CDX2 methylation may predict the prognosis of patients with lung cancer

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Keywords
lung cancer, prognosis, methylation, Cdx2

Abstract

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CDX2 methylation predicts poor prognosis in gastric cancer, squamous esophageal cancer and colorectal cancer. The present study was performed to investigate the roles of CDX2 methylation in lung cancer.

Material and methods
One hundred and sixty-seven patients with lung cancer were enrolled. Methylation-specific PCR (MSP) was performed to investigate the methylation status of CDX2. Sequencing of the CDX2 5\' CpG island was conducted as well. A 5-year follow-up was performed by a research nurse or dedicated physician. The primary endpoint was death related with lung cancer. Kaplan-Meier curve was used to analyze the survival situation of patients. Univariate and multivariate Cox analysis was performed to investigate the potential predictors for prognosis of patients with lung cancer.

Results
The patients were classified into two groups according to CDX2 status: methylation (n=75) and unmethylation (n=92). After the 5-year follow-up, we found that the survival rate of patients with methylation of CDX2 was much lower than those with unmethylation of CDX2 (56% vs. 84.8%, P=0.000). Among the smoking patients, methylation of CDX2 was related with poorer prognosis of patients with lung cancer (P=0.000). DFS of patients with CDX2 methylation was lower than those without CDX2 methylation (56.0% vs. 73.9%, P=0.009). Univariate and multivariate Cox analysis demonstrated that CDX2 methylation served as independent prognostic predictor of patients with lung cancer (univariate: HR=3.705, 95%CI=1.922-7.139; multivariate: HR=3.418, 95%CI=1.826-6.397).

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**Conclusions:** CDX2 methylation may serve as independent prognostic predictor for patients with lung cancer.

**Key words:** CDX2, methylation, prognosis, lung cancer.
Introduction

Lung cancer is regarded as the most common cause of cancer-related death with 1.3 million deaths worldwide each year. Smoking accounts for 50% and 80% of deaths from lung cancer for females and males, respectively [1]. The 5-year survival rate of lung cancer patients is merely 17% [2], largely due to the fact that most cases are already metastatic at diagnosis or recur after radiotherapy or initial surgery. Metastatic cases are incurable since intrinsic resistance to chemotherapy or acquired resistance after an initial response [3]. The prognosis of cancer patients is commonly affected by multiple factors [4-7]. To improve the clinical outcomes, a better understanding on the molecular pathogenesis of lung cancer is needed to identify novel therapeutic targets.

Genetic and epigenetic factors exhibit key roles in the initiation and progression of lung cancer. Genetic abnormalities in key components are tightly related to the pathogenesis of lung cancer [8,9]. CDX2 and CK20 are proteins related with intestinal development and differentiation, which are also useful markers to identify adenocarcinomas and normal intestinal epithelium of colorectal origin [10-12]. CDX2, an intestinal transcription factor, contributes to regulating the expression of many intestine-specific genes responsible for intestinal proliferation and differentiation [13-15]. It is regarded as a tumor suppressor gene [16-18] that is involved in Wnt/β-catenin signaling pathway [19-21]. Promoter CpG island methylation is a crucial mechanism of silencing tumor suppressor genes during the carcinogenic process. DNA methylation could result in silencing of CDX2 in gastric cancer and squamous esophageal cancer [22, 23]. Grimminger et al. reported that up regulation of CDX2 mRNA expression appeared to be associated with the pathogenesis of non small cell lung cancer [24]. In another study by Liu et al., the researchers found that CDX2 is frequently methylated in lung cancer and expression of CDX2 is regulated by promoter region hypermethylation [25]. In addition, enhanced CDX2 methylation was reported to be related with shorter survival of CRC patients [26]. However, the prognostic role of CDX2 methylation in lung cancer was not explored.
In the present study, methylation status of \textit{CDX2} was determined with methylation-specific PCR (MSP) method and then the prognostic role of \textit{CDX2} methylation in lung cancer was analyzed after a 5-year follow-up. Univariate and multivariate Cox analyses were performed to evaluate whether \textit{CDX2} methylation could serve as independent predictor for the prognosis of lung cancer patients.

\textbf{Materials and methods}

\textbf{Subjects}

A total of 167 patients with lung cancer were enrolled between April 2012 and October 2013 from Affiliated Hospital of Weifang Medical University. The patients comprised 98 men and 69 women. With biopsy, 167 tissue samples were collected from all patients. These patients had not undergone any treatment before surgery. The patients underwent surgery or chemotherapy according to diseases status.

