**ALKBH5 gene is a novel biomarker that predicts the prognosis of pancreatic cancer: A retrospective multicohort study**

Sung Hwan Cho¹⁺, Mihyang Ha²⁺, Yong Hoon Cho¹, Je Ho Ryu¹, Kwangho Yang¹, Kang Ho Lee¹, Myoung-Eun Han², Sae-Ock Oh²⁺†, and Yun Hak Kim²⁺†

¹Department of Surgery, Pusan National University Yangsan Hospital, ²Department of Anatomy, School of Medicine, Pusan National University, Yangsan, Korea

**Backgrounds/Aims:** Discovery of new prognostic factors for cases in which the pancreatic cancer scoring and staging system does not result in a clear definition is imperative. We examined the role of Human AlkB homolog H5 (ALKBH5) as a prognostic marker for pancreatic cancer.

**Methods:** Patient data were extracted from the International Cancer Genome Consortium (ICGC) and The Cancer Genome Atlas (TCGA). The prognostic value of ALKBH5 was confirmed via analysis of ALKBH5 and other clinical factors, such as age, sex, and stage, using the time-dependent area under the curve (AUC) of Uno's C-index, the AUC value of the receiver operating characteristics (ROC) at three years, the Kaplan-Meier survival curve, and multivariate analysis.

**Results:** ALKBH5 showed excellent prognosis prediction in comparison with existing markers in the two independent cohorts (n=262). Kaplan-Meier survival analysis showed that ALKBH5 expression was positively associated with overall survival (log-rank test, ICGC, \( p = 0.001 \); TCGA, \( p = 0.01 \)). Notably, comparison of C-index and AUC values in ROC analysis showed that ALKBH5 was associated with high C-index and AUC values compared with other clinical variables (C-index: ICGC, 0.621; TCGA, 0.614 and AUC at three years: ICGC, 0.609; TCGA, 0.558). Multivariate analysis demonstrated that ALKBH5 is an independent prognostic factor (ICGC, \( p = 0.0123 \); TCGA, \( p < 0.001 \)).

**Conclusions:** These findings contribute to the study of RNA methylation in pancreatic cancer. We believe that ALKBH5 is a new prognostic marker for pancreatic cancer. (Ann Hepatobiliary Pancreat Surg 2018;22:305-309)

**Key Words:** ALKBH5; RNA methylation; Pancreatic cancer; Prognosis

**INTRODUCTION**

Pancreatic neoplasms are one of the few cancers with a mortality rate approaching 100%. Furthermore, only 15-20% of patients are diagnosed during early stages because of nonspecific symptoms and approximately 50% present with distant metastasis at the time of diagnosis. As the etiology and screening test for this highly lethal disease have yet to be well defined, it is important to identify genetic factors that contribute to the development of this cancer.

N⁶-methyladenosine (m⁶A) is the most prevalent epitranscriptomic modification of the mRNA of higher eukaryotes. Human AlkB homolog H5 (ALKBH5) is a demethylase that oxidatively reverses several mRNAs, and significantly affects mRNA export and RNA metabolism, as well as the assembly of mRNA processing factors in nuclear speckles. This modification plays an essential role in the regulation of mRNA translation and RNA metabolism. Evidence strongly suggests that epigenetic alterations, especially methylation induce tumorigenesis-associated cellular changes, suggesting the role of oncogenic mechanisms beyond DNA mutations in cancer.

The methylation of related genes and mRNA has been strongly linked to the development and progression of pancreatic cancer. The regulation of the expression of oncogenic and tumor suppressor genes by demethylation or methylation mechanisms plays a significant role in identi-
fied tumorigenesis. Thus, studies investigating epigenetic changes associated with pancreatic cancer are very important for the development of new diagnostic and therapeutic methods.

Based on the function of *ALKBH5* as an mRNA methylation regulator, we hypothesized that *ALKBH5* influences the survival of patients with pancreatic cancer. Therefore, we examined the role of *ALKBH5* as a novel prognostic marker complementing the current deficient staging and scoring systems.

**MATERIALS AND METHODS**

**Data acquisition and characteristics**

Primary and processed data, including mRNA expression and clinical information, were downloaded from The Cancer Genome Atlas (TCGA) and International Cancer Genome Consortium (ICGC) in October 2017.

