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Abstract

Histopathology and immunohistochemistry (IHC) were performed on pancreatic tissue sections and tissue microarrays (TMAs). Tissues were fixed, embedded in paraffin, and sectioned for staining with hematoxylin and eosin (H&E). For IHC, sections were incubated with primary antibodies, followed by secondary HRP-conjugated antibodies and visualization with diaminobenzidine (DAB). Images were captured using light and fluorescent microscopy. TMA slides were analyzed for protein expression levels, and results were scored based on staining intensity and extent.

Histopathology and Immunohistochemistry

1 Pancreata were dissected and fixed in 4% paraformaldehyde in PBS and embedded in paraffin. 5 µm sections were prepared and stained with hematoxylin and eosin (H&E).

IHC was performed as described. Sections were incubated with primary antibodies diluted in PBS 0.5% Triton-X100, with 5% BSA for 1h at room temp, followed by incubation with 1:150 biotinylated secondary antibodies for 1h and 1:500 streptavidin-HRP for 1h. Bound peroxidase was visualized by 1-5 min incubation in a 3,3′-diaminobenzidine (DAB) solution (Vector Laboratories, SK-4100). Sections were washed, counterstained with hematoxylin, dehydrated, and mounted.
The following antibodies were used: rabbit anti-EZH2 (D2C9, Cell Signaling #5246; IHC: 1:100), rabbit anti-NQO1 (D6H3A, Cell Signaling #62262; IHC: 1:100), rabbit anti-NRF2 (ABclonal, A11159, IHC: 1:100) rabbit anti-Ki67, (GeneTex, GTX16667, IHC: 1:100), rabbit anti-3meH3K27 (ABclonal, A-2363, IHC: 1:100), anti-4 HNE (4-hydroxynonenal, Alpha Diagnostic, Cat. #HNE13-M, IHC: 1:200), rabbit anti-NOX1 (GeneTex, GTX103888; IHC: 1:150), goat anti-mouse cytokeratin 19 antibody (M17, Santa Cruz Biotechnology Cat. # sc-33111, IHC 1:100), rat anti-mouse cytokeratin 19 antibody (TROMA-III, DSHB, IHC: 0.25 µg/ml), rabbit anti-SOX9 (H-90, Santa Cruz Biotechnology Cat. # sc-20095, IHC: 1:100), rat
anti-mouse CD44 (Abdserotec, MCA4703, IHC: 1:100), rat anti mouse F4/80 (BM8, Invitrogen, # MF48000, IHC: 1:150), rabbit anti-ERK1/2 [Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Cell Signaling (D13.14.4E) #4370, IHC: 1:100], rabbit anti-RAS (G12D Mutant) (HL10, GeneTex, GTX635362, IHC: 1:100). Sirius Red (Abcam, ab150681) and Alcian blue (Abcam, ab150662) staining were performed using general guidelines indicated by the kit's manufacture.

Images were captured on an upright light/fluorescent Image A2 microscope with the AxioVision Release v.4.5 software (Zeiss) and the Olympus SLIDEVIEW VS200 research slide scanner.

Immunohistochemistry of OHSU Tissue Micro Array (TMA)Untitled section

2 Patient tissues from OHSU were obtained with informed consent in accordance with the Declaration of
Helsinki and were acquired through the Oregon Pancreas Tissue Registry under Oregon Health & Science University IRB protocol #3609. TMA containing tissue samples representing both adjacent PanINs (1-3) and PDAC tissues and normal control pancreas was designed and generated by the Brenden-Colson Center for Pancreatic Care at OHSU. Heat-induced was performed where slides were either immersed in target-retrieval solution: citric acid buffer (pH 6.0) for EZH2 or EDTA buffer (pH 8.0) for NRF2,

for 10 min at 121°C. Endogenous peroxide activity was blocked using 3% H_2O_2 solution for 15 min at room temperature (RT), followed by protein blocking using 3% FBS/goat serum for 30 min at RT. Next, TMAs were incubated either overnight at 4°C for EZH2 (Cell signaling, catalog #5246, (1:100)) or 1 hour at room temperature for NRF2 (abclonal, catalog #A11159 (1:300). After primary antibodies, TMAs were incubated with anti-rabbit horseradish peroxidase (HRP) conjugated secondary antibody (BD scientific, Catalog#55038, 1:150) for 1 hour and ABC Kit (Vector Laboratories) for 30 min at RT for EZH2 and incubated with SignalStain Boost
IHC Detection Reagent (Cell Signaling Technology) for 30 min at RT for NRF2, followed by detection using 3,3-Diaminobenzidine (DAB; Immunologic) according to manufacturer's instructions and counterstained using hematoxylin.
IHC expression of NRF2 and EZH2, was scored as: "0-absent" (–), "1-weak"(+), "2-moderate "(++), "3-strong"(+++). For EZH2, histoscores were generated as the product of the expression level multiplied by the percentage of staining positive tumor cells. Expression ≧1.5 was counted as positive, and for histoscore ≧150 was counted as positive.

Protocol references

1 Todoric,J. *et al.* Stress-Activated NRF2-MDM2 Cascade Controls Neoplastic Progression in Pancreas. *Cancer Cell* **32**, 824-839 e828, doi:10.1016/j.ccell.2017.10.011 (2017).