High mobility group AT-hook 2 and c-MYC as potential prognostic factors in pancreatic ductal adenocarcinoma

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Abstract. The present study investigated if c-MYC and high mobility group AT-hook 2 (HMGA2) expression was associated with prognosis of patients with pancreatic ductal adenocarcinoma (PDAC). A total of 102 patients undergoing surgery for PDAC were retrospectively reviewed. Immunohistochemistry was used to detect c-MYC and HMGA2 protein expression in PDAC and peritumoral tissue samples. Expression of c-MYC and HMGA2 was associated with clinicopathological characteristics and prognoses of patients with PDAC using multivariate analysis. HMGA2 and c-MYC protein expression was significantly higher in PDAC tissues compared with peritumoral tissue (P<0.001). HMGA2 and c-MYC expression was also significantly higher in patients with PDAC who had lymph node metastasis, invasion of regional tissues and tumor node metastasis (TNM) stage III or IV disease compared with those who had no lymph node metastasis, no invasion of regional tissues and TNM stage I or II disease (P<0.001). Multivariate logistic regression analysis was used to identify TNM stage (P=0.007) and invasion (P=0.003) as significant independent predictors of c-MYC expression (model AUC=0.8201), and lymph node metastasis (P=0.002) and invasion (P=0.003) as significant independent predictors of HMGA2 expression (model AUC=0.7638). Cox multivariate analysis showed that expression of c-MYC (P=0.019) and HMGA2 (P=0.001), TNM stage (P=0.014) and lymph node metastasis (P=0.032) were associated with reduced overall survival time. HMGA2 and c-MYC may be important biological markers and potential therapeutic targets involved in the tumorigenesis, metastasis, invasion and prognosis of PDAC.

Key words: pancreatic ductal adenocarcinoma, immunohistochemistry, c-MYC, high mobility group AT-hook 2

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the most common type of malignant pancreatic tumor and is the fourth leading cause of cancer-associated mortality worldwide in 2014 (1-3). Surgical resection remains the only optimal treatment regimen for patients with PDAC (4). Due to the paucity of symptoms in the early stages, most PDACs are diagnosed at advanced stages, resulting in low resectability (5,6). PDAC has a high recurrence rate, even in the small number of patients who undergo surgery, as it easily invades blood vessels and lymphatic tissue and has a tendency to disseminate along nerve fibers (7). In addition, PDAC produces dense desmoplastic stroma that consists of activated pancreatic stellate cells (PSCs) and proliferating fibroblasts surrounding the tumor cells which inhibits drug penetration and uptake (8-12). The overall survival (OS) time for patients with PDAC is ~1 year and the 5-year OS rate is <1.0% (13,14). The OS rate of patients with PDAC has not improved significantly despite intense research efforts being made to develop chemotherapy, radiotherapy and patient-targeted therapeutic strategies in recent years (15-17). There is an urgent need to find reliable prognostic biomarkers and new targets for future treatment.

c-MYC is one of the most frequently deregulated oncogenes and is located on the long arm of chromosome 8 (8q24), which encodes for the c-MYC protein, an important transcription factor involved in the regulation of protein synthesis, cellular metabolism and tumor growth and proliferation (18-20). Previous studies have demonstrated that abnormal expression of c-MYC is implicated in many malignancies such as Burkett's lymphoma, diffuse large B-cell lymphoma and breast cancer (21,22), c-MYC upregulation is frequently associated with poor clinical outcome (23). There have also been a few reports related to PDAC (18,24).

The high mobility group AT-hook 2 (HMGA2) protein is encoded by the HMGA2 gene, and is a member of the high mobility group (HMG) protein family and non-histone chromatin-binding protein family (25,26). HMGA2 protein has a DNA-binding domain located in the N-terminal region and three short basic repeats, the so-called AT-hooks, which bind to the minor groove of AT-rich DNA sequences (27,28). Once bound to DNA, HMGA2 interacts with various transcription factors to modulate gene transcription and alter...
chromatin structure, regulate cell growth, differentiation, apoptosis and DNA repair (29,30). HMGA2 protein is highly expressed during embryonic development and is expressed at low levels in adult tissues (26,31). High expression of HMGA2 has been detected in most human malignancies, including colorectal cancer, Wilms’ tumor and PDAC, and is associated with higher lymph node metastasis rates and poor tumor differentiation (32-34).

