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Reference:
Vandamme Timon, Beyens Matthias, Boons Gitta, Schepers A., Kamp K., Biermann K., Pauwels Patrick, De Herder W. W., Hofland L. J., Peeters Marc, ....- Hotspot DAXX, PTCH2 and CYFIP2 mutations in pancreatic neuroendocrine neoplasms
Full text (Publisher's DOI): https://doi.org/10.1530/ERC-18-0120
To cite this reference: https://hdl.handle.net/10067/1576050151162165141
Hotspot DAXX, PTCH2 and CYFIP2 mutations in pancreatic neuroendocrine neoplasms

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Short title: Recurrent DAXX, PTCH2 and CYFIP mutations in pNEN

Key words: Neuroendocrine tumors, Molecular genetics, Ultra-deep sequencing, Pancreatic neuroendocrine tumors, DAXX

Word count: 4602

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Abstract

Mutations in DAXX/ATRX, MEN1, and genes involved in the phosphoinositide-3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR) pathway have been implicated in pancreatic neuroendocrine neoplasms (pNENs). However, mainly mutations present in the majority of tumor cells have been identified, while proliferation-driving mutations could be present only in small fractions of the tumor. This study aims to identify high- and low-abundance mutations in pNENs using ultra-deep targeted resequencing. Formalin-fixed paraffin-embedded matched tumor-normal tissue of 38 well-differentiated pNENs was sequenced using a HaloPlex targeted resequencing panel. Novel amplicon-based algorithms were used to identify both single nucleotide variants (SNVs) and insertion-deletions (indels) present in >10% of reads (high-abundance) and in <10% of reads (low-abundance). Found variants were validated by Sanger sequencing. Sequencing resulted in 416,711,794 reads with an average target base coverage of 2663±1476. Across all samples, 32 high-abundance somatic, 3 germline and 30 low-abundance mutations were withheld after filtering and validation. Overall, 92% of high-abundance and 84% of low-abundance mutations were predicted protein-damaging. Frequently mutated genes were MEN1, DAXX, ATRX, TSC2, PI3K/Akt/mTOR and MAPK-ERK pathway-related genes. Additionally, recurrent alterations on the same genomic position, so-called hotspot mutations, were found in DAXX, PTCH2 and CYFIP2. This first ultra-deep sequencing study highlighted genetic intra-tumor heterogeneity in pNEN, by the presence of low-abundance mutations. The importance of the ATRX/DAXX pathway was confirmed by the first-ever pNEN-specific protein-damaging hotspot mutation in DAXX. In this study, both novel genes, including the pro-apoptotic CYFIP2 gene and hedgehog-signaling PTCH2, and novel pathways, such as the MAPK-ERK pathway, were implicated in pNEN.
Introduction

Neuroendocrine neoplasms of the pancreas (pNENs), originating from the islet cells, are considered rare, although incidence is increasing (Dasari, et al. 2017). pNENs can occur as part of genetic syndromes, such as multiple neuroendocrine neoplasia 1 (MEN1), Von-Hippel Lindau (VHL) and tuberous sclerosis complex (TSC). However, most pNENs are sporadic tumors without familial history of NENs (Crona and Skogseid 2016). Recently, non-familial pNENs have been genetically characterized using whole-exome and whole-genome sequencing in large cohorts of 40 to 102 patients (Jiao, et al. 2011; Sadanandam, et al. 2015; Scarpa, et al. 2017). These studies identified MEN1 as most frequently mutated gene, in frequencies ranging from 37% to 44% of all sequenced tumors. Additionally, DAXX was found to be mutated in 22% to 25% of all tumor samples, while ATRX was mutated in 10% to 17% of all tumors. Menin (the MEN1 protein), DAXX, and ATRX are epigenetic regulators. Menin is involved in histone modification, while ATRX and DAXX play a role in alternative telomere lengthening and chromatin remodeling (Elsasser, et al. 2011; Heaphy, et al. 2011). Additionally, mutations in genes involved in the phosphoinositide-3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR) pathway were found in 12% to 14% of tumors (Jiao et al. 2011; Scarpa et al. 2017). In the pivotal Radiant-3 trial, treatment with everolimus, an mTOR inhibitor, has demonstrated an improved progression-free survival in advanced pNENs (Yao, et al. 2011). Hence, alterations in the PI3K/Akt/mTOR pathway, such as mutations in PTEN, could have clinical implications. However, no molecular predictive biomarker for everolimus treatment has yet been identified. Frequent tumor-specific copy number alterations in MEN1, ATRX, DAXX and PI3K/Akt/mTOR genes implicate these core pathways further (Scarpa et al. 2017). Recent efforts to describe molecular subtypes, have led to the identification of five mutational signatures in pNEN, including the novel MUTYH signature.
(Scarpa et al. 2017). However, in more than 50% of all tumors no dominant mutational signature could be identified (Scarpa et al. 2017). RNA expression analysis revealed three expression subtypes, respectively the insulinoma, MEN-1-like/intermediate and metastasis-like (MLP) subtype (Sadanandam et al. 2015; Scarpa et al. 2017). Clinical utility of these expression subtypes is subject of further study, as expression subtypes show a variable association with tumor grade, mainly in WHO 2010 grade 1 and 2 tumors (Sadanandam et al. 2015). Moreover, within-patient and within-tumor heterogeneity in proliferation has been demonstrated in neuroendocrine neoplasm models and patients (Shi, et al. 2015; Vandamme, et al. 2016; Vandamme, et al. 2015a). In recent pNEN sequencing studies, average sequencing depth was 61 to 102-fold (Sadanandam et al. 2015; Scarpa et al. 2017). Although covering all of the genome, these studies might lack sequencing power to reliably detect mutations present in a fraction of the cells, as these rare alleles might be present in less than 1 in 100 sequencing reads on a given genomic position (Gerstung, et al. 2012). Therefore, current studies lack information on mutational heterogeneity. By increasing sequencing depth, mutations, present in a fraction of cells, can be reliably identified (Gerstung et al. 2012). This study is the first to use an ultra-deep targeted resequencing approach in pNENs to elucidate mutations, present in less than 10% of sequencing reads.

