ORIGINAL ARTICLE

Differential expressions of MDM2 and TAP73 in cancer and cancer-adjacent tissues in patients with non-small-cell lung carcinoma

B. Wanga,1, X. Liua,1, H. Liub,1, J. Guoa, T. Zhanga, N. Zhoua, Y. Ma, H. Yu, a, L. Chena, Z. Rena, K. Fan, X. Tianb,∗

a Department of Thoracic Surgery, Chinese PLA General Hospital, Beijing, China
b Department of Thoracic Surgery, PLA 309 Hospital, Beijing, China

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Abstract
Aim: To investigate the differences in mRNA and protein expressions of MDM2 (mouse double minute 2 homolog) and P73 in cancer and cancer-adjacent tissues in patients with non-small-cell lung carcinoma (NSCLC).

Materials and methods: We compared the protein expressions of MDM2 and P73 in lung cancer and cancer-adjacent tissues in NSCLC patients by IHC (immunohistochemistry) and WB (Western blot). We divided the NSCLC patients into two subgroups, adenocarcinoma and squamous carcinoma. The mRNA expressions of two main isoforms of P73, TAP73 and DNP73, as well as the ratio of DNP73/TAP73 were analyzed by qPCR (quantitative real-time PCR) in the two tissues in all NSCLC patients and in patients with adenocarcinoma or squamous carcinoma, respectively.

Results: WB results did not show significant differences in MDM2 and P73 protein expressions in lung cancer and cancer-adjacent tissues. However, IHC results indicated that MDM2 expression significantly increased in cancer tissues in female patients, but not male patients. In addition, TAP73 mRNA expression significantly increased in cancer tissues in all NSCLC patients (p = 0.002) and in patients with adenocarcinoma (p = 0.01); while there was no significant difference in DNP73 mRNA expression. Hence the fold-change of DNP73/TAP73 ratio significantly decreased (p = 0.0003) in cancer tissues in all NSCLC patients and in patients with adenocarcinoma.

∗ Corresponding author.
E-mail address: tianxiaodong301@163.com (X. Tian).
1 These three authors are co-first authors.

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Introduction

Lung cancer is a malignant tumor with the highest morbidity and mortality, and is a serious threat to human health. The etiology of lung cancer is the interaction of environment factors (smoking, air pollution, ionizing radiation, and diet) and genetic factors. Lung cancer can be classified into two major types, SCLC (small cell lung cancer) and NSCLC (non-small cell lung cancer), according to the histopathology. The most common types of NSCLC are SC (squamous carcinoma), adenocarcinoma, and large cell carcinoma. Adenocarcinoma accounts for 50% of all lung cancer cases. SC is more common in elderly men and is correlated with smoking. SC is sensitive to CT (chemotherapy) and RT (radiotherapy) treatments. The best treatment for patients with SC is surgical approach in a combination of CT and RT and the five-year survival rate is relative high in this context. Adenocarcinoma is more frequently observed in female patients and is not always with smoking. The morbidity of adenocarcinoma has risen in recent years and it has become the main type of lung cancer in some countries. Although the therapeutic methods have been improved, the overall-survival rate of lung cancer has not improved in recent years. Hence, a deeper understanding of the etiology of lung cancer is necessary for the development of new therapeutic approaches and the treatment of lung cancer.

TP53 is a classical tumor-suppressor gene resulting in the expression of mutant P53 proteins with single-amino-acid substitutions within the DNA-binding domain (DBD). Therefore, TP53 plays an important role in maintaining the genome integrity. P73 and P63 are two homologs of TP53. Unlike TP53, P63 and P73 regulate developmental processes rather than participate in the control of genome stability. P73 is located on human chromosome 1p36.3, and is consisted of 13 exons and 12 introns. It has been reported that P73 plays an important role in cancers. P73 is involved in the control of programmed cell death, and can be used as an indicator of cancer prognosis. P73 mutation is often resulted in a variety of tumors, including neurocytoma, CRC (colorectal cancer) and breast cancer.

