA)). After a 60 min incubation on ice, samples were spun at 12000 r/min for 10 min at 4 ℃ and supernatants were colleted for protein extraction. Protein concentrations of the resulting lysates were determined by Coomassie brilliant blue staining. Equivalent of 40μg protein in each sample was denatured at 95 ℃ for 8 min, loaded on a 10% SDS-polyacrylamide gel containing a 2.5% stacking gel, together with a molecular weight standard (GibcoBRL, USA), and then electrophoresed at 75 V for 1 h and in Tris-glycine running buffer (25 ml/l Tris-base, 250mol/l glycine, 0.1% SDS). Proteins were electroblotted onto a nitrocellulose membrane. The blotted membrane was blocked with 5% nonfat dry milk in PBST (PBS, pH 7.4, Tween 20) with gentle shaking at room temperature for 1h and then incubated overnight at room temperature in 1% bovine serum albumin (BAS)/PBST containing Ubc9 monoclonal antibodies (both diluted 1:1000, Santa Cruz Biotechnology Inc.) with gentle shaking. Following three washes in PBST, the membrane was then incubated for 1 h at room temperature in 1% BSA/PBST containing a 1:5000 dilution of the second antibody (mouse anti-goat IgG, Santa Cruz Biotechnology Inc.) with gentle shaking. The band intensities of Ubc9 protein expressions were measured using image analysis software (NIH Image).

1.5 Statistical Analysis

All statistical analyses were performed with the SPSS17.0 software and statistical differences at $P < 0.05$ were considered to be statistically significant. Experimental data were expressed as mean+SD. The Chi-square test was applied to analyze the association between Ubc9 and clinicopathological characteristics.

2 Results

2.1 The expression of Ubc9 mRNA detected by Quantitative real–time PCR analysis

In the present study, 52 male and 48 female patients with the mean age of 63 years (range 45-75 years) were included. Using Real-time PCR analyses, a statistical significance was observed for the higher Ubc9 mRNA expression in the cancer group compared to the normal group . The expression of Ubc9 mRNA was shown in Fig.1and Table1. Furthermore, for Ubc9, a significant elevation could be found in stage III compared to stages I and II tumors ($P< 0.05$).

2.2 The expression of Ubc9 protein detected by Immunohis–tochemistry

Using Immunohistochemistry analyses, Ubc9 protein was stained positively as brown granules in cytoplasm and nucleus of cancer cells. And nonuniform staining intensities were observed in the carcinoma tissues (Fig.2B). Compared with adjacent tissues, Ubc9 protein was expressed at a higher level with statistical significance in lung carcinoma tissues (Fig.2A).

2.3 The expression of Ubc9 protein detected by Western blot

Western blot results, as shown in Fig. 3A and B, reveal that Ubc9 protein was noticeably increased in cancer tissues. The results indicate an approximate 1-fold and 2-fold increase in Ubc9 protein expression in cancer tissues of TNM stage I-II and stage II-I-IV respectively as compared with normal tissue (Fig. 3A). Similar to RT-PCR results, increased Ubc9 protein could be found in poorly differentiated tumors as compared with well-moderately differentiated tumors ($P<0.05$), and in smoking compared with no smoking ($P<0.05$), as well as in lymph nodes metastasis compared with no lymph nodes metastasis ($P<0.05$). The results show Ubc9 protein expression in lung cancer was correlated with clinical characteristics.

Table1 Relative expression of Ubc9 mRNA in different samples (TNM-stage)

Sample	Result				
	GAPDH Ct	Ubc9 Ct	ΔCt	-ΔΔCt	$2^{-\Delta\Delta Ct}$
Normal	19.35	25.15	5.80	-----	-----
Normal	19.14	24.47	5.33	-----	-----
Normal	19.83	25.37	5.54	-----	-----
Normal	19.80	25.17	5.37	-----	-----
Normal	19.54	25.05	5.51	-----	-----
Ⅰ-Ⅱ	19.92	24.38	4.46	1.05	2.07
Ⅰ-Ⅱ	19.89	24.46	4.57	0.94	1.92
Ⅰ-Ⅱ	19.52	24.76	5.24	0.27	1.21
Ⅰ-Ⅱ	19.99	24.84	4.85	0.66	1.58
Ⅰ-Ⅱ	19.27	24.65	5.38	0.13	1.09
Ⅲ-Ⅳ	19.83	23.43	3.60	1.91	3.76
Ⅲ-Ⅳ	19.37	23.66	4.29	1.22	2.33
Ⅲ-Ⅳ	19.87	23.45	3.58	1.93	3.81
Ⅲ-Ⅳ	19.14	23.79	4.65	0.86	1.81
Ⅲ-Ⅳ	19.26	23.68	4.42	1.09	2.12

