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

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Abstract

Pancreatic Neuroendocrine Tumors (PanNETs) are a rare but clinically important form of pancreatic neoplasia. To explore the genetic basis of PanNETs, we determined the exomic sequences of ten non-familial PanNETs and then screened the most commonly mutated genes in 58 additional PanNETs. Remarkably, the most frequently mutated genes specify proteins implicated in chromatin remodeling: 44% of the tumors had somatic inactivating mutations in *MEN-1*, which encodes menin, a component of a histone methyltransferase complex; and 43% had mutations in genes encoding either of the two subunits of a transcription/chromatin remodeling complex consisting of DAXX (death-domain associated protein) and ATRX (alpha thalassemia/mental retardation syndrome X-linked). Clinically, mutations in the *MEN1* and *DAXX/ATRX* genes were associated with better prognosis. We also found mutations in genes in the mTOR (mammalian target of rapamycin) pathway in 14% of the tumors, a finding that could potentially be used to stratify patients for treatment with mTOR inhibitors.

PanNETs are the second most common malignancy of the pancreas. The ten-year survival rate of patients with PanNETs is only 40% (1-3). They are usually sporadic, but they can arise in multiple endocrine neoplasia type 1 and more rarely in other syndromes, including von Hippel-Lindau (VHL) syndrome and tuberous sclerosis (4). “Functional” PanNETs secrete hormones that cause systemic effects, while “Nonfunctional” PanNETs do not and therefore cannot always be readily distinguished from other neoplasms of the pancreas. Nonfunctional PanNETs grow silently and patients often present with either an

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asymptomatic abdominal mass or symptoms of abdominal pain secondary to compression by a large tumor. Surgical resection is the treatment of choice, but many patients present with unresectable tumors or extensive metastatic disease, and medical therapies are relatively ineffective in these cases.

There is currently insufficient information about this tumor to either predict prognosis of patients diagnosed with PanNETs or to develop companion diagnostics and personalized treatments to improve disease management. Biallelic inactivation of the *MEN1* gene, usually through a mutation in one allele coupled with loss of the remaining wild-type allele, occurs in 25-30% of PanNETs (5, 6). *MEN1* is a tumor suppressor gene which, when mutated in the germline, predisposes to multiple endocrine neoplasia type 1 syndrome. Chromosomal gains and losses and expression analyses have revealed candidate loci for genes involved in the development of PanNETs, but these have not been substantiated by genetic or functional analyses (7-9).

To gain insights into the genetic basis of this tumor type, we determined the exomic sequence of ~18,000 protein-coding genes in a Discovery set of ten well-characterized sporadic PanNETs. A clinically homogeneous set of tumors of high neoplastic cellularity is essential for the successful identification of genes and pathways involved in any tumor type. Thus, we excluded small cell and large cell neuroendocrine carcinomas and studied only samples that were not part of a familial syndrome associated with PanNETs (table S1) (1). We microdisected tumor samples to achieve a neoplastic cellularity of >80%. DNA from the enriched neoplastic samples and from matched non-neoplastic tissue from ten patients was used to prepare fragment libraries suitable for massively parallel sequencing. The coding sequences were enriched by capture with the SureSelect Enrichment System and sequenced using an Illumina GAIIx platform (10). The average coverage of each base in the targeted regions was 101-fold and 94.8 % of the bases were represented by at least 10 reads (table S2).

We identified 157 somatic mutations in 149 genes among the ten tumors used in the Discovery set. The mutations per tumor ranged from 8 to 23, with a mean of 16 (table S3). Of these mutations, 91 % were validated by Sanger sequencing. There were some obvious differences between the genetic landscapes of PanNETs and those of pancreatic ductal adenocarcinomas (PDAC, ref. 11). First, there were 60% fewer genes mutated per tumor in PanNETs than in PDACs. Second, the genes most commonly affected by mutation in PDACs (*KRAS, TGF-β* pathway, *CDKN2A, TP53*) were rarely altered in PanNETs and vice versa (Table 1). Third, the spectrum of mutations in PDAC and PanNET were different, with C to T transitions more common in PDACs than in PanNETs, and C to G transversions more common in PanNETs than in PDACs (table S4). This suggests that mutations in PanNETs and PDAC arise through different mechanisms, perhaps due to exposure to different environmental carcinogens or through the action of different DNA repair pathways.