P73 encodes two isoforms, TAP73 (transcriptionally active P73) and DNP73 (dominant negative P73). Studies show that P73 mRNA expression is higher in cancer tissues than in healthy tissues, suggesting that P73 might be an oncogene. Evidence indicates that TAP73 can suppress tumors formation while DNP73 can promote tumor formation. Studies have found that TAP73 and DNP73 are overexpressed in ovarian cancer, hepatocellular carcinoma and colon cancer, and their expression levels are correlated with the development and prognosis of cancers. Accumulating evidence suggests that the overexpression of DNP73 transcript is associated with adverse prognosis and chemotherapy failure in several human tumors. High DNP73/TAP73 ratio is associated with poor prognosis in acute promyelocytic leukemia (APL). The expression of TAP73 and DNP73 can be elevated simultaneously in lung cancer. Hence, TAP73 and DNP73 interact with each other and play complex roles in regulating the proliferation and apoptosis of cancer.

MDM2 is located on human chromosome 12q14.3-q15, and is one of the principal ubiquitin ligases that are responsible for P53 degradation. MDM2 can regulate the activity, stability and function of P53 and can also interact with P73. In MDM2-P53 system, P53 activation induces MDM2 transcription; while MDM2 activation inhibits P53 activity by binding to its activated area of transcription. However, it is unclear whether MDM2 can regulate P73 activity. Studies show that MDM2 and P73 can form heterodimers in vivo or in vitro. MDM2 does not promote P73 degradation, but it can suppress P73 protein expression by binding to the N terminal of the p300/CBP; while P73 can stimulate the expression of endogenous MDM2. Hence, MDM2 is a negative feedback regulator of P73, and form a negative feedback loop with P73. MDM2-P73 system plays an important role in the development of lung cancer. It has been reported that MDM2 overexpression and P73 deficiency can induce genome instability and tumor development. To date, no study has reported the expressions of MDM2 and P73 in different types of lung cancers. Hence, in this study, we investigated the relationship between MDM2 and P73 in lung cancers, as well as the functions of TAP73 and DNP73 in the development and prognosis of lung cancer.

Materials and methods

Patients and materials

We calculated the estimated sample size based on our preliminary data. We selected 45 patients with lung cancer in our hospital from June 2016 to October 2016. The inclusion criteria included: (1) The patients had not received chemotherapy (CT), radiotherapy (RT), biological drug treatment (drugs that could bind to the specific cancer site and kill the cancer cells) and surgery; (2) the patients did not have other tumors (such as carcinoma); (3) the patients were suitable for surgery; (4) the...
patients did not have other non-cancer diseases according to http://geneontology.org/ (such as aquaphobia). We collected the samples of cancer tissues and cancer-adjacent tissues from all 45 patients. The lung cancer tissues were further divided into different sub-groups according to the histopathology, including 10 cases of squamous carcinoma, 31 cases of adenocarcinoma and 4 cases of other cancer types. Meanwhile, we also collected the basic and important information of all the patients.

This study was approved by the Hospital Ethics Committee (No. 2015034), and all the patients signed the informed consent.

Methods and statistical analysis

Methods
We measured the MDM2 and P73 protein expressions in cancer and cancer-adjacent tissues by IHC (immunohistochemistry) and WB (Western blot), respectively. The main reagents and instruments were shown in Appendix Table 1 (Table A.1).

IHC. The frozen tissues were dehydrated at room temperature and fixed with 4% paraformaldehyde for 15 min. Tissues were paraffin-embedded and sectioned. The sections were incubated in 5% H2O2 at room temperature for 15 min. The sections were incubated in EDTA for 3 min at 140°C, washed 3 times in PBS, 5 min each. The sections were blocked with 5% goat serum for 20 min at 37°C and incubated with the primary antibody (1:100) overnight at 4°C. After washing, the sections were incubated with biotinylated secondary antibody for 30 min at room temperature. Sections were washed 4 times in PBS and dehydrated with sequential ethanol gradients (75%, 80%, and 100%). Images were acquired by optical microscopy.

