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ORIGINAL ARTICLE

Long non-coding RNA DANCR regulates MLL3 and thereby it determines the progression of pancreatic cancer

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Summary

Purpose: *Pancreatic cancer is a most lethal disease with low survival rates and therefore understanding its molecular level development for early diagnosis is key for designing improved therapeutic strategies. The long non-coding RNAs DANCR were reported as oncogenes, which are upregulated in many cancer types including pancreatic cancer. The role of DANCR and its correlation with tumour suppressor protein MLL3 expression are keen to understand different pathological stages of pancreatic cancer.*

Methods: *The role of long non-coding RNAs DANCR in correlation with MLL3 was studied using histology, in situ hybridization, immunohistochemistry and Western blotting. TM00314 strain of mutant mice in KRAS G12A and MPL gene were used since they are able to develop initial and advanced stage of pancreatic cancer after 3 and 5 months of growth.*

Results: *The initial pancreatic cancer tissue showed low grade of dysplasia with diffusion of the solid nature of cells and in advanced stages giant cells and foci formation was observed. The expression of DANCR showed gradual upregulated expression as pancreatic cancer progressed. However, the expression of MLL3 was upregulated in the initial pancreatic condition, but its expression was restricted in advanced stages of pancreatic cancer. Additionally, the signals for MLL3 RNA expression were more when compared with the context of protein expression.*

Conclusions: *The results show that MLL3 was overexpressed in the initial pancreatic cancer to restrict cancer progression and in which DANCR had no role in regulating MLL3 but in advanced stages it downregulated MLL3.*

Key words: *pancreatic cancer, MLL3, DANCR, KRAS G12A, MPL, histopathology*

Introduction

In global level the occurrence of pancreatic cancer is quite common and accounts for the 7th most lethal cancer mortality [1]. Early detection of pancreatic cancer and starting timely treatment is the key factor in reducing mortality associated with pancreatic cancer [2]. Mutation in susceptible genes like ATM, BRCA2, PALB2, BRCA1, CDKN2A, STK11, MLH1, MSH6, MSH2, and PRSS1 are known to contribute in the development of pancreatic cancer [3, 4]. In most cases (60-70%) pancreatic adenocarcinoma arises from the pancreatic head and the remaining occurs in the body (15%) and tail (15%) of the pancreatic gland. Secondly, at the time of pancreatic cancer diagnosis metastases are more common and especially nodal metastases [5]. In the present condition, early detection of pancreatic cancer is crucial for improving the therapeutic and clinical outcomes.

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The long non-coding RNAs (lncRNAs) are a group of transcribed RNA with length greater than 200 nucleotides that significantly play an important role in many human diseases and particularly in different types of cancers [6-8]. Differentiation antagonizing the non-protein coding RNA (DANCR) is one of the novel IncRNAs identified recently, that plays potential role in developing various cancers which includes pancreatic cancer [9-12]. DANCR, normally called oncogene, was significantly upregulated in pancreatic cancer tissue and pancreatic cancer cell lines and it was also observed that knockdown of DANCR inhibits growth and metastasis of this disease [9]. However, the complete biological function associated with different genes linked with pancreatic cancer is completely unrevealed.

Mixed Lineage Leukemia 3 (MLL3) is known for its tumour suppressor function and is involved in histone methylation, thereby it activates, or suppress specific gene functions [13]. Also, reports are available that MLL3 act as a tumour suppressor in pancreatic cancer inhibiting MLL3 function to promote tumour growth in mice model and cell lines [14]. MLL3 act as a co-activator of p53 and thereby it enhances the expression of many genes that co-ordinately activate p53 gene [15]. In the present study, the underlying link between IncRNAs DANCR in regulating MLL3 were revealed in different pathological stages of pancreatic cancer.

Methods

Mouse model developed with pancreatic cancer

The pancreatic mouse model TM00314 which possesses mutations in KRAS G12A and Myeloproliferative Leukaemia Gene (MPL) gene was purchased from Jackson Laboratory (Shanghai, China) and housed in the laboratory environment. KRAS gene mutation is able to turn the mice into oncogenic form which later aids in developing pancreatic cancer in mouse models [16]. By rearing the KRAS and MPL gene mutated mice for 3 months (n=12) and 5 months (n=12) respectively, the different pathological stages of pancreatic cancer mice were able to attain. All mice that were used in the present experiments and the protocol followed were pre-approved by the institutional ethical committee. All the mice were maintained in the cage at a laboratory temperature between 26 to 28°C. The mice were fed with regular laboratory feed and water ad libitum throughout the experimental period of 3 months and 5 months respectively and then sacrificed for further analysis.