To date, no systematic study has investigated the relationship between the expression of HMGA2 and c-MYC and PDAC. In the present study, the expression of c-MYC and HMGA2 in resected specimens, including adenocarcinoma and peritumoral tissue, was examined using immunohistochemistry. The association of c-MYC and HMGA2 levels with the prognosis of PDAC was evaluated. This study suggests that c-MYC and HMGA2 are promising prognostic biomarkers and potential therapeutic targets in PDAC.

Materials and methods

Case selection. A total of a 102 PDAC and 93 peritumoral tissues were obtained at the First Affiliated Hospital, Army Medical University (Chongqing, China) between March 2013 and September 2015. This study was pre-approved by the Ethics Committee for Human Study of Army Medical University (approval no. KY201802) and oral consent was previously obtained from the family members of the patients included in the study. The ethics committee waived the requirement for further written informed consent for this study. Clinical information collected included: Gender, age, tumor location, tumor size, degree of tumor differentiation, tumor staging, regional lymph node metastasis, invasion to surrounding organs and serum CA19-9 level. Tumor staging, regional lymph node metastasis, invasion and serum cancer antigen (CA) 19-9 level were based on the 8th edition American Joint Committee on Cancer (AJCC) standard criteria (35,36). Survival information was obtained through letters and phone calls from patients with PDAC. Peritumoral tissue (n=93) was collected ≥2 cm from the tumors. A flowchart of the process is presented in Fig. 1.

Immunohistochemistry. All tissues were treated with 10% formaldehyde for 24-48 h at room temperature, and embedded in paraffin. For immunohistochemistry, 3-µm thick sections were mounted on poly-L-lysine-coated slides, deparaffinized with xylene three times for 5 min, and hydrated through graded alcohol. The sections were washed with three changes of PBS for 15 min, and incubated with xylene three times for 5 min, and hydrated through graded alcohol. The sections were washed with three changes of PBS for 15 min, and incubated with horseradish peroxidase-conjugated goat anti-mouse/rabbit IgG compound (1:50; cat. no. KIT-9903; Fuzhou Maixin Biotech Co., Ltd) for 1 h at room temperature. Following three washes with PBS for 15 min, the sections were incubated with DAB solution (Beyotime Institute of Biotechnology) for 10 min at room temperature. Finally, samples were counterstained with hematoxylin (Beyotime Institute of Biotechnology) for 2 min at room temperature. A total of 500 cells from 10 random fields per section were examined by 2 independent observers. The mean of the percentages from these 2 observers was used for final evaluation. Cases with ≥25% positive cells were considered positive, whereas other cases were considered negative (positive controls were provided by Protein Tech Group, Inc.) (37). Results were visualized using an Olympus light microscope (Olympus Corporation) at x400 magnification.

Statistical analysis. All data were analyzed with SPSS 18.0 (SPSS, Inc.). Continuous variables were summarized as mean ± standard deviation (SD) and the categorical variables were described as percentage. Protein expression of c-MYC and HMGA2 was compared between PDAC and peritumoral tissue samples using McNemar’s test. The association of c-MYC and HMGA2 expression with histological or clinical factors was analyzed using the χ² test. The correlation between c-myc and HMGA2 expression was tested by the contingency coefficient test. Multiple logistic regression analysis was subsequently used to determine the association between histological or clinical factors and c-MYC and HMGA2 protein expression. In multivariate logistic regression analysis, factors with P<0.15 in the univariate model were entered into the initial model. The backward elimination method was used to select the final predictive model. At each step, factors with P>0.05 were eliminated. A receiver operating characteristic curve for the model was constructed and the area under the curve (AUC) was calculated. The OS of patients with PDAC was analyzed using Kaplan-Meier univariate survival analysis and log-rank tests. Multivariate analysis was performed with Cox proportional hazards model and 95% confidence intervals (CI) were calculated. P<0.05 was considered to indicate a statistically significant difference.

