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Performance of DAXX Immunohistochemistry as a Screen for DAXX Mutations in Pancreatic Neuroendocrine Tumors

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Abstract

Objectives: DAXX immunohistochemistry (IHC) is often used as a surrogate for sequencing. We aimed to elucidate the sensitivity of IHC for DAXX mutation.

Methods: All pancreatic neuroendocrine tumors (PanNETs) with DAXX mutations detected by sequencing and a subset of DAXX wild type PanNETs were analyzed for DAXX expression by IHC.

Results: Of 154 PanNETs with MSK-IMPACT testing, 36 (30%) harbored DAXX mutations. DAXX mutations were associated with TSC2 mutations (46% vs. 10%, $P < 0.0001$), tended to co-occur with MEN1 mutations (63% vs 49%, $P = 0.11$), and tended to be mutually exclusive with ATRX mutations (11% vs 25%, $P = 0.053$). Of 27 available DAXX mutant PanNETs, 23 lost DAXX expression (85.2%). All 4 DAXX mutants with retained expression harbored DAXX mutations within the SUMO-interacting motif of the last exon. Telomere-specific fluorescence in situ hybridization demonstrated alternative lengthening of telomeres in all 4 cases. Of 20 PanNETs with wild type DAXX, 19 retained DAXX IHC expression (95%).

Conclusions: The sensitivity and specificity of IHC for DAXX mutation is 85% and 95%, respectively. Last exon DAXX mutant PanNETs often show alternative lengthening of telomeres despite retained DAXX expression, likely due to escape of non-mediated decay.

Keywords

DAXX; pancreatic neuroendocrine tumor; immunohistochemistry; next generation sequencing; alternative lengthening of telomeres

INTRODUCTION

Pancreatic neuroendocrine tumors (PanNETs) are the second most common primary tumor of the pancreas and occur in approximately 1 in 100,000 persons annually.¹ These tumors

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have widely variable behavior ranging from incidental and indolent, to high grade metastatic neoplasms. While mitotic rate and proliferation index are commonly reported and used to manage patients, the status of two tumor suppressors has recently been reported to be important prognostically.²⁻⁵

Along with α -Thalassemia/ x linked mental retardation (*ATR**X*) gene, Death domain associated protein (*DAXX*) gene encodes a nuclear protein that regulates the deposition of histone H3.3 during the assembly of telomeric chromatin. *DAXX* function is paramount to various nuclear functions including transcription and cell cycle regulation (including apoptosis).²⁻⁴ Recently, it has been shown that over 40% of PanNETs harbor mutations in either *DAXX* or *ATR**X*, and that these mutations correspond with the presence of alternative lengthening of telomeres (ALT).⁵ *DAXX* and *ATR**X* IHC have since been used in several studies as surrogates for mutation analysis, but 3 studies have found that ALT is present in 6–21% of PanNETs with retained *DAXX* and *ATR**X* IHC.⁵⁻⁷ Here, we investigate the relationship of *DAXX* mutations with *DAXX* protein expression via immunohistochemical analysis and show that the sensitivity of *DAXX* IHC depends on the site of *DAXX* mutation.

MATERIALS AND METHODS

Retrospective analysis of *DAXX* (transcript NM_001141970) mutations was performed on a cohort of PanNETs that had undergone molecular sequencing with MSK-IMPACT, a hybridization-based capture assay that identifies somatic mutations, copy number changes, microsatellite instability, and select structural rearrangements in over 400 genes against a patient's matched normal sample.⁸ Domain location for mutant proteins was identified using the sequence based predicted modular organization of *DAXX*.³ *P* values for mutation associations were calculated using two tailed chi square tests with statistically significant findings of $P < 0.05/(\text{number of variables})$ to include Bonferroni correction.

