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PKR2 and β-catenin genes regulates pancreatic cancer chemosensitivity

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Abstract. – OBJECTIVE: Pancreas is a well developed glandular organ lying behind the stomach. Cancer arises in this organ are difficult to identify in the initial stages, even in advanced stages it shows non-specific symptoms, and it is difficult to prognosis. Since they are identified and treated in the last stage, they are less responsive to chemotherapy. Therefore, it is important to study the proteins that are involved in regulating chemosensitivity and chemoresistance.

MATERIALS AND METHODS: Initially, using KRAS mutant mice, we developed initial and advanced stage of pancreatic cancer. And we analyzed the expression of PKR2 and β-catenin in different pathological stages of pancreatic cancer using Immunohistology and Western blotting.

RESULTS: The histology of the tissue nature confirms and helps to categorize cancer, which shows enlarged nucleus in initial stages and shows clustering of cells in advanced stages. Immunohistological and Western blotting analyzes show prominent increasing in the expression of PKR2 and β-catenin as the tumor develops to the next stages. On the course of initial treatment with cisplatin we find out that PKR2 and β-catenin regulate the chemosensitivity with under-expression when compared with respective controls. In the advanced stages of pancreatic cancer with cisplatin treatment, we observed chemoresistance behavior with overexpression, especially for β-catenin.

CONCLUSIONS: The results conclude that using PKR2 and β-catenin we are able to assess the chemosensitivity and chemoresistance nature of pancreatic cancer.

Key Words:
PKR2, β-catenin, KRAS, Chemosensitivity.

Introduction

Pancreatic cancer is one of the leading forms of cancer that results with high mortality[1]. Once pancreatic cancer is identified, it is hard to treat because it is diagnosed mostly in the advanced stage. At that stage, the therapy lacks of efficacy[2]. The major reasons point out for developing pancreatic cancer are smoking[3], family history of pancreatic cancer[4], obesity, advanced ageing, high-fat diet, periodontal disease[5]. The 5-year survival rate of pancreatic cancer accounts for only 20%. In the advanced stages with metastasis, the survival rate[6] is only 2%. Pancreatic cancer occurs due to molecular level changes in cancer controlling genes and one particularly important gene in that is TP53 which codes for P53 protein. It was reported that 75% of pancreatic cancer are due to a mutation that accumulates in TP53 gene, that results with impaired P53 which lacks the ability to control cell proliferation and apoptosis[7,8].

Chemotherapy with multiple drug combination together with radiotherapy has an impressive result in increasing the survival of the patient[9,10]. But there is a problem associated with it regarding the drug availability and lack in large group studies in the view point of side effects[8]. Besides this the dose range of drugs in different stages of pancreatic cancer needs to be studied with a large group of patients in the different background are essential to access the chemosensitive and chemoresistance response to different drugs. The drug sensitivity for pancreatic cancer is achieved by key proteins associated with it, for example, SPARC helps in the delivery of paclitaxel thereby improving its sensitivity[11].

Prokineticin receptor 2 (PKR2) is a protein that binds with Prokineticin 2 and carries out various biological activities in the complicated microenvironment of tumorigenesis[12]. Particularly, it has a wide effect on gastrointestinal motility, circadian

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rhythm, neurogenesis, angiogenesis, nociception and hematopoiesis[13]. β-catenin play a critical role in development, homeostasis, cell-cell adhesion, activating transcriptional factors and also have a diverse function by interacting with non-Wnt signalling pathway[14]. Recently, it was revealed that developing chemoresistance against Wnt signalling is a major reason for developing pancreatic cancer[15]. But studies with a different experimental setup with a diverse pathological stage are critical to understand the molecular mechanism behind it.

Materials and Methods

Mouse Model with Pancreatic Cancer

The mice model (TM00314) with a mutation in KRAS G12A and MPL was purchased from Jackson Laboratory. The mutation in KRAS gene can develop pancreatic cancer[16]. To study pancreatic cancer in different pathological stages, one group of KRAS and MPL mice was allowed to grow up to two months and sacrificed to study the earlier effects associated with PKR2 and β-catenin. Another group of mice was maintained for four months and later sacrificed to study the changes in advanced stages. For treatment purposes and to study chemosensitivity, cisplatin was given for ten days for initial stages of pancreatic cancer and for fifteen days for aggressive cancer in a dose range of 8 mg/kg, and they are further analyzed. The animals subjected to the present investigation and the protocols followed were approved by the Animal Care Committee of the host organization.