Histology and immunohistochemistry

The dissected pancreatic gland obtained from different stages of pancreatic cancer developed mice were initially cut into smaller pieces and fixed with 10% formalin solution. Following dehydration and clearing step, the tissues were paraffin-embedded and subjected to histological sectioning (6µm). The tissue in the wax ribbon was processed and finally stained with hematoxylin and eosin for visualizing the abnormalities. For immunohistochemistry, the paraffin-embedded tissue was processed as described earlier [17]. After dewaxing, the thin sections sized 6µm had their endogenous peroxidase activity blocked using 10% H_2O_2 solution in methanol for 20 min. Before incubating with primary antibody, the non-specific sites were blocked using 4% bovine serum albumin (BSA) solution in 1X TBST buffer. The primary antibody anti-MLL3 (Abcam, Hong Kong, China ab40973; 1:400 dilution) diluted in 1X TBST buffer was placed over the slides and incubated for 6 h in 4°C. Following immunoreactivity, the non-specific binding of primary antibody was washed out and further incubated with secondary antibody (Abcam, Hong Kong, China ab205718; 1: 3000 dilution) for 2 h at room temperature. The quantitative signals against MLL3 were obtained by developing it with DAB solution, which gives brown colour signals against the MLL3 protein. The final images obtained were documented using light microscope.

In situ hybridization

The expression of Long non-coding RNA DANCR was probed using Digoxigenin (DIG)-labelled corresponding RNA probe. Initially, formalin-fixed and parrafin-embedded tissues were sliced to 4µm sized sections. For initiating antigen retrieval, protease 3 and CCI buffer were used. Next, DIG labelled DANCR probe or MLL3 probe were incubated for 6 h at 66°C in a RiboHybe diluent solution and after that the non-specific binding probes were eliminated by increasing the temperature to 70°C. Finally, HRP-conjugated anti-digoxigenin antibody (Abcam, Hong Kong, China ab51949; 1:100 dilution) was used to detect the Long non-coding RNA DANCR or MLL3 expression. For negative controls, DIG labelled sense strand against DANCR or MLL3 was used.

Western blot analysis

The dissected pancreatic tissue was immediately transferred to ice-cold condition and the protein samples were prepared by crushing them in 2X sample buffer along with protease inhibitor. Following Coomassie plus protein assay, the wells of stacking gel in the 10% SDS-PAGE gel were loaded with equal loading of 60µg of protein concentration and the protocol was followed as previously described [18]. The resolved protein in the gel was transferred to polyvinylidene difluoride membrane and blocked with 3% bovine serum albumin (BSA) solution in 1X TBST buffer for 2 h at room temperature. The membranes were then incubated with primary antibody against anti-MLL3 antibody (Abcam, Hong Kong, China, ab40973; 1:500 dilution) for 6 h at 4°C. After washing the membrane with 1X TBST for 4 times the membranes were further incubated with secondary antibody (Abcam, Hong Kong, China ab205718; 1: 4000 dilution) and developed with DAB solution.

Statistics

All of the experiments were performed in triplicate. The experimental data were analyzed through SPSS 25.0 statistical package and expressed as mean ± SD. The significance of the data was analysed using Student's t-test, followed by Tukey's *post hoc* test. P value<0.05 showed statistical significance.

Results

Histological features of pancreatic cancer developing mice

In KRAS and MPL gene mutated mice, the initiation of pancreatic cancer was observed histologically after 3 months of growth in laboratory environment. In the dissected control pancreatic tissue and the solid nature of tissue were maintained and the cells were tightly bound to the cellular matrix (Figure 1A) but in the initial stage of pancreatic cancer developed mice the cellular integrity was disturbed and observed with low grade dysplasia (Figure 1B). In particular, in the initial stage of pancreatic cancer the cell size increased and observed with diffusion of cells along with loss of matrix tissue (Figure 1B). The mice grown for 5 months in the laboratory were observed with high grade of dysplasia in which giant cells were identified with large vacuolization and nuclear atypia (Figure 1C).

Analysing the expression of long non-coding RNA DANCR and MLL3 through in situ hybridization

In order to understand the expression of long non-coding RNA DANCR and MLL3, *in situ* hybridization was carried out using digoxigenin labeled RNA probe as described in the method section of this manuscript. In normal pancreatic tissue the expression of long non-coding RNA DANCR was restricted to a very limited quantity (Figure 2A), but as the initial pancreatic cancer developed its expression was upregulated which was predominantly observed in abnormal cells (Figure 2B). In advanced-stage disease, the expression of long non-coding RNA DANCR was further upregulated in almost every cell with high intensity signals (Figure 2C). In order to understand the link between long non-coding RNA DANCR and MLL3 expression, *in situ* hybridization was also performed for MLL3. In the control tissue, the hybridization signal against MLL3 was observed in the inhibitory level with expression observed in some cells only (Figure 2D), but as pancreatic cancer initiated the expression of MLL3 it was altogether upregulated in the majority of cells (Figure 2E). In prolonged tumour condition of 5 months, the MLL3 signals were almost completely lost in the advanced pancreatic tissue structures (Figure 2F).