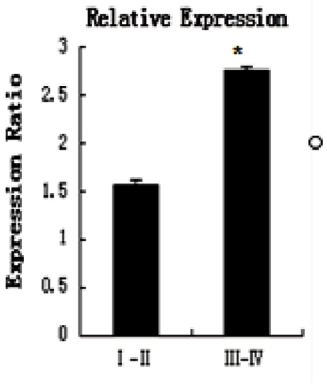

Fig.1 Real-time PCR analyses of Ubc9 mRNA. Ubc9 mRNA expression was elevated in in stage III compared to stages I and II tumors (*p < 0.05)

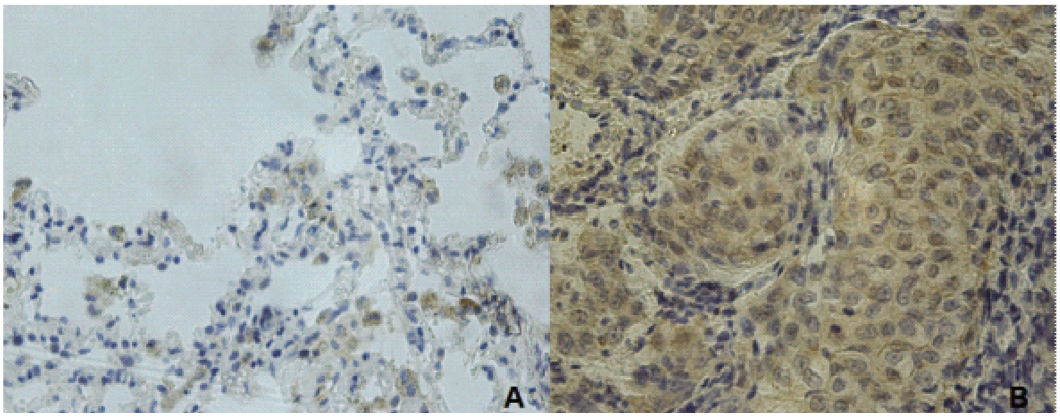

Fig.2 Ubc9 protein expression analysis by immunohistochemistry (400) (A) Negative and weak expression of Ubc9 in adjacent lung tissues. (B) Positive expression of Ubc9 with cellular location in cytoplasm and nucleus in carcinoma tissues.

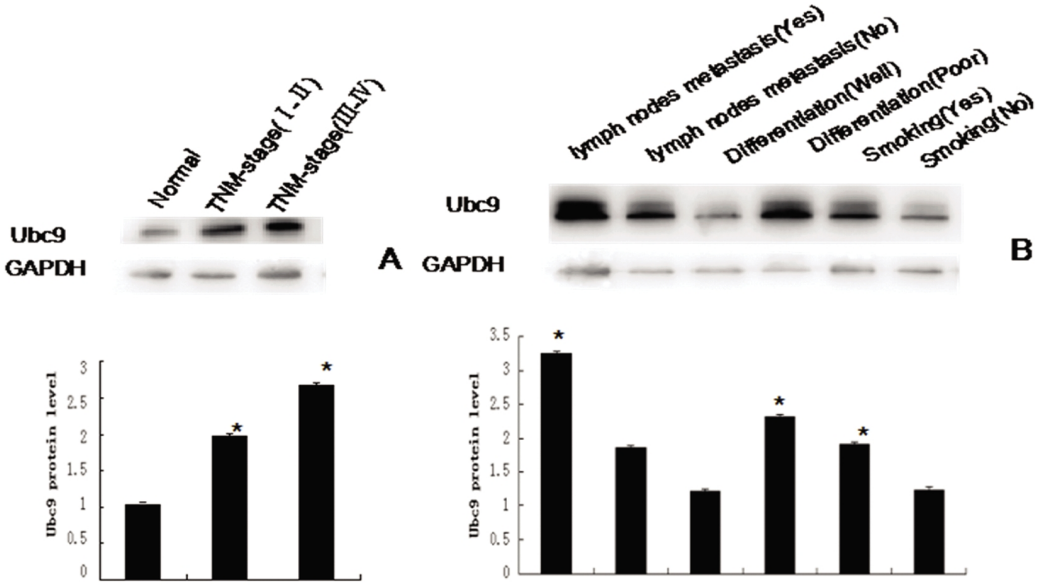

Fig.3 Western-blot analysis of Ubc9 protein. (A) The Ubc9 protein level is higher in lung cancer tissues compare to normal tissues (*p<0.05), and much higher level of Ubc9 protein expression were observed in TNM-stage Ⅲ-IV than stage Ⅰ-Ⅱ (*p < 0.05). (B) Higher expressin of Ubc9 in poorly differentiated tumors than well-moderately differentiated tumors (*p < 0.05), in smoking than no smoking (*p<0.05), as well as in lymph nodes metastasis than no lymph nodes metastasis (*p < 0.05).

