Supporting Online Material for

**DAXX/ATRX, MEN1, and mTOR Pathway Genes Are Frequently Altered in Pancreatic Neuroendocrine Tumors**


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Supplementary Materials and Methods

Preparation of Illumina Genomic DNA libraries
Fresh-frozen surgically resected tumor and normal tissues were obtained from patients under an Institutional Review Board protocol. Genomic DNA libraries were prepared following Illumina’s (Illumina, San Diego, CA) suggested protocol with the following modifications. (1) 3 micrograms (µg) of genomic DNA from tumor or normal cells in 100 microliters (µl) of TE was fragmented in a Covaris sonicator (Covaris, Woburn, MA) to a size of 100-500 bp. To remove fragments shorter than 150bp, DNA was mixed with 25µl of 5 x Phusion HF buffer, 416µl of ddH₂O, and 84µl of NT binding buffer and loaded into NucleoSpin column (cat# 636972, Clontech, Mountain View, CA). The column was centrifuged at 14000 g in a desktop centrifuge for 1 min, washed once with 600 µl of wash buffer (NT3 from Clontech), and centrifuged again for 2 min to dry completely. DNA was eluted in 45 µl of elution buffer included in the kit. (2) Purified, fragmented DNA was mixed with 40 µl of H₂O, 10 µl of End Repair Reaction Buffer, 5 µl of End Repair Enzyme Mix (cat# E6050, NEB, Ipswich, MA). The 100 µl end-repair mixture was incubated at 20°C for 30 min, purified by a PCR purification kit (Cat # 28104, Qiagen) and eluted with 42 µl of elution buffer (EB). (3) To A-tail, all 42 µl of end-repaired DNA was mixed with 5 µl of 10X dA Tailing Reaction Buffer and 3 µl of Klenow (exo-)(cat# E6053, NEB, Ipswich, MA). The 50 µl mixture was incubated at 37°C for 30 min before DNA was purified with a MinElute PCR purification kit (Cat # 28004, Qiagen). Purified DNA was eluted with 25 µl of 70°C EB. (4) For adaptor ligation, 25 µl of A-tailed DNA was mixed with 10 µl of PE-adaptor (Illumina), 10 µl of 5X Ligation buffer and 5 µl of Quick T4 DNA ligase (cat# E6056, NEB, Ipswich, MA). The ligation mixture was incubated at 20°C for 15 min. (5) To purify adaptor- ligated DNA, 50 µl of ligation mixture from step (4) was mixed with 200 µl of NT buffer and cleaned up by NucleoSpin column. DNA was eluted in 50 µl elution buffer. (6) To obtain an amplified library, ten PCRs of 50 µl each were set up, each including 29 µl of H₂O, 10 µl of 5 x Phusion HF buffer, 1 µl of a dNTP mix containing 10 mM of each dNTP, 2.5 µl of DMSO, 1 µl of Illumina PE primer #1, 1 µl of Illumina PE primer #2, 0.5 µl of Hotstart Phusion polymerase, and 5 µl of the DNA from step (5). The PCR program used was: 98°C 2 minute; 6 cycles of 98°C for 15 seconds, 65°C for 30 seconds, 72°C for 30 seconds; and 72°C for 5 min. To purify the PCR product, 500 µl PCR mixture (from the ten PCR reactions) was mixed with 1000 µl NT buffer from a NucleoSpin Extract II kit and purified as described in step (1). Library DNA was eluted with 70°C elution buffer and the DNA concentration was estimated by absorption at 260 nm.

Exome and targeted subgenomic DNA capture
Human exome capture was performed following a protocol from Agilent’s SureSelect Paired-End Version 2.0 Human Exome Kit (Agilent, Santa Clara, CA) with the following modifications. (1) A hybridization mixture was prepared containing 25 µl of SureSelect Hyb # 1, 1 µl of SureSelect Hyb # 2, 10 µl of SureSelect Hyb # 3, and 13 µl of SureSelect Hyb # 4. (2) 3.4 µl (0.5 µg) of the PE-library DNA described above, 2.5 µl of SureSelect Block #1, 2.5 µl of SureSelect Block #2 and 0.6 µl of Block #3; was loaded into one well in a 384-well Diamond PCR plate (cat# AB-1111, Thermo-Scientific, Lafayette, CO), sealed with microAmp clear adhesive film (cat# 4306311; ABI, Carlsbad, CA) and placed in GeneAmp PCR system 9700 thermocycler (Life Sciences Inc., Carlsbad CA) for 5 minutes at 95°C, then held at 65°C (with the heated lid on). (3) 25-30 µl of hybridization buffer from step (1) was heated for at least 5 minutes at 65°C in another sealed plate with heated lid on. (4) 5 µl of SureSelect Oligo Capture Library, 1 µl of nuclease-free water, and 1 µl of diluted RNase Block (prepared by diluting RNase Block 1: 1 with nuclease-free water) were mixed and heated at 65°C for 2 minutes in another sealed 384-well plate. (5) While keeping all reactions at 65°C, 13 µl of Hybridization Buffer from Step (3) was added to the 7 µl of
the SureSelect Capture Library Mix from Step (4) and then the entire contents (9 µl) of the library from Step (2). The mixture was slowly pipetted up and down 8 to 10 times. (6) The 384-well plate was sealed tightly and the hybridization mixture was incubated for 24 hours at 65°C with a heated lid.