Current smoking status was defined as an individual who had smoked continuously for 6 months (at least one cigarette per day). Former smoking status was defined as individual who smoked but not meet the standard of current smoking. All patients signed written informed consent and the study was approved by the review committee of Affiliated Hospital of Weifang Medical University.

\textbf{Methylation-specific PCR (MSP)}

Bisulfate modification of DNA was performed with a Bisul-Flash DNA Modification Kit (EpiGentek) following the manufacturer’s instructions. The methylation of \textit{CDX2} was assessed by methylation-specific PCR (MSP). The DNA treated by bisulfite was amplified with methylation-specific primers of \textit{CDX2}. The methylation-specific primers were: 5’-TTTTCGTGTTTTTCGGTAGTTTTAGC-3’ (methylation forward primer, MF) and 5’-ACTCAGTGTTACATAAAGGAAAATCCG-3’ (methylation reverse primer, MR). The unmethylation-specific primers were: 5’-TTTTTTGTGGTTTTTGGTAGTTTTTGT-3’ (unmethylation forward primer, UF)
and 5’TAACTCACATACATAATAACAAAAATCCA-3’ (unmethylation reverse primer, UR). The MSP products were analyzed with 3% agarose gels. All the MSP procedures were performed more than twice.

Endpoints and follow-up

The primary endpoint was death from any cause. A 5-year follow-up on all participants was performed by a research nurse or dedicated physician. Any clinical events were known by telephone contact and/or direct interview and/or medical records. Disease-free survival (DFS) was also analyzed to explain the prognostic role of CDX2 methylation.

Statistical analysis

All data was analyzed with SPSS 18.0 software. The difference in categorical variables between methylation and unmethylation status was compared by \( \chi^2 \) test. The Kaplan-Meier method was adopted to analyze cumulative probability of survival and statistical significance was determined with log-rank test. Univariate and multivariate Cox analysis was performed to investigate whether the participants’ characteristics (age, gender, smoking status, TNM, histology and CDX2) could predict the prognosis of overall survival (OS). \( P \) value less than 0.05 indicated statistically significant difference.

Results

Basic information of patients with lung cancer

MSP method was used to detect the methylation status of CDX2 of all patients. The patients were classified into two groups according to CDX2 status: methylation (n=75) and unmethylation (n=92) groups. The results indicated that there were no significant differences in age, gender, smoking status, TNM, and histology between two groups (\( P > 0.05 \), Table 1).

Kaplan-Meier analysis
The Kaplan-Meier curve was shown in Figure 1. There were 33 deaths for patients with methylation of CDX2 and 14 deaths for patients with unmethylation. After the 5-year follow-up, the survival rate of patients with methylation of CDX2 was 56%, while the survival rate of patients with unmethylation of CDX2 was 84.8%. The outcome indicated that the survival rate of patients with unmethylation of CDX2 was significantly higher than those with methylation of CDX2 (Log-rank: \( P=0.000 \)).

In addition, we also plotted Kaplan-Meier curve for patients who smoke currently or ever smoke (Figure 2). Among the smoking patients, methylation of CDX2 resulted in much lower survival rate (50.9%), compared to unmethylation of CDX2 (84.6%) (Log-rank: \( P=0.000 \)). As shown in Figure 3, DFS of patients with CDX2 methylation was lower than those without CDX2 methylation (56.0% vs. 73.9%, \( P=0.009 \)).

**Univariate and multivariate Cox analysis**

Univariate and multivariate Cox analysis were performed to analyze the potential predictors for prognosis of patients with lung cancer. In univariate Cox analysis, we found that age, gender, smoking, TNM, histology were all not independent predictors for prognosis of patients with lung cancer (Table 2).

Further analysis by multivariate Cox method indicated that CDX2 methylation served as an independent prognostic predictor (HR=3.418, 95%CI=1.826-6.397) (Table 3).

**Discussion**

CDX2 expression is a marker of intestinal differentiation, which is expressed in any gastrointestinal cancer [27]. According to accumulated evidence, CDX2 is generally used as a specific marker for adenocarcinoma of the lower digestive tract [28-31]. It plays an important role in some relevant digestive system cancers, including gastric and colon cancer. Recent studies adopted the methods of real-time PCR and immunohistochemistry and found that CDX2 expression were detectable in primary lung adenocarcinomas except for the metastasis of colorectal origin [32, 33]. In addition, CDX2 mRNA expression could be detected in normal lung tissues of
patients with non-small-cell lung cancer (NSCLC), the level of which is much lower compared to the matched tumor tissues. Downregulation of CDX2 gene may result in the loss of relevant differentiation function and it may interact with other genes in the pathogenesis of relevant tumors.