Immunohistochemistry and Antibody

The dissected pancreatic samples from control, initial and aggressive stages of pancreatic cancer are subjected to fixation with 10% formaldehyde solution. After washing the tissue they are subjected to dehydration, clearing and paraffin embedded as described in previous protocol[17]. The paraffin embedded tissues are sliced using microtome into 5 µm size and place on a slide warming table for dewaxing. To prevent non-specific signals, the endogenous peroxidase activity was blocked using 10% H_2O_2. Following treating with 4% bovine serum albumin (BSA), the tissue sections are incubated with an anti-PKR2 antibody, Biorbyt (orb336449) or anti β-catenin antibody, ab32572 (Abcam, Cambridge, UK) for overnight at 4ºC. After washing the tissue for two times, they are further incubated with suitable secondary antibody for 1 hour at room temperature. The slides are then washed to prevent non-specific binding of antibody and incubated with a diaminobenzidine (DAB) solution. The signals developed are visualized and documented using a microscope.

Western Blot Analysis

To examine the expression of PKR2 and β-catenin in various samples of control, earlier and advanced stage of pancreatic disease of mice tissue, Western blotting was performed. The dissected tissue was homogenized and resolved in 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel as already described [[18]]. The proteins that are resolved are transferred to a polyvinylidene fluoride (PVDF) membrane and blocked with 4% BSA solution to prevent non-specific binding of the antibody. After that, the membrane is incubated with primary antibody of anti-PKR2 antibody, Biorbyt (orb336449) or anti β-catenin antibody, ab32572 (Abcam, Cambridge, UK) for overnight at 4ºC. The membrane is then washed with 1X TBS-T (tris buffered saline-tween) and it is incubated with the corresponding secondary antibody for 2 hours at room temperature. After washing, the membrane is washed to develop the signal.

Statistical Analysis

The experiments are repeated for more than three times and statistical significant was achieved. The significant differences among the data were estimated using student's t test. The statistical data are represented as mean ± SEM. The p values were calculated and considered as statistically significant when p value < 0.05.

Results

Histological Variation Observed Between Control, Earlier and Advanced Stages of Pancreatic Cancer

The KRAS gene mutated mice was grown for two months and four months respectively and were histologically observed for any changes in the pancreatic tissue along with the control. The results with the control tissue of pancreas show the homogeneous arrangement of tissue pattern (Figure 1A). The mice at the end of the 2nd month show pancreatic tissue abnormalities with a disturbed tissue layer along with enlarged nucleus (Figure 1B). The morphology of the tissue emphasizes the initial pancreatic cancer. The mice that are maintained for up to four months shows