We next selected genes for further analysis that were well-documented components of a pathway that was genetically altered in more than one tumor, because alterations in these genes are most likely to be clinically relevant. Four genes were mutated in at least two tumors in the Discovery set: *MEN1* in five, *DAXX* in three, *PTEN* in two, and *TSC2* in two. *ATRX* was mutated in only one sample in the Discovery set, but its product forms a heterodimer with DAXX and therefore is part of the same pathway, so it was also evaluated in the Validation set. Similarly, *PIK3CA* was included because its product is part of the mTOR pathway that includes PTEN and TSC2 (12-14). The sequences of these genes were then determined by Sanger sequencing in a Validation set consisting of 58 additional PanNETs and their corresponding normal tissues (Fig. 1, A and B). In total, somatic

mutations in *MEN1*, *DAXX*, *ATRX*, *PTEN*, *TSC2*, and *PIK3CA* were identified in 44.1%, 25%, 17.6%, 7.3%, 8.8%, and 1.4% PanNETs, respectively (Table 2).

Of the 30 mutations in *MEN1*, 25 were inactivating mutations (18 insertions or deletions (indels), 5 nonsense and 2 splice-site mutations), while five were missense. At least 11 were homozygous; in the others, the presence of "contaminating" DNA from normal cells made it difficult to reliably distinguish heterozygous from homozygous changes. *MEN1* encodes menin, a nuclear protein that acts as a scaffold to regulate gene transcription by coordinating chromatin remodeling. It is an essential component of the MLL SET1-like histone methyltransferase (HMT) complex (15-19). Overall, *MEN1* was mutated in 30 of the 68 PanNETs used in the Discovery and Validation sets combined.

DAXX and *ATRX* were mutated in 17 and 12 PanNETs, respectively. No tumor with a mutation in *DAXX* had a mutation in *ATRX*, consistent with their presumptive function within the same pathway. Overall 29 of 68 PanNETs (42.6%) had a mutation in this pathway. There were 11 insertions or deletions (indels) and 4 nonsense mutations in *DAXX*, and six indels and 3 nonsense mutations in *ATRX*. The three *ATRX* missense mutations were within the conserved helicase domain while the *DAXX* missense mutations were non-conserved changes. Five *DAXX* and four *ATRX* mutations were homozygous, indicating loss of the other allele. The high ratio of inactivating to missense mutations in both genes establishes them as PanNET tumor suppressor genes. Loss of immunolabelling for DAXX and ATRX correlated with mutation of the respective gene (fig. S1, A and B, and table S5). From these data, we hypothesize that both copies of *DAXX* are generally inactivated, one by mutation and the other either by loss of the non-mutated allele or by epigenetic silencing. We also hypothesize that both copies of *ATRX* are inactivated, one by mutation and the other by chromosome X inactivation. Recently, it has been shown that DAXX is an H3.3-specific histone chaperone (20). ATRX encodes for a protein that at the amino-terminus has an ADD (ATRX-DNMT3-DNMT3L) domain and a carboxy-terminal helicase domain. Almost all missense disease causing mutations are within these two domains (21). DAXX and ATRX interact and both are required for H3.3 incorporation at the telomeres and ATRX is also required for suppression of telomeric repeat-containing RNA expression (22-24). ATRX was recently shown to target CpG islands and G-rich tandem repeats (25), which exist close to telomeric regions.