IPP (Image-pro plus 6.0) software was used to analyze the IHC images. The ratio of region of interest to overall area was calculated to analyze the difference between MDM2 and P73 protein expressions in cancer and cancer-adjacent tissues. The main principle and process were as follows: images from five different fields in each tissue were randomly acquired. The density of the background was adjusted to distinguish the background and target area. The region of interest (ROI, the brown staining area) on each image was defined and the total area was measured. The values from the five images were exported and the mean values were calculated.

The accumulated IOD (Integrated optical density) of the brown background in a selected field was measured, and the mean IOD was calculated by the formula: Mean IOD = IOD/Total area of the selected field.

WB. The proteins were extracted from the cancer and cancer-adjacent tissues of patients using RIPA lysis Buffer. The protein concentration was measured using BCA (Bicinchoninic acid) Kit according to the manufacturer’s instructions. 32 μg sample was loaded onto 10% SDS-PAGE, run at 90 V for 20 min, and 120 V for 50 min. After electrophoresis, protein samples were transferred onto PVDF membranes (0.45 μm). The membranes were incubated in ponceau and the protein bands were observed. The membranes were blocked in 5% BSA-TBST for 1 h, and then incubated with primary antibodies (1:500) overnight at 4°C. Next day, the membranes were washed 3 times with TBST, 10 min each. The membranes were incubated with secondary antibodies (1:10,000) for 40 min at room temperature. After washing, the membranes were developed using ECL and exposed to X-ray. Films were scanned by scanner and Gel-Pro analyzer was used to analyze the WB outcomes. Beta-actin was used as the internal control. The formula for the calculation of mean gray value was as follows: Mean gray value = object value/internal control value. The experiments were repeated three times and the values were calculated and averaged.

RT-PCR (reverse transcription PCR) and qPCR (quantitative real-time PCR). RT-PCR and qPCR was used to detect the mRNA expressions of TAP73 and DNP73 (two isoforms of P73) in cancer tissues and cancer-adjacent tissues from each patient. The primers were: TAP73 (Amplonc size: 111 bp) forward: 5′-GCCACCGTGTGAGCCTCT-3′, reverse: 5′-GCAATTGAACCTGCGCATGA-3′; DNP73 (Amplonc size: 123 bp) forward: 5′ACT AGC GCC GAG CCT CTC CC-3′, reverse: 5′GT GC T CA GCA GAT GAA CTG G-3′; H-ACTB (Amplonc size: 127 bp) forward: AGGCCAATGAAGACTCAAGAT CAT-3′, reverse: ACTCGTCACTCTCCGTTGC. Other reagents and instruments were shown in Appendix Table 1 (Table A.1). I. RNA isolation. 50 mg tissues were pulverized in liquid nitrogen, and transferred into centrifugal tubes. The tissues were homogenized in 1 mL Trizol and incubated at room temperature for 5 min. 0.2 mL trichloromethane was added, vortexed for 10 s, and incubated at room temperature for 5 min. The samples were centrifuged at 12,000 rpm for 15 min at 4°C, and 550 μL supernatants were collected. After adding the same volume of iso-propanol, the samples were incubated at −20°C for 20 min, and then centrifuged at 12,000 rpm for 15 min at 4°C. The RNA pellet was washed with 1 mL 75% ethyl alcohol and centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was removed and the RNA samples were air dry for 5 min. RNA was dissolved in 30 μL RNase-free water.

II. RT-PCR. After Dnase treatment RNA was reverse-transcribed into cDNA using HiFiScript kits. 10-μL reaction system included 1 μg RNA Template, 0.5 μL gDNA Eraser and 1 μL 10× g DNA Eraser Buffer. Samples were heated at 42°C for 2 min and cooled on ice. 1 μL HiFiScript (200 U/μL), 1 μL Primer Mix, 4 μL 5× RT Buffer and 4 μL RNase-free ddH2O were added. The thermal cycles were 42°C for 50 min, 85°C for 5 min, and 4°C forever.

III. Real-time PCR. Real-time PCR was carried out with KAPA SYBR FAST qPCR Kit Master Mix (2×) (KAPA Biosystems, KK4601). The 10 μL reaction system included 5 μL PCR Master Mix (2×), 0.2 μL mRNA forward primers (10 μM), 0.2 μL mRNA reverse primers (10 μM), 1 μL cDNA, 0.2 μL Dye (50×), and 3.4 μL ddH2O.