These processes were performed using the *cgdsr* package of R software version 3.5.0 (The R Foundation for Statistical Computing, 2018). The flow of this study described in Fig. 1.

**Survival analysis**

Survival analyses were performed to predict the overall survival (OS) using three methods: [1] Kaplan-Meier survival curves to evaluate the accuracy of the discrimination, [2] Uno’s C-index for the time-dependent area under the curve (AUC) analysis, and [3] AUC values in receiver operating characteristics (ROC) at three years. These analyses were performed using R packages *survival* and *survAUC*. C-indices and AUC values ≥0.6 were considered acceptable for survival predictions. Using Kaplan-Meier analyses, we determined the optimal cutoff value with the maximal Uno’s C-index by 5-fold cross-validation. We used univariate and multivariate Cox regression to compare the effect of *ALKBH5* on prognosis along with other clinical variables. Statistical analyses were performed using R software.

**RESULTS**

**Patient distribution**

In total, 172 TCGA and 90 ICGC patients were included in this study. Of the 172 patients in the TCGA...
Sung Hwan Cho, et al. *ALKBH5* gene is a novel biomarker for pancreatic cancer

Table 1. Patients’ information used in current research in the TCGA and ICGC cohorts

| Group          | ICGC | TCGA |
|----------------|------|------|
| *ALKBH5*       |      |      |
| All patients   | 90   | 172  |
| High expression| 61 (32) | 112 (54) |
| Low expression | 29 (26) | 60 (38) |
| Patients’ information |      |      |
| Male           | 47   | 94   |
| Female         | 43   | 78   |
| Stage I & II   | -    | 164  |
| Stage III & IV | -    | 8    |

**ALKBH5 survival curve**

To identify the discriminatory power of *ALKBH5* as a categorical variable, we analyzed Kaplan-Meier curves for *ALKBH5* gene expression and survival. High expression of *ALKBH5* was a significant predictor of overall survival in the TCGA and ICGC cohorts (*p*=0.01 and *p*=0.001, Fig. 2A, D). The prognostic value was further confirmed using multivariate analysis (ICGC, *p*=0.0123; TCGA, *p* <0.001, Table 2).

**ALKBH5 C-index and AUC values**

To evaluate the prognostic value of *ALKBH5*, we compared this gene with other clinical factors, such as age, stage, and sex, in both cohorts. We analyzed gene expression values as continuous variables using Uno’s C-indices and AUC values at three years. *ALKBH5* showed high C-index values in the two independent cohorts compared with the other factors (TCGA: 0.614, ICGC: 0.621, Fig. 2B, E). The three-year AUC value was also significantly higher than that of the other factors across the two cohorts (ICGC, 0.609; TCGA, 0.558, Fig. 2C, F).

![Fig. 2. Survival analyses of the ICGC and TCGA cohorts. (A, D) Kaplan-Meier estimates of overall survival of pancreatic cancer patients according to *ALKBH5* gene expression. (B, E) Time dependent area under the curve (AUC) according to *ALKBH5* gene expression. (C, F) Receiver operating characteristic (ROC) curve at three years according to *ALKBH5* gene expression.](image)
Table 2. Univariate and multivariate analysis of overall survival in each cohort

| Parameters | Univariate analysis | Multivariate analysis |
|------------|---------------------|----------------------|
|            | p       | HR 95 Cl     | p       | HR 95 Cl     |
| ICGC       |         |              |         |              |
| ALKBH5     | 0.00146** | 0.4187 0.2449 0.7157 | <0.001*** | 0.3974 0.2305 0.685 |
| Sex        | 0.421   | 1.24 0.7335 2.097 | 0.251   | 1.4027 0.7871 2.500 |
| Age        | 0.881   | 0.9979 0.9702 1.026 | 0.9429  | 1.0011 0.9709 1.032 |
| TCGA       |         |              |         |              |
| ALKBH5     | 0.011*  | 0.5812 0.3826 0.8829 | 0.0123* | 0.5847 0.3841 0.8902 |
| Stage      | 0.716   | 0.8072 0.2545 2.561 | 0.5214  | 0.6846 0.2150 2.1799 |
| Sex        | 0.39    | 0.8354 0.5543 1.259 | 0.4164  | 0.8426 0.5575 1.2735 |
| Age        | 0.0136* | 1.0261 1.005 1.047   | 0.0119* | 1.027 1.0508 1.0480 |

**DISCUSSION**

In this study, we investigated the survival rate of patients diagnosed with pancreatic cancer based on the ALKBH5 gene expression using TCGA and ICGC databases. Patients in the two cohorts were comparable in age and sex but not significantly. As ICGC is an ongoing project, additional patient information will be released in the future. Survival analysis demonstrated that high ALKBH5 expression showed good discriminatory power compared with age, sex, and stage.