It is well known that increased DNA methylation within the promoter regions of tumor suppressor genes has been associated with gene silencing in various cancers. It was reported that CDX2 methylation is frequently present in colorectal cancers and may play a key role in inactivating CDX2 expression [34]. The study by Wang et al. found that the methylation rate of the promoter region of CDX2 gene in normal colorectal tissue was 43.5%, whereas that in the lesion tissue of CRC was 78.5% [35]. Liu et al. reported that CDX2 is frequently methylated in lung cancer, and expression of CDX2 is regulated by promoter region hypermethylation [25].

Previous study by Jiang et al. reported that enhanced CDX2 promoter methylation is associated with enhanced lymph node metastases and shorter survival time in colorectal cancer [26]. Our study detected methylation status of CDX2 in patients with lung cancer with MSP method. The results showed that the methylated rate was 44.9%. Kaplan-Meier curve showed that methylation of CDX2 was related to poor prognosis of patients with lung cancer. Among the smoking patients, methylation of CDX2 resulted in much lower survival rate (50.9%), compared to unmethylation of CDX2 (84.6%). DFS of patients with CDX2 methylation was lower than those without CDX2 methylation (56.0% vs. 73.9%, P=0.009). Univariate and multivariate Cox analyses indicated that CDX2 methylation was an independent predictor for prognosis of patients with lung cancer. The results were similar with those of study by Bae et al. in colorectal cancer [36].

The study analyzed the prognostic role of CDX2 methylation in lung cancer among Chinese population. The results were credible and reliable, however, there were several limitations in the study. The function mechanism of CDX2 in lung cancer was not explored, which contributes to uncovering the pathogenesis of lung cancer. In addition, the related factors of CDX2 methylation were not analyzed, which might
provide potential therapeutic targets for lung cancer.

In conclusion, CDX2 methylation may serve as an independent predictor for poor prognosis of patients with lung cancer.

Disclosure

The authors report no conflicts of interest in this work.

References

1. Jemal A, Bray F, Center MM, et al. Global cancer statistics. CA Cancer J Clin 2011; 61:69-90.
2. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. CA Cancer J Clin 2013; 63:11-30.
3. Stewart DJ. Tumor and host factors that may limit efficacy of chemotherapy in non-small cell and small cell lung cancer. Crit Rev Oncol Hematol 2010; 75:173-234.
4. Kang K, Huang YH, Li HP, et al. Expression of UCA1 and MALAT1 long-chain non-coding RNAs in esophageal squamous cell carcinoma tissues is predictive of patient prognosis. Arch Med Sci 2018; 14: 752-9.
5. Li H, Wang R, M é ndez-S ánchez N, et al. Impact of spider nevus and subcutaneous collateral vessel of chest/abdominal wall on outcomes of liver cirrhosis. Arch Med Sci 2009; 15: 434-48.
6. Wang W, Song Z, Zhang Y. Efficacy of brain radiotherapy plus EGFR-TKI for EGFR-mutated non-small cell lung cancer patients who develop brain metastasis. Arch Med Sci 2018; 14: 1298-1307.
7. Özgen Arslan Solmaz. Prognostic significance of the tumor-stroma ratio in colon
carcinoma: a retrospective study. Archives of Medical Science - Civilization Diseases 2018; 3: e190-e194.

8. Brambilla E, Gazdar A. Pathogenesis of lung cancer signalling pathways: roadmap for therapies. Eur Respir J 2009; 33:1485-97.

9. Liu HN, Qie P, Yang G, et al. miR-181b inhibits chemoresistance in cisplatin-resistant H446 small cell lung cancer cells by targeting Bcl-2. Arch Med Sci 2018; 14: 745-51.

10. Silberg DG, Swain GP, Suh ER, et al. Cdx1 and cdx2 expression during intestinal development. Gastroenterology 2000; 119:961-971.

11. Moll R, Zimbelmann R, Goldschmidt MD, et al. The human gene encoding cytokeratin 20 and its expression during fetal development and in gastrointestinal carcinomas. Differentiation 1993; 53:75-93.

12. Werling RW, Yaziji H, Bacchi CE, et al. CDX2, a highly sensitive and specific marker of adenocarcinomas of intestinal origin: an immunohistochemical survey of 476 primary and metastatic carcinomas. Am J Surg Pathol 2003; 27:303-10.

13. Boudreau F, Rings EH, van Wering HM, et al. Hepatocyte nuclear factor-1 alpha, GATA-4, and caudal related homeodomain protein Cdx2 interact functionally to modulate intestinal gene transcription. Implication for the developmental regulation of the sucrase-isomaltase gene. J Biol Chem 2002; 277: 31909-17.