The reaction protocol was 3 min at 95°C activation, 40 cycles of 3 s at 95°C and 20 s at 60°C. Melting curve was constructed in the range of 60−95°C. The original data, amplification curve and solubility curve were exported to quantification software. The relative expression levels of target genes were analyzed using 2−ΔΔCt method. The fold-change of mRNA expression in cancer tissues relative to cancer-adjacent tissues was compared.
Statistical analysis
SPSS 19.0 software was used to analyze all the data. Mean value and standard error were used to present MDM2 and P73 protein expressions, as well as TAP73 and DNP73 mRNA expressions. One-way ANOVA (one-way analysis of variance) with Bonferroni–Dunnett corrections were used for multiple-group comparisons. $p < 0.05$ indicated a statistical significance.

Results
The summary of the patients’ information was as follows: (1) the age of the patients ranged from 40 to 70 years old. (2) 27 were male and 18 were female. (3) The cancer types were roughly divided into adenocarcinoma (22.2%), squamous carcinoma (68.9%) and others (8.9%). (4) TNM tumor stages varied among patients; however, no metastasis ($M = 0$) was found in all the patients we analyzed. The details of the patients’ information were shown in Table 1.

MDM2 and P73 protein expressions

IHC
According to the quantitative analysis using IPP software, we compared MDM2 and P73 protein expressions in cancer and cancer-adjacent tissues. We found that MDM2 ($p > 0.05$) and P73 ($p > 0.05$) expressions were similar in cancer and cancer-adjacent tissues in all patients (Appendix Fig. A.1). We also compared the MDM2 and P73 protein expressions in cancer and cancer-adjacent tissues in patients with squamous carcinoma or adenocarcinoma, respectively. However, we did not find any significant difference.

Next, we analyzed the MDM2 and P73 protein expressions based on gender and smoking history. Interestingly, we found that MDM2 expression significantly increased in cancer tissues in pregnant patients ($p = 0.01$, Fig. 1), but not in the male patients. Moreover, we found that the MDM2 was mainly expressed in the nucleus in the cancer-adjacent tissues; while MDM2 was simultaneously expressed in nucleus and cytoplasm in the cancer tissues. There was no significant difference in P73 expression in male and female patients. Moreover, there was no statistical significance in MDM2 and P73 expressions in smoking and non-smoking groups.

WB
We analyzed the MDM2 and P73 protein expressions using WB and the representative WB images are shown in Appendix Fig. A.2. We found that there was no significant difference in the MDM2 and P73 protein expressions between cancer and cancer-adjacent tissues in all patients ($p > 0.05$, Appendix Fig. A.3). We also analyzed the MDM2 and P73 protein expressions in patients with adenocarcinoma or squamous carcinoma, respectively. However, we did not find any significant difference.

Next, we analyzed the MDM2 and P73 protein expressions based on gender and smoking history. However, there was no significant difference in MDM2 and P73 expressions in male and female patients, and smoking and non-smoking groups. TAP73 and DNP73 mRNA expressions. We compared the fold change of TAP73 and DNP73 mRNA expression in cancer tissues relative to cancer-adjacent tissues in all lung cancer patients. We found that there was a significant increase in TAP73 mRNA expression in cancer tissues ($p = 0.035$, Fig. 2a); while there was no significant difference in DNP73 mRNA expression ($p = 0.415$, Fig. 2b). The ratio of DNP73/TAP73 significantly decreased in cancer tissues ($p = 0.0003$) (Fig. 2c).

Next we compared the TAP73 and DNP73 mRNA expressions in patients with adenocarcinoma or squamous carcinoma, respectively. We found that TAP73 mRNA expression was significantly higher in the cancer tissues in patients with adenocarcinoma ($p = 0.01$, Fig. 3a) while the TAP73 mRNA expression was similar between cancer and cancer-adjacent tissues in patients with squamous carcinoma. Moreover, there was no significant difference in the DNP73 mRNA expression in patients with adenocarcinoma or squamous carcinoma (Fig. 3b). The fold changes of the ratio of DNP73/TAP73 significantly decreased in cancer tissues in patients with adenocarcinoma ($p = 0.002$), but not in patients with squamous carcinoma (Fig. 3c).