Immunohistochemical analysis of *DAXX* in situ protein expression was performed on all *DAXX* mutant PanNETs with available tissue, and on 20 PanNETs with wild type *DAXX*. For the immunohistochemical detection of *DAXX*, rabbit monoclonal antibody (mAb) clone E94 (ab32140; Abcam, Cambridge, Mass) was used on all cases tested. Select *DAXX* mutant PanNETs were also tested with a second immunohistochemical antibody for *DAXX*: rabbit mAb clone 25C12 (#4553; Cell Signaling Technology, Danvers, Mass). While the immunogen for mAb E94 was not further specified, mAb 25C12 was generated with a synthetic peptide surrounding aa 259 of the *DAXX* protein. All assays were performed on a Leica Bond-III automated staining platform (Leica, Buffalo Grove, Ill). E94 was optimized on panels of normal tissue and mutated PanNETs. Best staining was achieved at a dilution of 1:300 (0.8 ug/ml) using a 30 minutes heat-based antigen retrieval step employing a high pH buffer solution (ER2, Leica). A polymer-based secondary kit (Refine, Leica) was used for the detection of the primary. Immunohistochemical interpretation was based nuclear staining: complete loss of nuclear staining was considered 'lost' or 'negative'. Non-neoplastic lymphocytes served as positive internal controls for each sample.

In cases with *DAXX* mutation and retained IHC, alternative lengthening of telomeres was assessed by Telomere-specific FISH. Cy3-labeled peptide nucleic acid (PNA) probe

(TTAGGGTTAGGGTTAGGG 3') from Applied Biosystems was used and samples hybridized according to manufacturer's protocol with few modifications. Briefly, paraffin sections were dewaxed, microwaved in 10 mM sodium citrate buffer, pretreated with pepsin, washed, and dehydrated in cold ethanol series. The PNA Probe and target DNA were co-denatured for 8 minutes, incubated overnight at 37°C, washed twice in 2XSSC+0.1% Tween20, rinsed with sterile distilled water and counterstained with antifade medium containing DAPI (Vectashield, Vector Laboratories). Slides were scanned using a Zeiss Axioplan 2i epifluorescence microscope equipped with a megapixel CCD camera (CV-M4+CL, JAI) controlled by Isis 5.5.9 imaging software (MetaSystems Group Inc, Waltham, Mass). Metafer and VSlide module within MetaSystems were used to generate virtual image of the tissue sections (DAPI and H&E); in all, corresponding H&E section was used to identify tumor regions.

The entire section was hybridized and scanned through 63X for the presence of ALT-positive cells. Cells exhibiting the characteristic large, ultrabright, intranuclear signal (telomere sequence aggregates) were considered ALT-positive. A case was considered ALT-positive if five or more 63X fields contained ALT-positive cell(s). In all, normal stromal elements/regions were also analyzed to confirm the restriction of ALT-signals to tumor cells.

RESULTS

DAXX Mutations: Molecular Profiling

Out of 154 PanNETs with MSK-IMPACT results, 46 (30%) had *DAXX* mutation(s) (Fig. 1). *DAXX* mutations were significantly correlated with *MEN1* and *TSC2* mutations and trended to be mutually exclusive with *ATRX* mutations. Consistent with its role as a tumor suppressor, no recurrent hotspots were identified. Sixty-four mutations were identified in 46 PanNETs, including 25 nonsense mutations, 20 frameshift deletions, 17 missense mutations, 1 splice site mutation, and 1 in frame deletion.

Of the 46 PanNETs with *DAXX* mutations, 5 had *ATRX* mutations, 29 had *MEN1* mutations or deletions, and 21 had *TSC2* mutations or deletions. Of the 108 PanNETs with wild type (WT) *DAXX*, 27 had *ATRX* mutations, 53 had *MEN1* mutations or deletions, and 11 had *TSC2* mutations or deletions (Table 1). *TSC2* mutations were significantly enriched in *DAXX* mutant PanNETs ($P = 0.0001$) while *ATRX* mutations trended to be mutually exclusive with *DAXX* mutations without reaching statistical significance ($P = 0.053$).