Methods

Sample collection and clinical data

Patients, diagnosed between 1997 and 2013 with a reported WHO 2010 grade 1 or 2 pancreatic neuroendocrine neoplasm were retrospectively included in this study (Bosman, et al. 2010). Patients with a familial syndrome were excluded. Formalin-fixed paraffin-embedded (FFPE) samples of tumor and matched distant normal tissue, if available, of all
patients was collected at the Erasmus Medical Center (Rotterdam, the Netherlands) and the
Antwerp University Hospital (Antwerp, Belgium). All samples were reviewed by a dedicated
pathologist for histology, Ki67 index, mitoses per high power field and tumor purity. Only
samples with estimated tumor purity >60% on hematoxylin and eosin (H&E) stained
histological slide, after macrodissection, were included. Data on age, sex, TNM stage, age at
diagnosis, secretion status, received treatments, disease-free survival and overall survival
was collected. The study was approved by the institutional human ethics review board of the
Antwerp University Hospital. Informed consent was obtained from all patients for the use of
excess tissue material for scientific research, based on the opt-out registry to document the
objection of patients (as specified in Belgian and Dutch law).

Targeted gene panel development

A custom HaloPlex enrichment panel (Agilent Technologies, Santa Clara, CA, VS) was
developed to sequence all exons of 20 genes (table 1). Genes were selected based upon
patient and cell line sequencing results (Jiao et al. 2011; Vandamme, et al. 2015b). PI3K/Akt/mTOR pathway-related genes MTOR, PTEN, PIK3CA and TSC2 mutations have been
found in pNEN patients and could have therapeutic implications (Grabiner, et al. 2014; Jiao
et al. 2011; Scarpa et al. 2017). The gene panel was extended with the PI3K-related PIK3C2A
gene, as it was found mutated in pNEN cell line data (Vandamme et al. 2015b). Epigenetic
modifiers could play a role in pNEN, as demonstrated by MEN1, ATRX and DAXX mutations
identified previously in pNEN (Jiao et al. 2011; Scarpa et al. 2017). Next to these genes,
KANSL1 and PIF1, two epigenetic modifiers found in pNEN cell line data, were included in the
panel (Vandamme et al. 2015b). As preclinical data suggests a role for the MAPK/ERK
pathway in pNEN, KRAS, MAP4K2, MAPK9 and MAPKBP1 were added to the panel
The SMAD4 gene has been found mutated in neuroendocrine neoplasms, both in patient material and in cell lines (Bartsch, et al. 1999; Vandamme et al. 2015b). TP53 and CYFIP2 are two genes involved in apoptosis that are frequently implicated in oncogenesis. Both genes were found mutated in pNEN cell line data and were hence added to the panel (Vandamme et al. 2015b). Finally, the panel was extended with three genes (KISS1, PTCH2, WNT1) in known cancer-related pathways. All genes contained mutations in pNEN cell line data (Vandamme et al. 2015b). In our custom HaloPlex primer design, 99.59% of the 70,715 target kilobases of selected genes were covered by developed primers. The primer design was optimized for use with FFPE material and for sequencing on Illumina technology with a read length of 150 base pairs by increasing the number of amplicons to 19,838.