3 Discussion

Lung cancer is the most frequent cancer worldwide[12] and the leading cause of cancer related deaths [13]; its five-year survival rate is next to the lowest of all cancers[14]. An important reason for poor outcomes of the patients is that lung cancer is often detected after it has spread beyond the primary site. To find an effective indicators for early diagnosis of lung cancer is the key to prolong surial of the patients with lung cancer for early treatment and also the spot problem in research.

SUMOylation, a dynamic post-translational modification process, requires E1 (SAE1/SAE2), E2 (Ubc9) and multiple E3s (e.g. Siz and PIAS in vertebrates) to carry out covalent conjugation of SUMO (e.g. SUMO1, SUMO2 and SUMO3 in mammalian cells) to target proteins, and a number of de-SUMOylation enzymes for rapid deconjugation[15]. Like other post-translational modifications, SUMOylation has been shown to be involved in many cellular processes, such as cell cycle regulation, proliferation, apoptosis, DNA repair and mitosis[16,17]. SUMOylation exclusively utilizes the E2 conjugating enzyme, namely, SUMO ubiquitin-conjugating enzyme 9 (Ubc9). Ubc9 is highly conserved from yeast to humans and contains a core ubiquitin-conjugating catalytic (UBCc) domain, which contains a conserved cysteine residue (Cys93). During SUMOylation, SUMO is transferred onto the active Cys93 by the activating enzyme E1[18], Ubc9 then transports SUMO onto the substrate, and an isopeptide bond forms between the double-Gly residues of SUMO and the Å-amino group of a substrate lysine residue [19]. In vitro assays have confirmed that E1activating enzyme and E2 conjugating enzyme are sufficient for substrate SUMOylation proteins [17,18]. Many proteins involved in cell cycle regulation, proliferation, apoptosis have been demonstrated to be the targets for sumoylation [20,21]. Ectopic expression of Ubc9 enhances tumor growth in animal models, suggesting that Ubc9 protein plays a critical role in multiple tumor suppressive functions, such as growth inhibition, apoptosis, replicative senescence, suppression of oncogenic transformation, and inhibition of migration and angiogenesis.Thus understanding of its mechanisms is important for the prognosis and intervention of diseases. In this study, the expression of Ubc9 at mRNA and protein levels were siginificantly elevated in the human lung cancer than that in the adjacent tissues.

As we have known that the survival rate and prognosis of the patients with lung cancer are much correlated with TNM stage, lymph nodes metastases, and differentiation, we evaluated the relationship of the expression of Ubc 9 in lung cancer with TNM stage, lymph nodes metastases, differentiated degree and smoking. Our results show that the higher expressions of the Ubc9 in the cancer tissues correlated strongly with the TNM stages, lymph nodes metastasis, grades of differentiation and smoking. There were stronger expressions of Ubc 9 mRNA and protein in the tumors with poorly differentiation , in the stage III, with lymph nodes metastasis or with smoking. However, our results show no statistically significant difference of Ubc9 expression among the cancer of different pathologic types or from patients of different ages. The observed correlation between the expression of Ubc9and the grade of differentiation in human lung cancer suggests that Ubc9 might be a promising novel biomarker of cell differentiation in human lung cancer.

References

[1] Ministr of Healthy of the People's Republic of China.Major situation of the third survey of national death causes[J]. China Cancer, 2008, 17 (5): 344-345

[2] Mulshine JL, van Klaveren RJ. Lung cancer screening: what is the benefit and what do we do about it?[J]. Lung Cancer, 2011, 71(2): 247-248

[3] Gazder A, Franklin W.A, Brambilla E, et al. In World Health Organization Classification of Tumors[J]. Pathology and Genetics of Tumors of the Lung, Pleura and Heart, 2004, 18(5): 21-23

[4] Ciervid R, Dhage S, Guth A, et al. Five year outcome of 145 patients with ductal carcinoma in situ (DCIS) after accelerated breast radiotherapy[J]. Int Radiat Oncol Biol Phys, 2012, 26(83): 159-164