Discussion
In this study we compared the differences in MDM2 and P73 expressions between the cancer and cancer-adjacent tissues in patients with NSCLC. Interestingly, TAP73 mRNA expression, an isomorf of P73, significantly increased in the cancer tissues in all NSCLC patients and in patients with adenocarcinoma; while there was no difference in DNP73 mRNA expression. Therefore, fold changes of the ratio of DNP73/TAP73 significantly decreased in cancer tissues in all NSCLC patients and in patients with adenocarcinoma.
Some studies show that TAP73 is a tumor-suppressor gene. Irwin MS et al. demonstrated that TAP73 can trans-activate P53 target genes, such as Bax, Puma, and P21, inducing apoptosis and cell cycle arrest; however, Deepa Subramanian reported that TAP73 plays a vital role in activation of activator protein-1 (AP-1) target genes, leading to enhanced activation of other AP-1 family members and increased cellular growth. These results suggest that TAP73 may have different functions in different cells. Moreover, we found that TAP73 mRNA expression significantly increased in cancer tissues in patients with adenocarcinoma, but not in patients with squamous carcinoma. These results may reflect the heterogenic pathology of different types of tumors. Squamous carcinoma is the most common type of NSCLC, and is highly associated with smoking, which is different from adenocarcinoma.

We speculate that elevated TAP73 mRNA expression in cancer tissues may change the interaction between CDK
The mRNA expression of TAp 73 between cancer and paracancer tissues in all NSCLC patients.

The mRNA value of DNp73/TAp 73 between cancer and paracancer tissues in all NSCLC patients.

The expression of DNp73 between cancer and paracancer tissues in all NSCLC patients.

Figure 2. Box-plots show the mRNA expression of TAP73 (a), DNp73 (b) and the ratio of DNp73/TAP73 (c) between cancer and cancer-adjacent tissues in all NSCLC patients. n. s: no significant; C: cancer; P: paracancer; small circles: abnormal values; small starlets: significantly abnormal values; figures: the number of abnormal values.

(cell cyclin-dependent kinase) and Cyclins, thereby promoting cell proliferation. However, the underlying mechanisms remain unknown. Moreover, as isoforms of P73, TAP73 and DNp73 exhibit a complex relationship, which have important effects on the function of genes (e.g. P21) that modulate tumor development.

We further compared the MDM2 and P73 protein expressions in the cancer tissues and cancer-adjacent tissues in all NSCLC patients, in patients with adenocarcinoma or squamous carcinoma alone, in male and female patients and in patients with and without smoking history. We found that there was no difference in MDM2 expression when it was analyzed in all NSCLC patients, in patients with adenocarcinoma or squamous carcinoma alone, and in patients with and without smoking history. Interestingly, we found that the MDM2 expression significantly increased in the cancer tissues in only female patients, but not in male patients by IHC, suggesting the expression of MDM2 may be affected by gender. Studies show that women have a higher risk of adenocarcinoma than squamous carcinoma, suggesting that the expression of MDM2 is higher in adenocarcinoma. Moreover, MDM2 overexpression is associated with gynecological cancers, indicating that the MDM2 expression in tumor tissues might be regulated by estrogen. However, our WB results did not show a significant difference in the MDM2 expression. The discrepancy between the IHC and WB results might be due to the different expression patterns of MDM2 in the cancer tissues and cancer-adjacent tissues. For examples, MDM2 is mainly expressed in the nucleus in the cancer-adjacent tissues, while MDM2 is simultaneously expressed in nucleus and cytoplasm in the cancer tissues. The difference in the MDM2 expression pattern can be detected using IHC, but not by WB. Moreover, we used the whole tissues for WB, some of the areas may not have MDM2 expression, which may cause false negative results. There was no difference in P73 protein expression when it was analyzed in all NSCLC patients or in patients with adenocarcinoma or squamous carcinoma alone, in male and female patients, and in patients with and without smoking history. Studies show that MDM2 expression is higher in cancer tissues than cancer-adjacent tissues, and P73 can act as a tumor-suppressor gene or an oncogene. In lung cancer, Di Vinci A et al. found that both DNp73 and TAP73 increased, and the overexpression of TAP73 deteriorates the tumor prognosis, which is similar to the finding of Wen Hong Toh et al. in gastrointestinal carcinomas. These findings suggest that a complex regulatory mechanism of P73 may also exist in lung cancer. In our study we demonstrate that TAP73 mRNA expression significantly increased in the lung cancer tissues, which provide new information on the roles of P73 in lung cancer.