We identified five *PTEN* mutations, two indels and three missense; six *TSC2* mutations, one indel, one nonsense and four missense; and one *PIK3CA* missense mutation. Previously published expression analyses have indicated that the expression of genes in the mTOR pathway is altered in most PanNETs (26,27). Our data suggest that, at least at the genetic level, only a subset of PanNETs have alterations of this pathway. This finding may have direct clinical application through prioritization of patients for therapy with mTOR pathway inhibitors. Everolimus (Afinitor, RAD-001, 40-O-(hydroxyethyl)-rapamycin) has been shown to increase progression free survival in a subset of PanNET patients with advanced disease (28). If the mutational status of genes coding for proteins in the mTOR pathway predicts clinical response to mTOR inhibitors, it should be possible to select patients who would benefit most from an mTOR inhibitor through analysis of these genes in patients' tumors (29, 30).

All 68 tumors evaluated in this study were from patients undergoing aggressive intervention (table S6) and included patients undergoing curative resection as well as those with metastatic disease. Interestingly, mutations in *MEN1*, *DAXX/ATRX* or the combination of both *MEN1* and *DAXX/ATRX* were associated with prolonged survival relative to those patients whose tumors lacked these mutations (Fig. 1, C and D and table S7). This was particularly evident in patients with metastatic disease and with mutations in both *MEN1* and

DAXX/ATRX: 100% of patients with PanNETs that had these mutations survived at least ten years while over 60% of the patients without these mutations died within five years of diagnosis (Fig. 1D). One possible explanation for the difference in survival is that mutations in *MEN1* and *DAXX/ATRX* identify a biologically specific subgroup of PanNETs.

In summary, whole exome sequencing of pancreatic neuroendocrine tumors has led to the identification of novel tumor suppressor genes and illuminated the genetic differences between the two major neoplasms of the pancreas. The mutations may serve to aid prognosis and provide a way to prioritize patients for therapy with mTOR inhibitors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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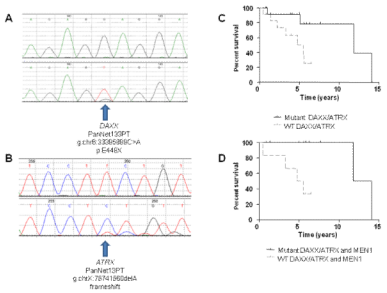

Figure 1.

Table 1

Comparison of commonly mutated genes in PanNETs and PDAC[c]

Genes[a]	PanNET	PDAC[b]
MEN1	44%	0%
DAXX, ATRX	43%	0%
Genes in mTOR pathway	15%	0.80%
TP53	3%	85%
KRAS	0%	100%
CDKN2A	0%	25%
TGFBR1, SMAD3, SMAD4	0%	38%

[a] Includes point mutations and indels.

[b] Data from Jones *et al* ., *Science* **321**, 1801 (2008).

[c] Based on 68 PanNETs and 114 PDACs.

Table 2

Mutations in *MEN1*, *DAXX*, *ATRX*, *PTEN*, *TSC2*, *PIK3CA*, and *TP53* in Human Pancreatic Neuroendocrine Tumors.