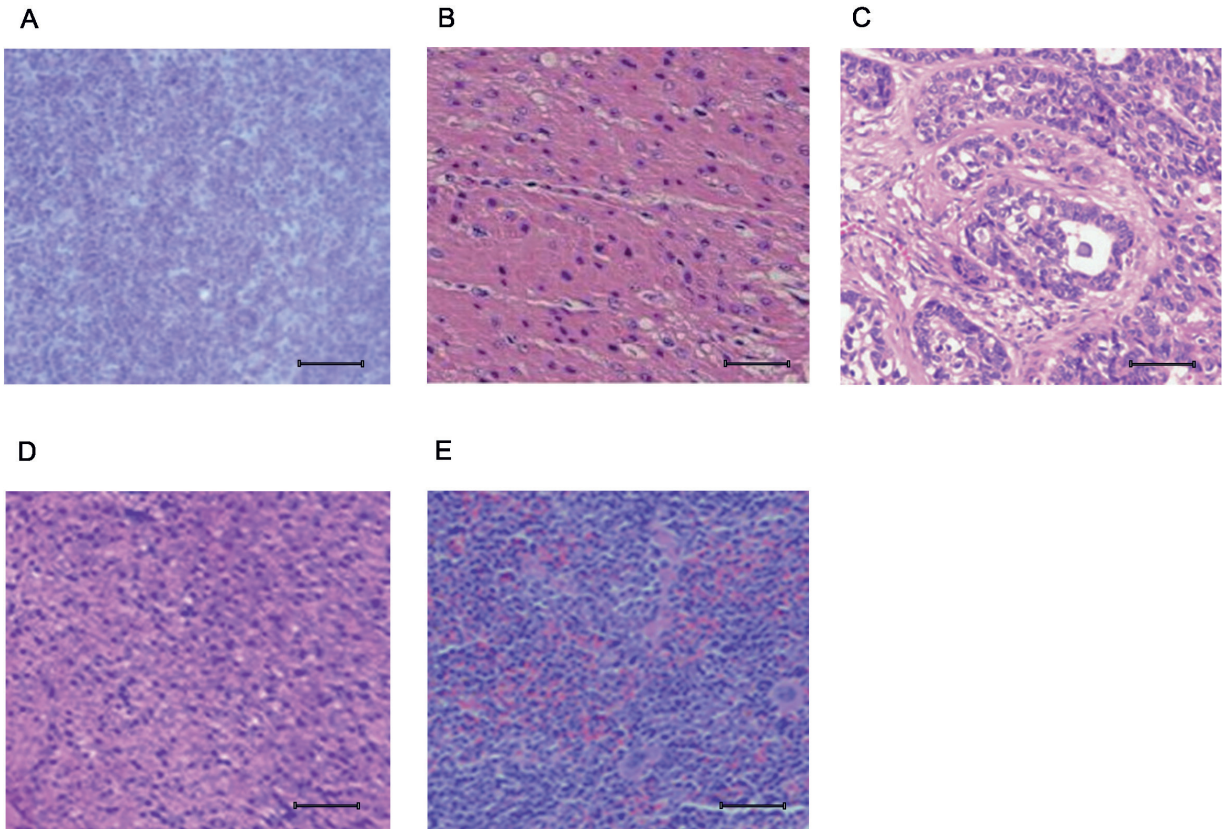

Figure 1. Mice model with pancreatic cancer. ***A,*** Histological image of control pancreatic tissue with a regular pattern of cell arrangement. ***B,*** Histology of initial stages of pancreatic cancer tissue with enlarged nucleus. ***C,*** Histology of aggressive pancreatic cancer tissue with intense lesions together with clustered cells. ***D,*** Histology of the initial stages of pancreatic cancer treated with cisplatin shows prominent improvement with rearrangement of cells. ***E,*** Histology of aggressive pancreatic cancer tissue treated with cisplatin shows minimum recovery with slight spreading of clustered cells Scale Bar denotes 50 μm size.

adverse changes in the tissue pattern with clustering of cells that result with tissue hardening (Figure 1C). Following treatment with cisplatin the initial form of pancreatic cancer revert as it begins to rearrange the tissue pattern as evident from the histological data (Figure 1D). The mice in advanced stage show minimal recovery as it dislocate clustering of cells, but shows clustering in minute pockets of cells (Figure 1E).

Assessing the Expression of PKR2 and β-Catenin in Different Pathological Stages of Pancreatic Cancer

To analyze the role of PKR2 and β-catenin expression in different stages of pancreatic cancer the mice were sacrificed and their dissected pancreatic tissue was subjected to Immunohistological analysis by following the protocol as mentioned in materials and methods. PKR2 mediate signal transaction associate with the binding of prokineticins and it is involved in activating many intracellular signalling pathways[19,20]. Here we find out that the expression of PKR2 shows prominent expression in the control tissue (Figure 2A) because lacking of which results with many abnormalities include the irregular development of reproductive and olfactory system[21]. Although PKR2 is essential to regulate the normal developmental process, we observed that its elevated expression is associated with initiation of pancreatic cancer that is clearly observed in the KRAS gene mutated mice that are maintained for 2 months (Figure 2B). The PKR2 expression shows the magnitude of overexpression in the advanced stage of pancreatic cancer mice (Figure 2C). On the course of treatment with cisplatin, the initial form of cancer responds well and its chemosensitivity is assessed with decreased with the expression of PKR2 (Figure 2D). On the other hand, the PKR2 shows threshold decrease in expression in the cisplatin-treated mice with advanced pancreatic cancer (Figure 2E).