After hybridization, five steps were performed to recover and amplify captured DNA library: (1) Magnetic beads for recovering captured DNA: 50 µl of Dynal MyOne Streptavidin C1 magnetic beads (Cat # 650.02, Invitrogen Dynal, AS Oslo, Norway) was placed in a 1.5 ml microfuge tube and vigorously resuspended on a vortex mixer. Beads were washed three times by adding 200 µl of SureSelect Binding buffer, mixed on a vortex for five seconds, then removing and discarding supernatant after placing the tubes in a Dynal magnetic separator. After the third wash, beads were resuspended in 200 µl of SureSelect Binding buffer. (2) To bind captured DNA, the entire hybridization mixture described above (29 µl) was transferred directly from the thermocycler to the bead solution and mixed gently; the hybridization mix/bead solution was incubated an Eppendorf thermomixer at 850rpm for 30 minutes at room temperature. (3) To wash the beads, the supernatant was removed from beads after applying a Dynal magnetic separator and the beads was resuspended in 500 µl SureSelect Wash Buffer #1 by mixing on vortex mixer for 5 seconds and incubated for 15 minutes at room temperature. Wash Buffer #1 was then removed from beads after magnetic separation. The beads were further washed three times, each with 500 µl pre-warmed SureSelect Wash Buffer #2 after incubation at 65°C for 10 minutes. After the final wash, SureSelect Wash Buffer #2 was completely removed. (4) To elute captured DNA, the beads were suspended in 50 µl SureSelect Elution Buffer, vortex-mixed and incubated for 10 minutes at room temperature. The supernatant was removed after magnetic separation, collected in a new 1.5 ml microcentrifuge tube, and mixed with 50 µl of SureSelect Neutralization Buffer. DNA was purified with a Qiagen MinElute column and eluted in 17 µl of 70°C EB to obtain 15 µl of captured DNA library. (5) The captured DNA library was amplified in the following way: 15 PCR reactions each containing 9.5 µl of H₂O, 3 µl of 5 x Phusion HF buffer, 0.3 µl of 10 mM dNTP, 0.75 µl of DMSO, 0.15 µl of Illumina PE primer #1, 0.15µl of Illumina PE primer #2, 0.15 µl of Hotstart Phusion polymerase, and 1 µl of captured exome library were set up. The PCR program used was: 98°C for 30 seconds; 14 cycles of 98°C for 10 seconds, 65°C for 30 seconds, 72°C for 30 seconds; and 72°C for 5 min. To purify PCR products, 225µl PCR mixture (from 15 PCR reactions) was mixed with 450 µl NT buffer from NucleoSpin Extract II kit and purified as described above. The final library DNA was eluted with 30 µl of 70°C elution buffer and DNA concentration was estimated by OD260 measurement.

Somatic mutation identification by massively parallel sequencing
Captured DNA libraries were sequenced with the Illumina GAIIx Genome Analyzer, yielding 150 (2 X 75) base pairs from the final library fragments. Sequencing reads were analyzed and aligned to human genome hg18 with the Eland algorithm in CASAVA 1.6 software (Illumina). A mismatched base was identified as a mutation only when (i) it was identified by more than three distinct tags; (ii) the number of distinct tags containing a particular mismatched base was at least 16% of the total distinct tags; and (iii) it was not present in >0.5% of the tags in the matched normal sample. SNP search databases included http://www.ncbi.nlm.nih.gov/projects/SNP/ and http://browser.1000genomes.org/index.html.

Evaluation of genes in additional tumors and matched normal controls.
For the ATRX, DAXX, MEN1, PIK3CA, PTEN, TP53 and TSC2 genes, the coding region was sequenced in a validation Set, comprising a series of additional pancreatic neuroendocrine tumors and matched controls. PCR amplification and Sanger sequencing were performed following protocols described previously (1) using the primers listed in table S8.
**Immunohistochemistry**

Immunohistochemical labeling for ATRX and DAXX proteins was performed on formalin-fixed, paraffin-embedded sections of PanNETs. Heat-induced antigen retrieval was performed in a steamer using citrate buffer (pH 6.0) (Vector Laboratories) for 30 min followed by 10 min of cooling. Endogenous peroxidase was blocked for 10 min with dual endogenous enzyme-blocking reagent (Dako). Serial sections were then incubated with primary antibody; anti-ATRX (1:400 dilution; catalog no. HPA001906, Sigma-Aldrich) and anti-DAXX (1:75 dilution; catalog no. HPA008736, Sigma-Aldrich) for 1 h at room temperature. The sections were then incubated for 30 min with secondary antibody (Leica Microsystems) followed by detection with 3,3′-Diaminobenzidine (Sigma-Aldrich) for 8 min. Sections were washed with phosphate-buffered saline with 0.1% Tween-20. Finally, sections were counterstained with Harris hematoxylin, subsequently rehydrated and mounted. Only nuclear labeling of either protein was considered positive. At least 50% of the cells needed to have nuclear labelling for the marker to be considered positive. Internal controls included islets of Langerhans and endothelial cells (including within intra-tumoral vessels) which demonstrated strong nuclear labeling for both ATRX and DAXX.

**Clinical correlations**

Clinical information on the patients evaluated in this study were obtained from the Johns Hopkins Hospital and the Memorial Sloan-Kettering Comprehensive Cancer Center in the context of approved IRB protocols. Clinical data were collected retrospectively and compared with mutational status. Overall survival was calculated from the time of diagnosis until death. Patients who were alive at the time of analysis were censored at the date of last observation. Survival curves were plotted by the Kaplan-Meier method and compared using the Mantel-Cox log-rank test (Prism, GraphPad Software, La Jolla, CA).

**References**

Figure S1. A. Immunohistochemical labeling with antibody against DAXX shows lack of nuclear labeling in neoplastic cells (arrows). Labeling in the non-neoplastic cells (endothelial and stromal cells) served as an internal control (arrow-head). B. Similar labeling of another tumor with an antibody against ATRX protein. In both A and B, nuclei that don’t react with antibodies are blue because of the counterstain, while those that do react are brown.