Sample [#]	Gene	Transcript Accession	Nucleotide (genomic) [*]	Nucleotide (cDNA)	Amino acid (protein) [$]	Mutation type
PanNET3PT	*ATRX*	CCDS14434.1	g.chrX:76716462G>A (hom)	c.6235C>T(hom)	p.R2079X	Nonsense
PanNET5PT	*ATRX*	CCDS14434.1	g.chrX:76742636G>A	c.5620C>T	p.Q1874X	Nonsense
PanNET13PT	*ATRX*	CCDS14434.1	g.chrX:76741560delA	c.5932delT	fs	Indel
PanNET27PT	*ATRX*	CCDS14434.1	g.chrX:76700959_76700962delATAA	c.6338_6341delTTAT	fs	Indel
PanNET35PT	*ATRX*	CCDS14434.1	g.chrX:76806893_76806909delAATTTCTTCTAAAAGCA	c.3824_3840delTGCTTTTAGAAGAAATT	fs	Indel
PanNET52PT	*ATRX*	CCDS14434.1	g.chrX:76796337_76796340delCTTT	c.4221_4224delAAAG	fs	Indel
PanNET59PT	*ATRX*	CCDS14434.1	g.chrX:76761014C>A	c.5364G>T	p.Q1788H	Missense
PanNET78PT	*ATRX*	CCDS14434.1	g.chrX:76665406C>T	c.6829G>A	p.E2277K	Missense
PanNET85PT	*ATRX*	CCDS14434.1	g.chrX:76794404dupC	c.4414dupG	fs	Indel
PanNET98PT	*ATRX*	CCDS14434.1	g.chrX:76700832T>A(hom)	c.6468A>T(hom)	p.Q2156H	Missense
PanNET100PT	*ATRX*	CCDS14434.1	g.chrX:76762518_76762521delCACT(hom)	c.5270_5272delAGTG(hom)	fs	Indel
PanNET112PT	*ATRX*	CCDS14434.1	g.chrX:76826041T>A(hom)	c.1363A>T(hom)	p.K455X	Nonsense
PanNET25PT	*DAXX*	CCDS4776.1	g.chr6:33394939delT	c.1976delA	fs	Indel
PanNET31PT	*DAXX*	CCDS4776.1	g.chr6:33394935delC(hom)	c.1980delG(hom)	fs	Indel
PanNET44PT	*DAXX*	CCDS4776.1	g.chr6:33394795delG	c.2120delC	fs	Indel
PanNET56PT	*DAXX*	CCDS4776.1	g.chr6:33397319delG	c.211delC	fs	Indel
PanNET77PT	*DAXX*	CCDS4776.1	g.chr6:33396614G>A	c.916C>T	p.R306X	Nonsense
PanNET84PT	*DAXX*	CCDS4776.1	g.chr6:33395309delG	c.1766delC	fs	Indel
PanNET87PT	*DAXX*	CCDS4776.1	g.chr6:33397141A>C	c.389T>G	p.L130R	Missense
PanNET93PT	*DAXX*	CCDS4776.1	g.chr6:33396641C>G	c.889G>C	p.A297P	Missense
PanNET94PT	*DAXX*	CCDS4776.1	g.chr6:33394872_33394873insA	c.2042_2043insT	fs	Indel
PanNET95PT	*DAXX*	CCDS4776.1	g.chr6:33397221_33397224delCGCC	c.306_309delGGCG	fs	Indel
PanNET96PT	*DAXX*	CCDS4776.1	g.chr6:33396167delC	c.1219delG	fs	Indel
PanNET97PT	*DAXX*	CCDS4776.1	g.chr6:33395838C>A(hom)	c.1393G>T(hom)	p.E465X	Nonsense
PanNET102PT	*DAXX*	CCDS4776.1	g.chr6:33397515T>A	c.166A>T	p.K56X	Nonsense
PanNET103PT	*DAXX*	CCDS4776.1	g.chr6:33397579delA	c.102delT	fs	Indel
PanNET104PT	*DAXX*	CCDS4776.1	g.chr6:33396604_33396605insACT(hom)	c.925_926insAGT(hom)	p.L309QF	Missense
PanNET108PT	*DAXX*	CCDS4776.1	g.chr6:33395828delT(hom)	c.1403delA(hom)	fs	Indel