DAXX Immunohistochemistry

Twenty-seven PanNET cases with known *DAXX* mutation were available for immunohistochemical analysis. Of these *DAXX* mutant PanNETs, 23 showed loss of expression while 4 had retained expression (Figs. 2A, 2B; Table 2). All 6 *DAXX* mutant PanNETs tested with the second immunohistochemical antibody (clone 25C12) were concordant. Each of the 4 cases with retained expression harbored *DAXX* mutations that occurred in the small ubiquitin like modifier (SUMO)-Interaction motifs (SIMs) in the last exon, 2 of which were nonsense mutations that would not result in transcription of the affected SIM sequence (Fig. 2C, Table 2). Consequently, the sensitivity of DAXX loss for

DAXX mutations was 85.2% and dependent on the presence of mutations in other than the last exon.

Twenty PanNETs with wild type *DAXX* also underwent immunohistochemical staining for *DAXX*. Nineteen of 20 (95%) PanNETs with WT *DAXX* displayed nuclear immunopositivity indicating retained expression, yielding a specificity of 95%. The single case with loss of *DAXX* expression and WT *DAXX* did not harbor any coding mutations, structural variants, deletions, or intronic mutations in *DAXX*. The *DAXX* staining on this case was technically satisfactory; positive *DAXX* labeling was observed in both internal (lymphocytes) and external controls.

Telomere-specific FISH—The only PanNETs with discordant *DAXX* mutation status and protein expression status were 4 PanNETs with *DAXX* mutations in the last exon, WT *ATRX*, and retained *DAXX* IHC. Telomere specific FISH was performed in all 4 cases, and all 4 cases were positive for ALT (Fig. 2D).

DISCUSSION

In this study, we show that *DAXX* IHC is specific (95%) for *DAXX* mutation in PanNETs, and sensitivity is approximately 85%, with retained *DAXX* expression present in all 4 of 4 PanNETs with mutations in the last exon.

Previous studies have shown the clinical importance of ALT and its association with *DAXX* or *ATRX* loss. Specifically, reduced survival and metastatic disease have been associated with *DAXX* loss.^{5,7} Interestingly, VandenBussche et al, Marinoni et al and Singhi et al each separately found that 21%, 15% and 6%, respectively, of PanNETs positive for ALT had retained *DAXX* and *ATRX* expression on IHC.^{5–7} These results suggest that *DAXX*/ *ATRX* IHC are not 100% sensitive for identifying PanNETs with ALT.

The *DAXX* gene is 8 exons and 752 amino acids in length. In our study, all cases harboring mutations in exons 1–7 were detected by immunohistochemical loss of expression. Interestingly, the 4 cases with retained *DAXX* protein expression all harbored mutations in the last exon. Consequently, the sensitivity of *DAXX* protein expression analysis depends on the site of the mutation. A likely reason for the retained expression of *DAXX* in the 4 cases of *DAXX* mutant PanNETs is escape of nonsense mediated decay. All 4 cases of *DAXX* mutant PanNET with retained *DAXX* expression harbored mutations in the last exon, and it is known that nonsense mediated decay is inefficient for nonsense mutations in the last exon.⁹

While *DAXX* expression was retained in all PanNETs with *DAXX* mutations in the last exon, all 4 of these mutations (including 2 nonsense mutations) occurred in an important area: the SUMO- interacting motif. This domain has important roles in *DAXX* function, including sensitizing cells to stress-induced apoptosis² and enhancing interactions between PML and *DAXX* which is important for apoptosis.¹⁰ The fact that ALT was present on telomere-specific FISH in all 4 cases with *DAXX* mutations in the last exon, retained *DAXX*, and WT *ATRX* also supports the likely scenario that presence of *DAXX* protein is

not a fully sensitive marker of adequately functioning DAXX protein when *DAXX* mutations occur in the last exon.

There was a single case of *DAXX* WT PanNET with loss of DAXX on IHC. Lymphocytes served as an internal positive control for DAXX IHC in this case. While there was not an indication of a genetic *DAXX* abnormality on MSK-IMPACT, the gene likely was inactivated in another way: either by epigenetic silencing or an intronic event not covered by MSK-IMPACT.

Several limitations to this study exist, including a sample size too small for survival and response analysis according to *DAXX* mutation, as well as a relatively small number of *DAXX* mutations in the last exons.

In conclusion, we show that DAXX IHC has a sensitivity of approximately 85% for the presence of *DAXX* mutations in PanNETs. The discordant cases are largely a reflection of the site of mutation since mutations in the last exon are not concordant with IHC results.