Less than 10% of patients diagnosed with pancreatic cancer present with resectable status and the overall 5-year survival is <4%.3,13 Patients with small, surgically resectable cancers have a realistic chance of a cure and exhibit 5-year survival rates of approximately 20-30%.3,13-16 Although many studies tried to predict the prognosis of patients with pancreatic cancer based on clinical and genetic variables, CA-19-9 is the only clinical biomarker approved by the United States Food and Drug Administration.17,18 The poor prognosis of pancreatic cancer is attributed to its late presentation and lack of accurate predictive biomarkers for early diagnosis and prognosis.19 In the current study, ALKBH5 facilitated the classification of patients into two risk groups in two independent cohorts. In addition, it also scored higher C-index compared with stage and age that are important prognostic factors. In particular, the AUC value of ALKBH5 remained constant regardless of the follow-up time, which provides a stable prediction of prognosis of the patient.

The past several years have witnessed a vast increase in our knowledge regarding epigenetic features in human cancers.20,21 M6A RNA methylation is regulated by several molecules such as methylases (METTL3, METTL14, WTAP) and demethylases (FTO, ALKBH5)20-23 that act as oncogenes and/or tumor suppressor genes in various cancers. High expression of ALKBH5, a well-known m6A demethylase, leads to oncogenic progression in glioblastoma and breast cancer by stabilizing FOXM1, NANOG, and KLF4 mRNA. In breast cancer, hypoxia in the cancer progenitor cells induces high expression of ALKBH5 via activation of hypoxia-inducible factors. Thus, the m6A modification level is reduced, which increases the stability and expression of NANOG and KLF4, which are involved in the development of breast cancer stem cells. In the case of glioblastoma, m6A modification modulates methylases such as METTL3 or METTL14, which increases the expression of oncogenes such as KLF4. ALKBH5 regulates FOXM1 gene expression and the altered expression affects GSC tumorigenesis via the FOXM1 axis.20-23 The discovery of new biomarkers via elucidation of these RNA methylation mechanisms is expected to facilitate pancreatic cancer diagnosis and prognosis by complementing the current chromosomal and nucleotide methods.

With increasing evidence suggesting the role of epigenetic alterations especially methylation in triggering tumorigenesis-associated cellular changes, the field of cancer research has evolved to incorporate oncogenic mechanisms beyond DNA mutations. In particular, the importance of m6A in mRNA has attracted attention due to its role in epitranscriptomic modification of cellular differentiation and pluripotency. However, the effect of modification of m6A mRNA on the complex process of tu-
morigenesis is unclear.

In conclusion, we found that the survival rate of patients with pancreatic cancer was enhanced under high ALKBH5 expression. Despite the unclear mechanism of action of ALKBH5 in tumorigenesis, it has been suggested that ALKBH5 may have a positive effect on the development and progression of pancreatic cancer. We believe that these findings will contribute to the study of RNA methylation in pancreatic cancer.

ACKNOWLEDGEMENTS

This study was supported by Busan Cancer Center Research Grant (2018), Pusan National University Hospital. This work was supported by grants from Basic Science Research Program through the National Research Foundation of Korea (NRF grant funded by the Korea government (MOE, NRF-2016R1A6A3A11931738, NRF-2016R1A2B4014593).