Sample [#]	Gene	Transcript Accession	Nucleotide (genomic)[*]	Nucleotide (cDNA)	Amino acid (protein)[$]	Mutation type
PanNET133PT	*DAXX*	CCDS4776.1	g.chr6:33395889C>A(hom)	c.1342G>T(hom)	p.E448X	Nonsense
PanNET3PT	*MEN1*	CCDS8083.1	g.chr11:64331709C>A(hom)	c.689G>T(hom)	p.G230V	Missense
PanNET5PT	*MEN1*	CCDS8083.1	g.chr11:64332046A>G(hom)	c.562T>C(hom)	p.W188R	Missense
PanNET6PT	*MEN1*	CCDS8083.1	g.chr11:64333812_64333828delCACGGCTGGAGACACCC	c.329_345delGGGTGTCTCCAGCCGTG	fs	Indel
PanNET10PT	*MEN1*	CCDS8083.1	g.chr11:64334105_64334108delTCGT(hom)	c.50_53delACGA(hom)	fs	Indel
PanNET23PT	*MEN1*	CCDS8083.1	g.chr11:64331233_64331234delAG(hom)	c.832_833delCT(hom)	fs	Indel
PanNET29PT	*MEN1*	CCDS8083.1	g.chr11:64334070C>A(hom)	c.88G>T(hom)	p.E30X	Nonsense
PanNET31PT	*MEN1*	CCDS8083.1	g.chr11:64328587G>T	c.1643C>A	p.S548X	Nonsense
PanNET39PT	*MEN1*	CCDS8083.1	g.chr11:64333993_64333999delAGGGATG(hom)	c.159_165delCATCCCT(hom)	fs	Indel
PanNET44PT	*MEN1*	CCDS8083.1	g.chr11:64333955delG	c.203delC	fs	Indel
PanNET45PT	*MEN1*	CCDS8083.1	g.chr11:64330370G>C	c.974C>G	p.P325R	Missense
PanNET52PT	*MEN1*	CCDS8083.1	g.chr11:64333876delG	c.282delC	fs	Indel
PanNET57PT	*MEN1*	CCDS8083.1	g.chr11:64334002delG(hom)	c.156delC(hom)	fs	Indel
PanNET59PT	*MEN1*	CCDS8083.1	g.chr11:64329234G>A	c.1213C>T	p.Q405X	Nonsense
PanNET61PT	*MEN1*	CCDS8083.1	g.chr11:64334049_64334201delGGAGCACCAGGTCCGGCTCCTCT CGGCCCAGCTCGGCAGCAAACAGGCGCACCACGTCGTCGATGGAGC GCAGCGGGAACAGCGTCTTCTGGGCGGCCTTCAGCCCCATGGCGGC GGGCGGTGGGCGGCGGCCTGCAAGGCAAGCCGGGGGAG(hom)	c.1_109delATGGGGCTGAAGGCCGCCCAGAA GACGCTGTTCCCGCTGCGCTCCATCGACGACG TGGTGCGCCTGTTTGCTGCCGAGCTGGGCCGA GAGGAGCCGGACCTGGTGCTCC(hom)	fs	Indel
PanNET64PT	*MEN1*	CCDS8083.1	g.chr11:64333781delC	c.377delG	fs	Indel
PanNET69PT	*MEN1*	CCDS8083.1	g.chr11:64330291delA	c.1053delT	fs	Indel
PanNET77PT	*MEN1*	CCDS8083.1	g.chr11:64334063_64334079delGGCTCCTCTCGGCCCAG	c.79_95delCTGGGCCGAGAGGAGCC	fs	Indel
PanNET78PT	*MEN1*	CCDS8083.1	g.chr11:64332045C>T	c.563G>A	p.W188X	Nonsense
PanNET83PT	*MEN1*	CCDS8083.1	g.chr11:64330369delG	c.975delC	fs	Indel
PanNET84PT	*MEN1*	CCDS8083.1	g.chr11:64334139G>A	c.19C>T	p.Q7X	Nonsense
PanNET85PT	*MEN1*	CCDS8083.1	g.chr11:64332011_64332012insCTGT	c.596_597insACAG	fs	Indel
PanNET93PT	*MEN1*	CCDS8083.1	g.chr11:64333906_64333909delAGAC	c.249_252delGTCT	fs	Indel
PanNET94PT	*MEN1*	CCDS8083.1	g.chr11:64333906_64333909delAGAC	c.249_252delGTCT	fs	Indel
PanNET95PT	*MEN1*	CCDS8083.1	g.chr11:64332032delC(hom)	c.576delG(hom)	fs	Indel
PanNET96PT	*MEN1*	CCDS8083.1	g.chr11:64331269T>C	c.IVS799-2A>G	c.IVS799-2A>G	SpliceSite
PanNET99PT	*MEN1*	CCDS8083.1	g.chr11:64331938C>A	c.IVS669+1G>T	c.IVS669+1G>T	SpliceSite
PanNET100PT	*MEN1*	CCDS8083.1	g.chr11:64331940_64331941delCG(hom)	c.667_668delCG(hom)	fs	Indel
PanNET102PT	*MEN1*	CCDS8083.1	g.chr11:64332102G>A	c.506C>T	p.A169V	Missense