Results

Characteristics of the study population. The 102 PDAC specimens were obtained from 58 males (56.9%) and 44 females (43.1%), with a mean age of 53.20±9.96 years. Preoperative computerized tomography (CT) imaging showed that 62 PDACs (60.8%) were located at the head of the pancreas and 40 (39.2%) at the body or tail of the pancreas. The diameter of the lesions was ≤3 cm in 21 cases (20.6%), 3-5 cm in 55 cases (53.9%) and >5 cm in 26 cases. The histopathological subtypes included 67 poorly-differentiated adenocarcinomas (65.7%), 35 moderately-differentiated adenocarcinomas (34.3%) and 0 well-differentiated adenocarcinomas. Of the cases, 58 (56.9%) were stage T1+T2 and 44 (43.1%) were stage T3+T4. Of the patients, 43 (42.2%) had regional lymph node metastasis, 53 (52.0%) had invasion to surrounding organs and tissues and 72 (70.6%) had serum CA19-9 level >37 U/ml (Table I).

Protein expression of c-MYC and HMGA2 in PDAC and peritumoral tissues. Representative preoperative CT images of PDAC and immunohistochemical staining of tissues are presented in Fig. 2. Only 93 paired PDAC and peritumoral tissue were included. Of the PDAC tissues, 50 (53.8%) were c-MYC positive and 47 (50.5%) were HMGA2 positive. Of the peritumoral tissue, 23 (24.7%) were c-MYC positive and
25 (26.9%) were HMGA2 positive. The positive rates for c-MYC and HMGA2 protein expression were significantly higher in PDAC tissue samples compared with peritumoral tissue samples (P<0.001, respectively; Fig. 3A).

**Association of c-MYC and HMGA2 protein expression with clinicopathological characteristics of patients with PDAC.** The protein expression of c-MYC and HMGA2 exhibited no significant association with age, sex, tumor differentiation, size and location and serum CA19-9 level (P>0.05; Table II). The positive rate of c-MYC and HMGA2 expression was significantly higher in PDAC patients with lymph node metastasis, invasion to surrounding tissues and organs and TNM stage III or IV disease compared with PDAC patients with no lymph node metastasis, no invasion, and TNM stage I and II disease (P≤0.001; Table I). Expression of c-MYC was positively correlated with HMGA2 (the contingency coefficient is 0.210, P=0.030). Multivariate logistic regression analysis revealed that TNM stage [odds ratio (OR), 5.097; 95% CI, 1.546-16.805; P=0.007] and invasion (OR, 5.249; 95% CI, 1.734-15.886; P=0.003) were predictors of c-MYC protein expression. The AUC was 0.8201 (95% CI, 0.7345-0.9056; P<0.0001; Table II; Fig. 3B). Lymph node metastasis (OR, 4.147; 95% CI, 1.653-10.407; P=0.002) and invasion (OR, 3.811; 95% CI, 1.556-9.336; P=0.003) were predictors of HMGA2 protein expression compared with no lymph node metastasis and no invasion. The AUC for predicting HMGA2 expression was 0.7638 (95% CI, 0.6705-0.8572; P<0.0001; Table II; Fig. 3C).

**HMGA2 and c-MYC protein expression and clinicopathological characteristics associated with OS in patients with PDAC.** OS time ranged from 1-30 months, with a mean of 9.86±7.99 months. Kaplan-Meier survival analysis revealed that lymph node metastasis, TNM stage, tumor invasion, c-MYC and HMGA2 protein expression were significantly associated with reduced OS time of patients with PDAC (P<0.01; Table III). The mean OS time for c-MYC or HMGA2-positive patients was significantly lower than for c-MYC or HMGA2-negative patients (P<0.01; Fig. 3D-F). Cox multivariate showed that with stepwise regression analysis, TNM stage, lymph node metastasis, and C-MYC and HMGA2 protein high expression were negatively associated with the mean OS time (Table IV).