14. Sakaguchi T, Gu X, Golden HM, et al. Cloning of the human claudin-2 5'-flanking region revealed a TATA-less promoter with conserved binding sites in mouse and human for caudal-related homeodomain proteins and hepatocyte nuclear factor-1alpha. J Biol Chem 2002; 277: 21361-70.

15. Suh E, Wang Z, Swain GP, et al. Clusterin gene transcription is activated by caudalrelated homeobox genes in intestinal epithelium. Am J Physiol Gastrointest Liver Physiol 2001; 280: G149-56.
16. Bonhomme C, Duluc I, Martin E, et al. The Cdx2 homeobox gene has a tumour suppressor function in the distal colon in addition to a homeotic role during gut development. Gut 2003; 52:1465-71.

17. Gross I, Duluc I, Benameur T, et al. The intestine-specific homeobox gene Cdx2 decreases mobility and antagonizes dissemination of colon cancer cells. Oncogene 2008; 27:107-15.

18. Aoki K, Tamai Y, Horiike S, et al. Colonic polyposis caused by mTOR-mediated chromosomal instability in Apc+/Delta716 Cdx2+/- compound mutant mice. Nat Genet 2003; 35:323-30.

19. Guo RJ, Huang E, Ezaki T, et al. Cdx1 inhibits human colon cancer cell proliferation by reducing beta-catenin/T-cell factor transcriptional activity. J Biol Chem 2004; 279: 36865-75.

20. Ezaki T, Guo RJ, Li H, et al. The homeodomain transcription factors Cdx1 and Cdx2 induce E-cadherin adhesion activity by reducing beta- and p120-catenin tyrosine phosphorylation. Am J Physiol Gastrointest Liver Physiol 2007; 293: G54-65.

21. Guo RJ, Funakoshi S, Lee HH, et al. The intestine-specific transcription factor Cdx2 inhibits beta-catenin/TCF transcriptional activity by disrupting the beta-catenin-TCF protein complex. Carcinogenesis 2010; 31:159-66.

22. Guo M, House MG, Suzuki H, et al. Epigenetic silencing of CDX2 is a feature of squamous esophageal cancer. Int J Cancer 2007; 121:1219-26.

23. Yuasa Y, Nagasaki H, Akiyama Y, et al. Relationship between CDX2 gene methylation and dietary factors in gastric cancer patients. Carcinogenesis 2005; 26:193-200.

24. Grimminger P, Ling FC, Neiss S, Vallböhmer D, et al. The role of the homeobox genes BFT and CDX2 in the pathogenesis of non-small cell lung cancer. Anticancer Res 2009; 29:1281-6.
25. Liu X, Zhang X, Zhan Q, et al. CDX2 serves as a Wnt signaling inhibitor and is frequently methylated in lung cancer. Cancer Biol Ther 2012; 13: 1152-7.

26. Jiang G, Luo C, Sun M, et al. Methylation of CDX2 as a predictor in poor clinical outcome of patients with colorectal cancer. Genet Test Mol Biomarker 2016; 20: 710-4.

27. Sullivan LM, Smolkin ME, Frierson HF Jr, et al. Comprehensive evaluation of CDX2 in invasive cervical adenocarcinomas: immunopositivity in the absence of overt colorectal morphology. Am J Surg Pathol 2008; 32:1608-12.

28. Kaimaktchiev V, Terracciano L, Tornillo L, et al. The homeobox intestinal differentiation factor CDX2 is selectively expressed in gastrointestinal adenocarcinomas. Mod Pathol 2004;17:1392-99.

29. Moskaluk CA, Zhang H, Powell SM, et al. Cdx2 protein expression in normal and malignant human tissues: an immunohistochemical survey using tissue microarrays. Mod Pathol 2003; 16: 913-9.

30. Barbareschi M, Murer B, Colby TV, et al. CDX-2 homeobox gene expression is a reliable marker of colorectal adenocarcinoma metastases to the lungs. Am J Surg Pathol 2003; 27:141-9.

31. Werling RW, Yaziji H, Bacchi CE, et al. CDX2, a highly sensitive and specific marker of adenocarcinomas of intestinal origin: an immunohistochemical survey of 476 primary and metastatic carcinomas. Am J Surg Pathol 2003; 27: 303-10.

32. Moskaluk CA, Zhang H, Powell SM, et al. Cdx2 protein expression in normal and malignant human tissues: an immunohistochemical survey using tissue microarrays. Mod Pathol 2003; 16: 913-9.