Acknowledgments

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FIGURE 1.

Distribution of *DAXX* mutations across the gene. *DAXX* has 6 regions of conserved sequences. The first and last correspond to SUMO-interaction motifs (SIMs), then there are 2 helical segments including the DAXX helix bundle (DHB) domain. Towards the C terminal, the “acidic” region is present, which contains 80% Glu/Asp residues, followed by segments rich in Ser/Pro/Glu (SPE) and Ser/Pro/Thr (SPT) residues. Mutation were distributed throughout the gene without hotspots. Black dots represent truncating mutations, green dots represent missense mutations, and the brown dot represents an in frame indel.

FIGURE 2.

Retained expression of DAXX in a distal *DAXX* mutant pancreatic neuroendocrine tumor positive for alternating lengths of telomeres (ALT). A, Microscopic sections showed an intermediate grade pancreatic neuroendocrine tumor with solid architecture, vascularization, and moderate to abundant cytoplasm. B, DAXX IHC from the same tumor shows retention of expression in nuclei with strong, uniform intensity. C, On Integrated Genomics Viewer, the tumor from (2A) and (2B) a nonsense mutation is present on the minus strand at 72%, signifying loss of heterogeneity, within the distal SUMO-interaction motif. D, Large, ultrabright, intranuclear FISH signals (orange) indicative of ALT (arrows) (scale bar = 5μm).

TABLE 1.Recurrent Mutations in Pancreatic Neuroendocrine Tumors and Their Relationship to *DAXX* Mutation Status

	<i>MEN1</i> Mutant, n (%)	<i>TSC2</i> Mutant, n (%)	<i>ATRX</i> Mutant, n (%)
<i>DAXX</i> mutant (n = 46)	29 (63)	21 (46)	5 (11)
<i>DAXX</i> WT (n = 108)	53 (49)	11 (10)	27 (25)
<i>P</i>	0.11	0.0001	0.053

TABLE 2.DAXX IHC Results According to *DAXX* Mutation

DAXX IHC (mAb E94)	AA	NT	Exon	DOMAIN
Negative/lost	Y71 *	213C>G	2	DHB
Negative/lost *	Q89 *	265C>T	3	DHB
Negative/lost	E116 *	346G>T	3	DHB
Negative/lost *	C118 *	354C>A	3	DHB
Negative/lost	L146P	437T>C	3	DHB
Negative/lost	H175fs	524_527delACCT	3	
Negative/lost	Q205 *	613C>T	3	Helical
Negative/lost	G262C; E466 *	784G>T; 1396G>T	3, 5	Helical
Negative/lost	N280S	839A>G	3	Helical
Negative/lost	N280K	840C>G	3	Helical
Negative/lost *	Q320 *; G531R	958C>T; 1591G>A	3, 6	Helical, Helical
Negative/lost	R330 *	988C>T	3	Helical
Negative/lost	C350R	1048T>C	3	Helical
Negative/lost *	Q395 *	1183C>T	4	Helical
Negative/lost	D419fs	1255delIG	4	
Negative/lost *	E466 *	1396G>T	5	
Negative/lost	M482_Q483delinsI *	1446_1447delinsAT	5	SPE
Negative/lost	R602Gfs *12	1803delC	6	
Negative/lost	F625Lfs *31	1875_1878delCAAT	6	
Negative/lost	Q650 *	1948C>T	6	
Negative/lost *	Y660 *	1977-1_1979dupGCTA	6	
Negative/lost	H723Ifs *55	2156delC	7	SPT
Medium/retained	E743 *	2227G>T	8	SIM
Medium/retained	S749 *	2246C>G	8	SIM
Medium/retained	D750fs	2247dupA	8	SIM
Medium/retained	D750V	2249A>T	8	SIM

* Also tested with clone 25C12 with concordant results.

DHB indicates DAXX helix bundle; SPE, domain rich in Ser/Pro/Glu residues; SPT, domain rich in Ser/Pro/Thr residues; SIM, sumo-interaction motif.