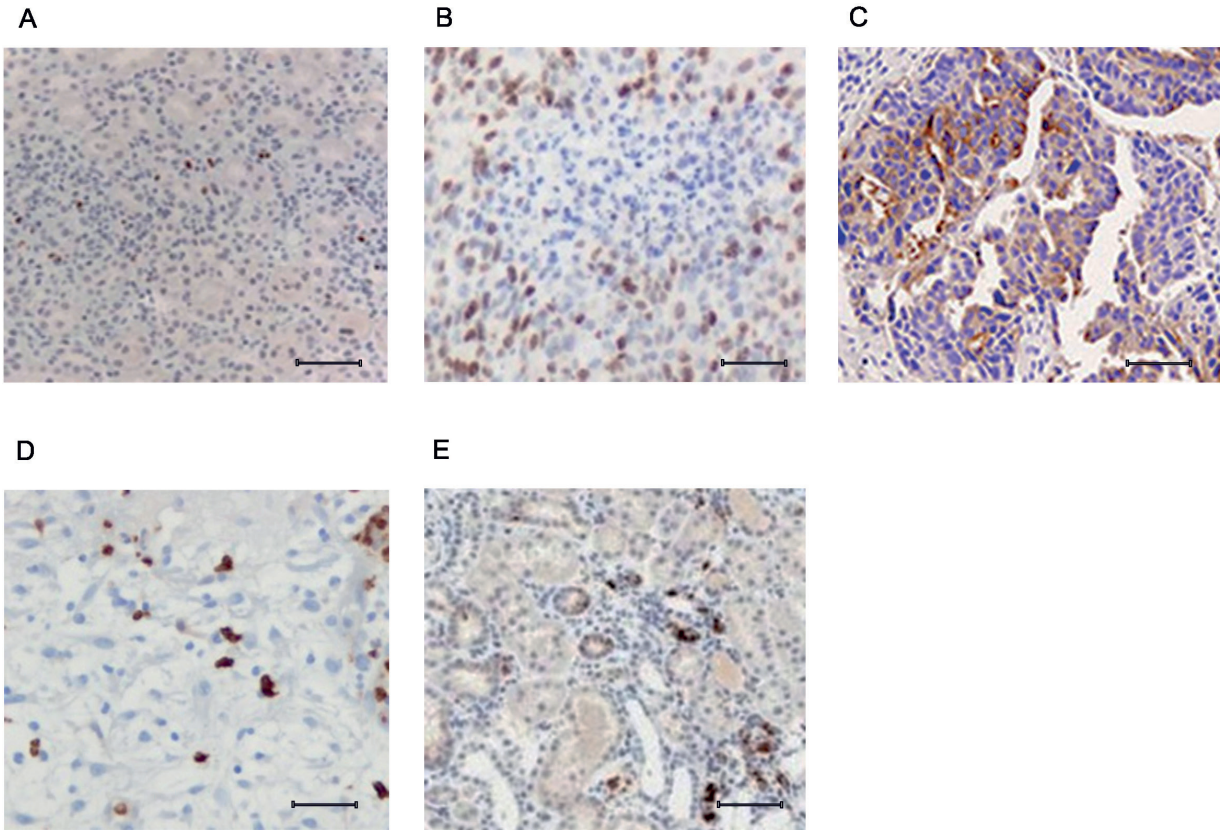

Figure 2. Expression of PKR2 in different pathological stages of pancreatic cancer. ***A,*** PKR2 that regulates normal functions of the pancreas shows slight expression in the control tissue. ***B,*** Initial pancreatic cancer with increased expression of PKR2. ***C,*** Aggressive pancreatic cancer shows over expression of PKR2. ***D,*** Initial stage of pancreatic cancer treated with cisplatin shows decreased expression of PKR2. ***E,*** Aggressive stage of pancreatic cancer treated with cisplatin shows over expression of PKR2. Scale Bar denotes 50 μm size.