**Discussion**

PDAC remains a major therapeutic challenge with a poor prognosis due to a limited understanding of the molecular and genetic mechanisms and the potential therapeutic targets of PDAC. Though some targets have been investigated, no effective treatment for PDAC has been discovered. A previous study investigated the interaction of PDAC with its microenvironment. The stroma surrounding the tumor and its cellular components, PSCs, provides a protumorigenic microenvironment associated with tumor hypoxia, hypovascularization and epithelial-mesenchymal transition (8). The
stroma also lowers the concentration of chemotherapeutic agents in the tumor, confers chemoresistance and affects tumor metabolism (38). Other studies have identified heterogeneity in PDAC, as well as the presence of cancer stem cells, which may lead to primary resistance to chemotherapeutic drugs and may be a key factor for tumor recurrence (39,40). These factors have led to the failure of PDAC to respond to most conventional chemotherapeutic drugs (13). Therefore, KRAS was once considered as a potential therapeutic target in PDAC. Unfortunately, there is no therapeutic intervention that can target the KRAS mutation that leads to activation and subsequently block the downstream pathways (41,42).

Epidermal growth factor receptor (EGFR) is a member of the ERBB receptor tyrosine kinase (TK) family. EGFR contains an extracellular N-terminal ligand-binding domain, a transmembrane region and a C-terminal intracellular domain that includes the kinase domain and multiple phosphorylation sites (43). Several ligands, including EGF-α, transforming growth factor (TGF)- and amphiregulin, can bind to EGFR, which can then homodimerize or heterodimerize with other ERBB receptors resulting in autophosphorylation of specific TK residues on the receptor (44-46). The EGFR pathway is associated with different cancer-associated cellular features, such as proliferation, adhesion, neoangiogenesis and apoptosis. EGFR plays an important role in carcinogenesis, is upregulated in 30-89% of PDACs and tends to predict poor

| Variable               | c-MYC expression | HMGA2 expression |
|------------------------|------------------|------------------|
| Age, years             |                  |                  |
| ≤45                    | 37               | 59.0%            |
| >45                    | 65               | 56.9%            |
| Sex                    |                  |                  |
| Male                   | 58               | 51.7%            |
| Female                 | 44               | 59.1%            |
| Differentiation        |                  |                  |
| Moderately             | 35               | 60.0%            |
| Poorly                 | 67               | 52.2%            |
| Tumor size, cm         |                  |                  |
| ≤3                     | 21               | 61.9%            |
| 3-5                    | 55               | 56.4%            |
| >5                     | 26               | 46.2%            |
| Location               |                  |                  |
| Head                   | 62               | 59.7%            |
| Body/tail              | 40               | 47.5%            |
| Lymph node metastasis  |                  |                  |
| No                     | 59               | 39.0%            |
| Yes                    | 43               | 76.7%            |
| Invasion               |                  |                  |
| No                     | 49               | 26.5%            |
| Yes                    | 53               | 81.1%            |
| Tumor node metastasis  |                  |                  |
| stage                  | 30               | 18.9%            |
| Serum cancer antigen   |                  |                  |
| 19-9, U/ml             | 0.042            | 0.839            |

Table I. Association of c-MYC and HMGA2 protein expression with clinicopathological characteristics of pancreatic ductal adenocarcinoma.
In view of this, therapeutic targeting of EGFR seems to be a promising strategy, however, so far results have not been optimistic. A clinical trial demonstrated that patients receiving erlotinib plus gemcitabine had a median survival time of 6.24 months compared with 5.91 months in the gemcitabine plus placebo arm, with an absolute difference in median survival time <1 month (47). This may be due to induction of EGFR-independent tumor-induced angiogenesis, activation of alternative TK receptors that bypass the EGFR signaling pathway, mutations in EGFR or loss of the target, or many other resistance mechanisms to EGFR inhibitors (48). New targets are urgently needed to guide clinical treatment. The present study demonstrated that c-MYC and HMGA2 upregulation are significantly associated with progression and prognosis of PDAC.