MLL3 expression as pancreatic cancer progress 0

To understand the MLL3 expression at the protein level and localize its expression within the cell immunohistochemistry experiments were carried out in different pathological condition of pancreatic cancer. In the control tissue, the MLL3 expression was observed in limited cells of the pancreatic tissue (Figure 3A) but in the early stages of tumorigenic condition, the MLL3 was observed to increase to a critical level (Figure 3B). But the condition of MLL3, a tumour suppressor protein lacked its upregulated expression pattern as pancreatic cancer progressed to the next advanced level (Figure 3C). Interestingly, overall, we observed MLL3 expression in abnormal cells in the boundary of loci. To further confirm the results Western blotting experiments were performed against anti-MLL3 antibody. In the pancreatic tissue extracts the MLL3 showed

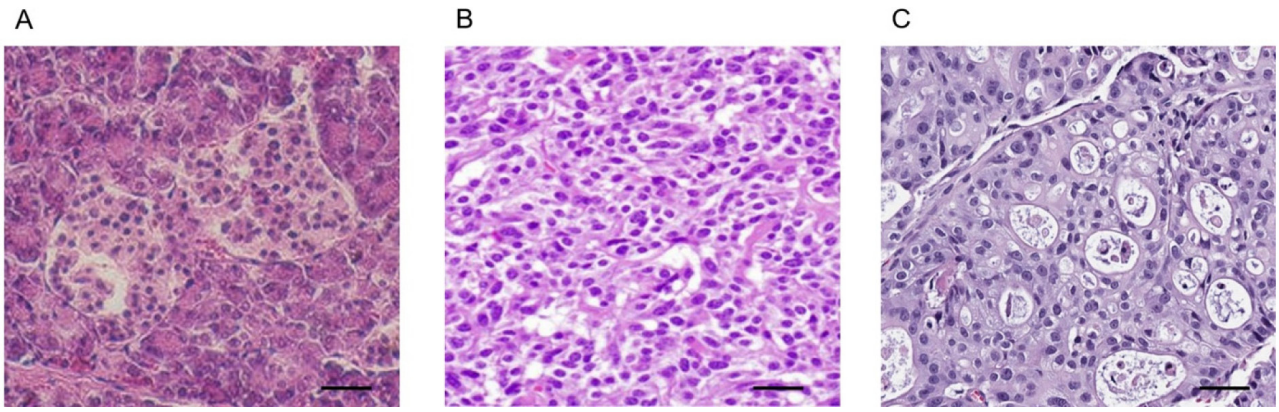

Figure 1. Histopathological variations observed in KRAS G12A and MPL mutated mice. **A:** Control mice show no abnormal arrangement in pancreatic tissue pattern. **B:** Initial stage of pancreatic cancer shows cellular abnormalities with a low grade of dysplasia. **C:** Advanced stage of pancreatic cancer shows high grade of dysplasia. H & E stain. Scale bar-50 µm.

a similar expression pattern with that of immunohistochemistry results. The control pancreatic tissue showed an optimal level of MLL3 expression in the tissue extract, but as pancreatic cancer progressed to initial stages the expression of MLL3 was uplifted (3.2 folds) with maximum expression level (Figure 4). However, the expression of MLL3 appeared to a restricted level in advanced stages of pancreatic cancer (Figure 4).

Discussion

The key aspects of mice models for any cancer studies is that it effectively help assess the disease genetics, variations and help assess the severity of the disease conditions as much as very close to clinical data. Pancreatic cancer induction in KRAS mutated mice generally appears with aggressive cancer, which is difficult to recover [19,20]. There-