Similarly, the β-catenin expression also shows a prominent increasing pattern as the tumor advanced to the next level. The control tissue shows a prominent expression of β-catenin to maintain normal cellular activity in control tissue (Figure 3A) and on tumor stimulation its expression gets upregulated constantly as shown in the initial (Figure 3B) and advanced stages (Figure 3C). After treatment with cisplatin in the initial stage the cell responds well with chemosensitive nature along with downregulated expression of β-catenin (Figure 3D) but its overexpression is documented in advanced stages of cancer with chemoresistance behavior (Figure 3E).

Western Blotting Analysis

To significantly confirm the expression of PKR2 and β-catenin at various stages of pancreatic cancer Western blotting was performed. We find out as the cancer progress to each next stage the PKR2 and β-catenin show overexpression (Figure 3, Lane 1-3) and our results superimpose with immunohistological data. Following the treatment procedure, the PKR2 and β-catenin expression sharply downregulated at critical stages of chemosensitivity for cisplatin treatment (Figure 3, Lane 4). Interestingly, we observed overexpression of β-catenin in the advanced stage of cancer that are treated with cisplatin, but PKR2 shows decreased expression when compared with the respective controls (Figure 3, Lane 5).

Discussion

Pancreatic cancer is a life threading disease that is difficult to prognosticate in the early stages[22,23]. Another reason for therapeutic failure in treating pancreatic cancer is developing drug resistance against the drug[2]. Therefore, identifying and treating patients in the initial stage is more vital to improve the survival rate and it is also es-