33. Yatabe Y, Koga T, Mitsudomi T, et al. CK20 expression, CDX2 expression, K-ras mutation, and goblet cell morphology in a subset of lung adenocarcinomas. J Pathol 2004; 203: 645-52.
34. Kawai H, Tomii K, Toyooka S, et al. Promoter methylation downregulates CDX2 expression in colorectal carcinomas. Oncol Rep 2005; 13: 547-51.

35. Wang Y, Li Z, Li W, et al. Methylation of promoter region of CDX2 gene in colorectal cancer. Oncol Lett 2016; 12: 3229-33.

36. Bae JM, Lee TH, Cho NY, et al. Loss of CDX2 expression is associated with poor prognosis in colorectal cancer patients. World J Gastroenterol 2015; 21: 1457-67.
Figure legends

Figure 1. Overall survival rate of patients grouped by the methylation status of CDX2.

Figure 2. Overall survival rate of smoking patients grouped by methylation status of CDX2.

Figure 3. Disease free survival of patients grouped by the methylation status of CDX2.
Table 1. Clinicopathological characteristics of all participants.

| Characteristic          | CDX2 status         | P value |
|-------------------------|---------------------|---------|
|                         | Methylation, n(%)   | Unmethylation, n(%) |         |
| No.                     | 75 (44.91)          | 92 (55.09) |         |
| Age (years)             |                     | 0.780   |
| $\leq$ 60              | 44 (58.67)          | 52 (56.52) |         |
| $\geq$ 60              | 31 (41.33)          | 40 (43.48) |         |
| Gender                  |                     | 0.205   |
| Male                    | 40 (53.33)          | 58 (63.04) |         |
| Female                  | 35 (46.67)          | 34 (36.96) |         |
| Smoking status          |                     | 0.434   |
| Never                   | 21 (28.00)          | 27 (29.35) |         |
| Current                 | 7 (9.33)            | 4 (4.35) |         |
| Former                  | 47 (62.67)          | 61 (66.30) |         |
| TNM                     |                     | 0.825   |
| I                       | 19 (25.33)          | 25 (27.17) |         |
| II                      | 24 (32.00)          | 30 (32.61) |         |
| III                     | 31 (41.33)          | 34 (36.96) |         |
| IV                      | 1 (1.33)            | 3 (3.26) |         |
| Histology               |                     | 0.897   |
| Squamous cell carcinoma | 38 (50.67)          | 45 (48.91) |         |
| Adenocarcinoma          | 22 (29.33)          | 30 (32.61) |         |
| Small-cell lung cancer  | 15 (20.00)          | 17 (18.48) |         |

$^1$χ² test; TNM, tumor, nodes, metastases; $P<0.05$ is of noticeable difference.
Table 2. Univariate Cox analysis.

| Variables                        | HR    | 95%CI       | P value |
|----------------------------------|-------|-------------|---------|
| **Age** (≥60 vs. <60)            | 0.707 | 0.382-1.307 | 0.268   |
| Gender (female vs. male)         | 0.835 | 0.432-1.617 |         |
| **Smoking**                      |       |             |         |
| Never                            | Reference | Reference |         |
| Current                          | 1.678 | 0.361-7.789 | 0.509   |
| Former                           | 1.345 | 0.644-2.810 | 0.430   |
| **TNM**                          |       |             |         |
| I                                | Reference | Reference |         |
| II                               | 1.566 | 0.710-3.452 | 0.266   |
| III                              | 1.181 | 0.532-2.621 | 0.683   |
| IV                               | 2.612 | 0.533-12.797| 0.236   |
| **Histology**                    |       |             |         |
| Squamous cell carcinoma          | Reference | Reference |         |
| Adenocarcinoma                   | 1.103 | 0.546-2.230 | 0.784   |
| Small-cell lung cancer           | 0.792 | 0.334-1.880 | 0.597   |
| **CDX2** (methylation vs. unmethylation) | 3.705 | 1.922-7.139 | <0.001  |

TNM, tumor, nodes, metastases; HR, hazards ratio; CI, confidence interval; P<0.05 is of noticeable difference.
Table 3. Multivariate Cox analysis (LR).

| Variables                          | HR   | 95%CI      | P value |
|------------------------------------|------|------------|---------|
| CDX2 (methylation vs. unmethylation)| 3.418| 1.826-6.397| <0.001  |

Note: The analysis was performed with forward method (LR). HR, hazards ratio; CI, confidence interval; P<0.05 is of noticeable difference.
Overall survival

Time (months)

P=0.000

CDX2
- methylation
- unmethylation
- methylation-censored
- unmethylation-censored
CDX2

- methylation
- unmethylation
- methylation-censored
- unmethylation-censored

P=0.009

Disease free survival vs Time (months)