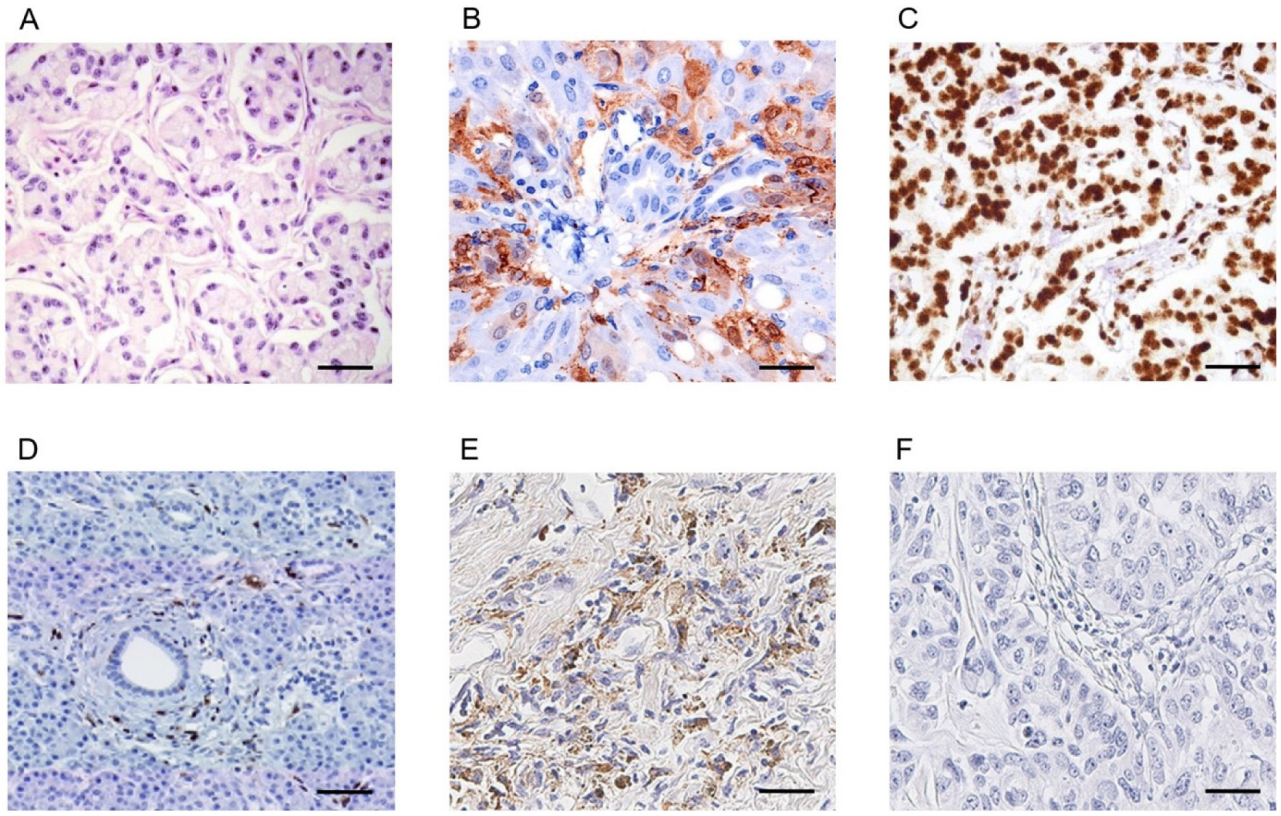

Figure 2. *In situ* hybridization with DANCR and MLL3. **A:** Portion of pancreatic tissue shows an optimal level of DANCR expression in control mice. **B:** Initial conditions of pancreatic cancer developed mice shows a gradual increase in DANCR expression. **C:** Advanced stage of pancreatic cancer shows elevated expression of DANCR. **D:** Portion of pancreatic tissue shows an optimal level of MLL3 expression in control mice. **E:** Initial stage of pancreatic cancer shows upregulated expression of MLL3. **F:** Advanced stage of pancreatic cancer shows no expression level of MLL3. H&E stain. Scale bar-50μm.