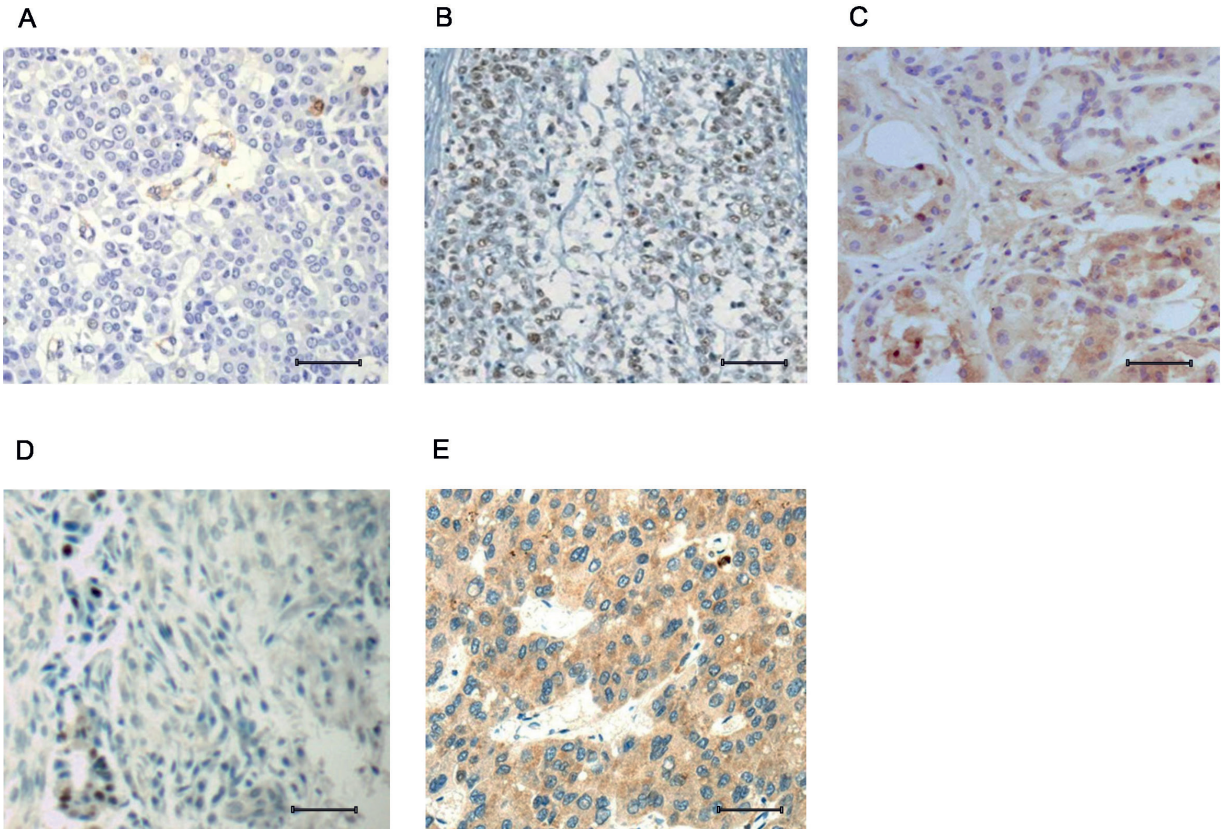

Figure 3. Expression of β-catenin in different pathological stages of pancreatic cancer. ***A,*** β-catenin that regulates normal functions of the pancreas shows slight expression in the control tissue. ***B,*** Initial pancreatic cancer with increased expression of β-catenin. ***C,*** Aggressive pancreatic cancer shows over expression of β-catenin. ***D,*** initial stage of pancreatic cancer treated with cisplatin shows decreased expression of β-catenin. ***E,*** Aggressive stage of pancreatic cancer treated with cisplatin shows over expression of β-catenin. Scale Bar denotes 50 μm size.

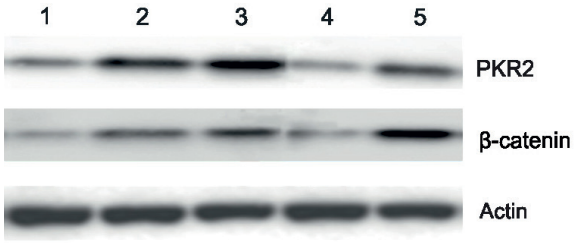

Figure 4. Western blot analysis. Lane 1 (row1 & 2) represents the PKR2 & β-catenin expression in control pancreatic tissue. Lane 2 band represents the PKR2 & β-catenin expression in initial pancreatic cancer. Lane 3 shows PKR2 & β-catenin expression in aggressive pancreatic cancer. Lane 4 implies PKR2 & β-catenin expression in initial pancreatic cancer tissue treated with cisplatin. Lane 5 implies PKR2 & β-catenin expression in advanced pancreatic cancer tissue treated with cisplatin. Actin was used as a loading control.

sential to study the genes associated with chemosensitivity and chemoresistance. Genetic changes are the key in developing drug resistance mechanism and changes in some vital genes can lead to multiple drug resistance[24]. In this study, we used cisplatin to assess the relation of PKR2 and β-catenin genes that are linked with chemosensitivity.

In the present study, we used a mouse model with a mutation in KRAS gene, which provides a potent model to active oncogene[16]. The mutant mice respond well, and it develops initial stage of pancreatic cancer on 2nd months and with aggressive on 4th months which are evident with the histological sections. The histological sections with harden tissue implies the solid and firm nature of pancreatic cancer[8]. The drug cisplatin responds well for initial cancer, but it shows no progress in treating an advanced form of pancreatic cancer. One possible reason may be, it shows morphologically unassessable for the drug due to the solid form of cancer in advanced stages.

Previously studies[25] reported that some genes are involved in regulating chemosensitivity, e.g., β-tubulins. Here we point out that PKR2 and β-catenin

are involved in chemoresistance and chemosensitivity which are clearly observed through immunohistochemical data. Peculiarly, we observed that in the advanced stage of pancreatic cancer, which is treated with cisplatin, the β-catenin overexpressed which helps to conclude the drug resistance ability.

Conclusions

We successfully use KRAS mutated mice develop initial and advanced stage of pancreatic cancer. And using PKR2 and β-catenin, we assess the chemosensitivity and chemoresistance nature of the drug cisplatin, in which will be helpful in understanding the chemoresistance behavior.

Acknowledgement

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Conflict of interest

The authors declare no conflicts of interest.

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