REFERENCES

1. Lowenfels AB, Maisonneuve P. Epidemiology and risk factors for pancreatic cancer. Best Pract Res Clin Gastroenterol 2006;20:197-209.
2. Eto S, Ishikawa M, Asanoma M, Tashiro Y, Matsuyama K, Oshio T. A long-term survival case of advanced biliary cancer with repeated resection due to recurrence in the pancreaticoduodenectomy site after pancreaticoduodenectomy. Ann Hepatobiliary Pancreat Surg 2018;22:173-177.
3. Kim HS, Jang JY, Han Y, Lee KB, Joo I, Lee DH, et al. Survival outcome and prognostic factors of neoadjuvant treatment followed by resection for borderline resectable pancreatic cancer. Ann Surg Treat Res 2017;93:186-194.
4. Wei P, Tang H, Li D. Insights into pancreatic cancer etiology from pathway analysis of genome-wide association study data. PLoS One 2012;7:e46887.
5. Jaffrey SR, Kharas MG. Emerging links between m6A and mis-regulated mRNA methylation in cancer. Genome Med 2017;9:2.
6. Zheng G, Dahl JA, Niu Y, Fdorecsak P, Huang CM, Li CJ, et al. ALKBH5 is a mammalian RNA demethylase that impacts RNA metabolism and mouse fertility. Mol Cell 2013;49:18-29.
7. Xu C, Liu K, Tempel W, Demetriades M, Aik W, Schofield CJ, et al. Structures of human ALKBH5 demethylase reveal a unique binding mode for specific single-stranded N6-methyladenosine RNA demethylation. J Biol Chem 2014;289:17299-17311.
8. Neureiter D, Jäger T, Ocker M, Kiesslich T. Epigenetics and pancreatic cancer: pathophysiology and novel treatment aspects. World J Gastroenterol 2014;20:7830-7848.
9. Feng C, Liu Y, Wang G, Deng Z, Zhang Q, Wu W, et al. Crystal structures of the human RNA demethylase Alkbh5 reveal basis for substrate recognition. J Biol Chem 2014;289:11571-11583.
10. Cancer Genome Atlas Research Network, Weinstein JN, Collisson EA, Mills GB, Shaw KR, Ozenberger BA, et al. The Cancer Genome Atlas Pan-Cancer analysis project. Nat Genet 2013;45:1113-1120.
11. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov 2012;2:401-404.
12. Zhang J, Baran J, Cros A, Guberman JM, Haider S, Hsu J, et al. International Cancer Genome Consortium Data Portal—a one-stop shop for cancer genomics data. Database (Oxford) 2011;2011:bart026.
13. Kleef J, Michalski C, Friess H, Büchler MW. Pancreatic cancer: from bench to 5-year survival. Pancreas 2006;33:111-118.
14. Sato N, Goggins M. The role of epigenetic alterations in pancreatic cancer. J Hepatobiliary Pancreat Surg 2006;13:286-295.
15. Kern S, Hruban R, Hollingsworth MA, Brand R, Adrian TE, Jaffe E, et al. A white paper: the product of a pancreas cancer think tank. Cancer Res 2001;61:4923-4932.
16. Yeo CJ, Cameron JL, Lillemoe KD, Sitzmann JV, Hruban RH, Goodman SN, et al. Pancreatocutaneous ductoencanctomy for cancer of the head of the pancreas. 201 patients. Ann Surg 1999;221:721-731.
17. Winter JM, Yeo CJ, Brody JR. Diagnostic, prognostic, and predictive biomarkers in pancreatic cancer. J Surg Oncol 2013;107:15-22.
18. Fong ZV, Winter JM. Biomarkers in pancreatic cancer: diagnostic, prognostic, and predictive. Cancer J 2012;18:530-538.
19. Nalls D, Tang SN, Rodova M, Srivastava RK, Shankar S. Targeting epigenetic regulation of miR-34a for treatment of pancreatic cancer by inhibition of pancreatic cancer stem cells. PLoS One 2011;6:e24099.
20. Cui Q, Shi H, Ye P, Li L, Qu Q, Sun G, et al. m6A RNA methylation regulates the self-renewal and tumorigenesis of glioblastoma stem cells. Cell Rep 2017;18:2622-2634.
21. Batista PJ. The RNA modification N6-methyladenosine and its implications in human disease. Genom Proteom Bioinform 2017;15:154-163.
22. Zhang S, Zhao BS, Zhou A, Lin K, Zheng S, Lu Z, et al. m6A demethylase ALKBH5 maintains tumorigenicity of glioblastoma stem-like cells by sustaining FOXM1 expression and cell proliferation program. Cancer Cell 2017;31:591-606.e6.
23. Zhang C, Zhi WI, Lu H, Samanta D, Chen I, Gabrielson E, et al. Hypoxia-inducible factors regulate pluripotency factor expression by ZNF217- and ALKBH5-mediated modulation of RNA methylation in breast cancer cells. Oncotarget 2016;7:64527-64542.