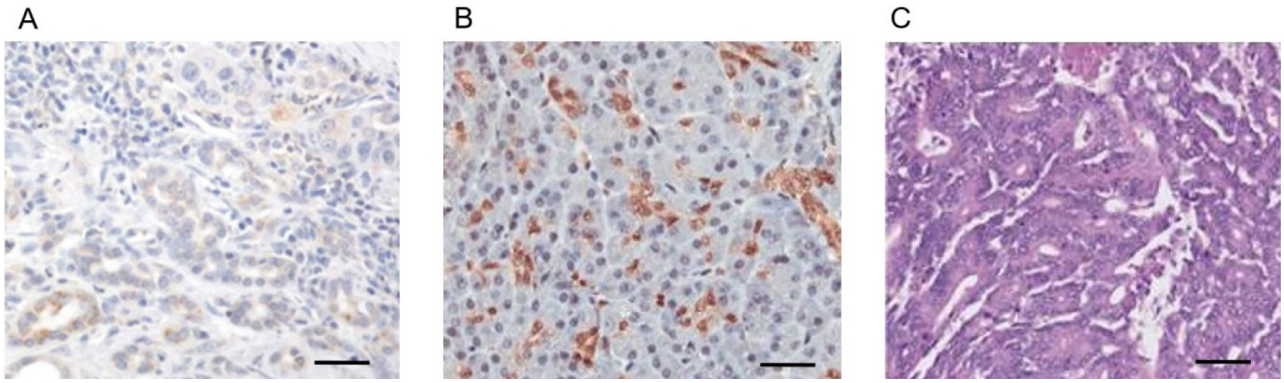

Figure 3. Immunohistochemistry with MLL3 protein. **A:** Normal pancreatic tissue shows an optimal level of MLL3 expression in control mice. **B:** Initial stage of pancreatic cancer shows upregulated expression of MLL3. **C:** Advanced stage of pancreatic cancer shows restricted expression level of MLL3. H&E stain. Scale bar-50μm.

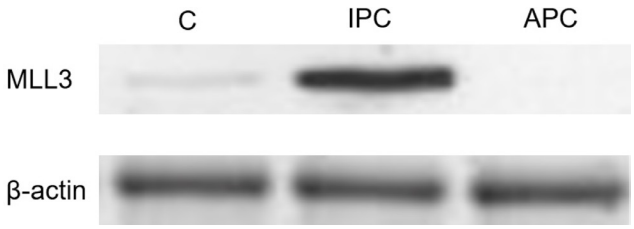

Figure 4. Analysing MLL3 expression pattern using Western blotting. Lane 1 represents the minimal expression of MLL3 in control tissue. Lane 2 shows a significant increase in MLL3 in initial stage of pancreatic cancer. Lane 3 represents quantification of MLL3 in advanced stage of pancreatic cancer which represents a downregulated expression pattern. For loading control β-actin was used. IPC represents "initial stage pancreatic" cancer and APC represents "advanced stage pancreatic cancer".

fore, the results obtained in the present study effectively prove the depth of long non-coding RNA DANCR and MLL3 expression as a natural occurrence of cancers. The genetically engineered TM00314 which possesses mutations in KRAS G12A and MPL helps understand the complex nature of pancreatic cancer with respect to long non-coding RNA DANCR and MLL3 expression.

With increase in a 3-5 month growth condition of mice, the pancreatic cancer grading was also complexed which was revealed through histopathological studies. The histomorphology variations like low and high grade of dysplasia, giant cells, nature of solid tissue were less helping to assess the progressive nature of pancreatic cancer [21]. The expression of long non-coding RNA DANCR was reported as an oncogene in a variety of human cancers [10,22]. In the present study to understand its molecular level expression in different pancreatic cancer stages, *in situ* hybridization experiments were carried out which revealed its constant upregulation pattern as cancer progressed. The *in vivo* experimental results showed similar results with recent findings [9] and in the present experiments we studied DANCR expression in different pathological conditions of pancreatic cancer which showed gradual upregulation. Also, the present investigation helped assess the long non-coding RNA DANCR expression in linking with MLL3 protein.

MLL3 acts as a tumour suppressor protein and it was also reported that it acts as a DNA repair components in cancer [23]. The expression of MLL3 was upregulated in initial pancreatic cancer which implies its role in restricting the tumour development and involved in regulating its DNA repair in abnormal cancer cells. Our investigation clearly showed that the MLL3 is expressed predominantly in abnormal cells, which revealed its key role as a tumour suppressor. But in advanced stage of pancreatic cancer the MLL3 expression was downregulated to critical level, which implies its lack of control over advanced pancreatic cancer. When comparing MLL3 expression in *in situ* hybridization and immunohistochemical studies, we observed MLL3 signals were more on transcription level, but restricted in translational level and from the results it was concluded that a different mechanism is operating to regulate the MLL3 protein expression. The results obtained through immunobiological studies were further cross-verified in the tissue extract by performing Western blotting, which also led to similar MLL3 expression.

Overall, using genetically modified strain of mice (TM00314), we developed an effective pancreatic cancer model which was evaluated through histopathological studies. Long non-coding RNA DANCR showed gradual overexpression pattern as pancreatic cancer progressed to initial and advanced stages. MLL3 expression in RNA and protein level showed upregulated expression in the initial condition of pancreatic cancer, but its expression was restricted as prostatic cancer progressed. Overall, we revealed the long non-coding RNA DANCR had less control over MLL3 in initial pancreatic cancer.

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Conflict of interests

The authors declare no conflict of interests.

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