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**c-MYC** is a proto-oncogene that encodes a nuclear transcription factor. c-MYC protein regulates expression of many genes involved in cell cycle progression and cell growth, and drives the cell cycle by promoting progression from G1 to S phase and G2 to M phase (49,50). c-MYC protein expression and gene activation by amplification have been described in a wide variety of malignancies and tend to predict poor prognosis, especially in lymphoma (21,22,50). Other studies have shown that c-MYC plays an important role in the aggressiveness of PDAC (18,51). The present study demonstrated that the rate of positive c-MYC expression was significantly higher in...
In PDAC, positive c-MYC expression was significantly associated with lymph node metastasis, invasion to surrounding tissues and organs, and high TNM stage. Patients with PDAC who had positive c-MYC expression had a worse survival compared to those with negative expression. Similarly, HMGA2 expression was also associated with poor prognosis, with patients having positive HMGA2 expression having a shorter mean survival time. These findings highlight the importance of c-MYC and HMGA2 expression as potential biomarkers for PDAC, as well as potential targets for therapeutic intervention.
c-MYC expression had a shorter OS time compared with patients who had negative c-MYC expression.

HMGA2 is a non-histone protein that acts as a transcription factor by altering chromatin architecture to regulate gene transcription (52,53). A previous study has shown that HMGA2 protein plays a role in malignant cell transformation and the progression of several tumor types, and is associated with poor prognosis (54). Another study demonstrated that HMGA2 may be a direct transcriptional target of TGF-β, and may influence epithelial-mesenchymal transition and participate in tumor invasion and metastasis (55). Several studies have shown that HMGA2 has oncogenic activity, and can enhance the self-renewal capability of tumor stem cells and promote tumorigenesis (56,57). Another study has shown that HMGA2 upregulation is significantly associated with tumor dedifferentiation (58). Previous studies on HMGA2 expression and PDAC have shown that HMGA2 upregulation is associated with poor tumor differentiation, lymph node metastasis and invasion (34,58). The present study demonstrated that the rate of positive HMGA2 expression in PDAC tissues was significantly higher compared with peritumoral tissues. HMGA2 expression was not associated with tumor differentiation, but positive expression was significantly associated with lymph node metastasis, tumor invasion and high TNM stage. Patients with PDAC who had positive HMGA2 expression had shorter survival times compared with patients who had negative HGMA2 expression.

The present study had some limitations. Firstly, the sample size was small and there were no cases of well-differentiated adenocarcinoma, which could have affected the results. Secondly, the possible synergistic effect between c-MYC and HMGA2 expression was not thoroughly investigated. Thirdly, this study only considered c-MYC and HMGA2 protein expression at the immunohistochemical level and did not investigate mRNA expression. These limitations should be addressed in future studies.

In summary, expression of c-MYC and HMGA2 maybe important predictive factors for the prognosis of patients with PDAC. c-MYC and HMGA2 may be useful biomarkers and potential therapeutic targets in PDAC.

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Availability of data and materials
The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors’ contributions
KL and JY were major contributors in writing the manuscript and analyzing the patient data. JC, YS, ZZ and WC made substantial contributions to study design, data analysis and interpretation and manuscript organization. All authors read and approved the final manuscript.

Ethics approval and consent to participate
This study was approved by the Human Ethics Committee of the Army Medical University (Chongqing, China) (approval
no. KY201802). All information is stored in the databases of the First Affiliated Hospital of Army Medical University and utilized for research purposes. Oral consent was obtained from the family members of the patients included in the study. The ethics committee waived the requirement for further written informed consent for this study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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