Sample [#]	Gene	Transcript Accession	Nucleotide (genomic)[*]	Nucleotide (cDNA)	Amino acid (protein)[$]	Mutation type
PanNET108PT	*MEN1*	CCDS8083.1	g.chr11:64331200_64331251delGCAGCCTGGCCACTTCCCTCTACTGACCTTTCCAGATGTCCCAGGTCATAGA(hom)	c.815_837delTCTATGACCTGGGACATCTGGAA(hom)	del exon and intron	Indel
PanNET109PT	*MEN1*	CCDS8083.1	g.chr11:64334093A>C	c.65T>G	p.L22R	Missense
PanNET10PT	*PIK3CA*	CCDS43171.1	g.chr3:180418785G>A	c.1633G>A	p.E545K	Missense
PanNET10PT	*PTEN*	CCDS31238.1	g.chr10:89707693delG	c.738delG	fs	Indel
PanNET31PT	*PTEN*	CCDS31238.1	g.chr10:89682819T>G	c.323T>G	p.L108R	Missense
PanNET29PT	*PTEN*	CCDS31238.1	g.chr10:89710790_89710791insTGACAAGGAATATCTAGTACTTACTTTAA	c.961_c.962insTGACAAGGAATATCTAGTACTTACTTTAA	fs	Indel
PanNET96PT	*PTEN*	CCDS31238.1	g.chr10:89675287T>C(hom)	c.202T>C(hom)	p.Y68H	Missense
PanNET104PT	*PTEN*	CCDS31238.1	g.chr10:89701856G>A(hom)	c.494G>A(hom)	p.G165E	Missense
PanNET24PT	*TP53*	CCDS11118.1	g.chr17:7518284G>A	c.722C>T	p.S241F	Missense
PanNET91PT	*TP53*	CCDS11118.1	g.chr17:7519210delA(hom)	c.445delT(hom)	fs	Indel
PanNET100PT	*TP53*	CCDS11118.1	g.chr17:7520101delT(hom)	c.311delA(hom)	fs	Indel
PanNET2PT	*TSC2*	CCDS10458.1	g.chr16:2070191C>T	c.3422C>T	p.A1141V	Missense
PanNET31PT	*TSC2*	CCDS10458.1	g.chr16:2074957G>A	c.4498G>A	p.V1500M	Missense
PanNET44PT	*TSC2*	CCDS10458.1	g.chr16:2074337_2074338delTG	c.4113_c.4114delTG	fs	Indel
PanNET70PT	*TSC2*	CCDS10458.1	g.chr16:2078571C>T	c.5383C>T	p.R1795C	Missense
PanNET93PT	*TSC2*	CCDS10458.1	g.chr16:2038643C>A	c.26C>A	p.S9X	Nonsense
PanNET112PT	*TSC2*	CCDS10458.1	g.chr16:2076836A>G	c.4952A>G	p.N1651S	Missense

(hom): these mutations appear homozygous

[*] Coordinates refer to human reference genome hg18 release (NCBI 36.1, March 2006).

[#] Samples PanNET3, PanNET7, PanNET10, PanNET21, PanNET23, PanNET24, PanNET25, PanNET31, PanNET36, and PanNET93 were used for the initial (Discovery Set) screen.

[$] Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S,Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.