[5] Oji Y, Kitamura Y, kamino E, et al. WT1 IgG antibody for early detection of non small cell lung cancer and as its prognostic factor[J]. Int Cancer, 2009, 125(2): 381-387

[6] Mü ller S, Hoege C, Pyrowolakis G, et al. SUMO.ubiquitins mysterious cousin [J]. Nat Rev Mol Cell Biol, 2001, 2(3): 202-210

[7] G.GSUMO and ubiquitin in the nucleus different functions similar mechanism[J]. GenesDev, 2007, 18(17): 2046-205

[8] Hay RT.SUMO: a history of modification [J]. Mol Cel1, 2005, 18(1): 1-12

[9] Lin D, Tatham MH, Yu B, et al. Identification of a substrate recognition site on Ubc9[J]. Biol Chem, 2002, 277(24): 21740-21748

[10] Punturieri A, Szabo E, Croxton TL, et al. Lung cancer and chronic obstructive pulmonary disease: needs and opportunities for integrated research[J]. Natl Cancer Inst, 2009, 101(22): 554-559

[11] Jemal A, Siegel R, Xu J, et al. Cancer statistics [J]. CA Cancer Clin, 2010, 60(9): 277-300

[12] Mathers CD, Loncar D. Projections of global mortality and burden of disease from 2002 to 2030[J]. PLoS Med, 2006, 12(3): e442

[13] Wu F, Zhu S, Ding Y, et al. MicroRNA-mediated regulation of Ubc9 expression in cancer cells [J]. Clin Cancer Res, 2009, 15 (19): 1550-1557

[14] Hay RT. SUMO: a history of modification [J]. Mol Cell, 2005, 18 (11):1-12

[15] AzumaY, Arnaoutov A, Dasso M. SUMO-2/3 regulates topoisomerase II in mitosis[J]. CellBiol, 2003, 163(13): 477-487

[16] Fernandez-miranda G, DE Castro IP, Carmena M, et al. SUMOylation modulates the function of Aurora-B kinase [J]. Cell Sci, 2010, 9 (16): 2823-2833

[17] Hay RT. Protein modification by SUMO [J]. Trends Biochem.Sci, 2007, 5(26): 332-333

[18] Desterro JM, Rodriguez MS, Kemp GD, et al. Identification of the

enzyme required for activation of the small ubiquitin-like protein SUMO-1[J]. Biol Chem, 2008, 11(274): 10618-10624

[19] Moschos SJ, Smith AP, Mandic M, et al. SAGE and antibody array analysis of melanoma-infiltrated lymph nodes:identificationof of Ubc9 as an important molecule inadvanced-stage melanom as [J]. Oncogene, 2007, 26(29): 4216-4225

[20] Deng H, Lin Y, Badin M, et al. Over-accumulation of nuclear IGF-1 receptor in tumor cells requires elevated expression of the receptor and the SUMO-conjugating enzymeUbc9 [J]. Biochem Biophys Res Commun, 2011, 404(2): 667-671

[21] Yu F, Deng H, Yao H, et aI. Mi r-30 reduction rrlai r/lains sell renewaI and Inhibits apoptosis In b reast LJmor-lnitiatili [J]. Cells Oncogene, 2010, 29(129): 4194-4204

UBC9在人肺癌组织中高表达及其临床意义*

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摘要 目的:由于Ubc9在肿瘤的发生于恶化中发挥巨大作用,本研究目的是对泛素结合酶(Ubc9)mRNA和Ubc9蛋白在非小细胞肺癌组织中表达进行检测,评估Ubc9在肺癌预后中的指导意义。**方法:**通过荧光定量PCR、免疫组织化学法、Western-blot检测100例非小细胞肺癌病人中Ubc9 mRNA、蛋白水平的表达进一步研究Ubc9的表达与肺癌临床特征的关系。**结果:**实验结果显示在肺癌细胞中Ubc9阳性表达显示为黄棕色颗粒,与癌旁组织比较,Ubc9 mRNA、蛋白在肺癌组织中高表达,并且与肺癌的临床分型(分期、淋巴结转移、吸烟、分化)有关。Ubc9 mRNA、蛋白在肺癌中的表达癌组织高于癌旁、有淋巴结转移的高于无转移、吸烟高于不吸烟者、低分化组织高于高分化组织。**结论:**由此可见Ubc9 mRNA、蛋白水平的高表达可能对肺癌临床特征的评估,以及预测术后生存率都有重要指导意义。

关键词:肺癌; Ubc9;临床特征

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