It has been reported that the positive rate of MDM2 protein expression is closely correlated with lymph node metastasis, TNM stages, degree of tumor cell differentiation, and tumor recurrence. Higashiyama et al. demonstrate that MDM2 protein expression detected by
Different expression of MDM2 and TAp73 in NSCLC

The mRNA expression of TAp73 between cancer and paracancer tissues in patients with adenocarcinoma or squamous carcinoma.

The mRNA value of DNp73/TAp73 between cancer and paracancer tissues in patients with adenocarcinoma or squamous carcinoma.

The mRNA expression of DNp73 between cancer and paracancer tissues in patients with adenocarcinoma or squamous carcinoma.

Figure 3 Box-plots show the mRNA expression of TAP73 (a), DNp73 (b) and the ratio of DNp73/TAP73 (c) between cancer and cancer-adjacent tissues in patients with squamous carcinoma or adenocarcinoma. A: adenocarcinoma; S: squamous carcinoma.

IHC can be used as a marker for NSCLC. In our study we found that MDM2 protein expression significantly increased in female lung cancer patients only, which is different from some other studies. However, IHC can reveal more details about the expression of MDM2 in cancer and cancer-adjacent tissues than WB. Previous studies have shown that P73 can act with MDM2, and MDM2 is involved in the occurrence of a variety of tumors. However, we did not discover significant correlation between MDM2 and P73 expressions in our study.

This study has some limitations. Firstly, our samples were mainly from patients with adenocarcinoma or squamous carcinoma, and did not include many other types of lung cancer. Therefore, we did not analyze the MDM2 and P73 expressions in other types of lung cancers. Secondly, because we did not find significant difference in P73 protein expression when it was analyzed by IHC, but not WB. TAP73 mRNA expression significantly increased in cancer tissues in all NSCLC patients and in patients with adenocarcinoma; while there was no change in the DNp73 mRNA expression. Therefore, the fold change of DNp73/TAP73 ratio significantly decreased in cancer tissues in all NSCLC patients and in patients with adenocarcinoma. There was no significant difference in P73 expression between cancer and cancer adjacent tissues. There was no correlation between smoking history and MDM2 and P73 expressions.

Conclusion

In conclusion, MDM2 protein expression significantly increased in cancer tissues only in female NSCLC patients when it was analyzed by IHC, but not WB. TAP73 mRNA expression significantly increased in cancer tissues in all NSCLC patients and in patients with adenocarcinoma; while there was no change in the DNp73 mRNA expression. Therefore, the fold change of DNp73/TAP73 ratio significantly decreased in cancer tissues in all NSCLC patients and in patients with adenocarcinoma. There was no significant difference in P73 expression between cancer and cancer adjacent tissues. There was no correlation between smoking history and MDM2 and P73 expressions.

Authors’ contribution

BW, XL, HL, JG, and TZ participated in data extraction and drafted the manuscript. NZ, YM, HY, and KF carried out the data analysis. LC, ZR, and XT participated in the design of the study. BW, XL, HL, and XT conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All the authors contributed to the interpretation of the results and the proof reading of the manuscript.

Conflicts of interest

The authors have no conflicts of interest to declare.
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Appendix A. Supplementary material

Supplementary material associated with this article can be found in the online version available at doi:10.1016/j.rppnen.2017.08.008.

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