DNA isolation, HaloPlex enrichment and sequencing

After macrodissection, 10 slides of 5 µm of both tumor and normal tissue were used as input for DNA isolation using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany), following manufacturer’s instructions. The concentration of the isolated DNA was quantified using the Qubit 2.0 fluorometer with the dsDNA Broad Range Assay (Thermo Scientific, Wilmington, USA). To determine the amount of input DNA for HaloPlex enrichment, quality and degradation of the DNA were checked following the FFPE-Derived DNA Quality Assessment-protocol (Agilent Technologies, Santa Clara, CA, VS) using the LabChip GX (PerkinElmer, Waltham, MA, VS). Next, all tumor samples and three representative normal samples were prepared for targeted resequencing using a custom HaloPlex Design enrichment, optimized for FFPE sample enrichment, following the FFPE-optimized protocol according to manufacturer’s
instructions. The enriched samples were hybridized, amplified and sequenced on two lanes of a paired-end flow cell using HiSeq 1500 (Illumina, San Diego, USA) platform in rapid run mode.

Read alignment, variant calling and filtering

Raw sequencing reads were analyzed using an in-house developed Perl-based workflow. First, FastQC software (version 1.0) was used to assess quality of the raw data (Andrews 2010). Adapters and low-quality bases were trimmed using Cutadapt (version 1.2.1) and an in-house developed paired-end read quality trimmer (Vandeweyer, et al. 2014), respectively. Paired-end reads were then aligned to the human reference genome (hg19, NCBI Build 37) using Burrows-Wheeler Aligner (BWA mem, version 0.7.3a) (Li and Durbin 2009). Picard (version 1.88) was used to mark and remove duplicates. Afterwards, the three aligned normals were merged using Samtools (version 0.1.18)(Li, et al. 2009). After merging, somatic variant calling was performed on the tumor aligned data with VarScan2 (version 2.3.9) using the three merged normals as one merged normal (Koboldt, et al. 2012). Alignments with mapping quality lower than 17 or nucleotides with base quality lower than 17 were ignored. The max per-BAM depth was set on 30,000 avoiding excessive memory usage. No correction for tumor purity was set during the somatic calling, purities were kept on default (100%). The variant calling files were first filtered using pyAmpli to eliminate false positive variants introduced by amplicon-based enrichment (Beyens, et al. 2017). All variants were annotated with ANNOVAR (Wang, et al. 2010), and filtered using VariantDB (Vandeweyer et al. 2014) according to different criteria (Results section). Identified variants were validated in tumor and its matching normal tissue, if available, with Sanger sequencing on the 3130xl Genetic Analyser (Applied Biosystems Inc., Foster City, VS) platform and analyzed using CLC DNA
Workbench v5 software (CLC Bio, Aarhus, Denmark). Data were visualized using the Maftools package (Mayakonda and Koeffler 2016) in R (version 3.3.3). Survival statistics were generated using the survival and survminer package for R (version 3.3.3).

Results

Patient characteristics

38 pNEN patients were included, of which 51% were male. Of the 38 included patients, 13 patients had a functional tumor (11 insulinomas, one gastrinoma and one glucagonoma). Mean age at diagnosis was 53 ± 14 years. Of all patients, 9 (16%) had metastatic disease at diagnosis (suppl. table S1). Tumor tissue was available for all included patients, while matched normal tissue was collected in 27 patients (71%). 24 patients were diagnosed with WHO grade 1 disease, while 13 patients had a WHO grade 2 tumor. Upon central pathology review, one patient was reclassified as a WHO 2010 grade 3 tumor, given the Ki67 index of 30%. Median follow-up time was 6.3 years (range: 1.9-19.2 years). Median overall survival was 13 years [95% confidence interval 11 years – not reached].

Genetic alterations in pancreatic neuroendocrine neoplasms

Targeted resequencing of the selected HaloPlex enrichment 20-gene panel (see materials and methods) resulted in a total of 416,711,794 reads, passing quality filtering, across 38 tumor samples and three normal samples. Of all reads, an average 80.4 ± 5.0% mapped on target regions. Average target base coverage was 2663 ± 1476 and 94.7 ± 1.9% of all target bases was covered at least 30 times. Using VarScan2, variants in the tumor were called
against three merged normal samples. Afterwards, the amplicon-based filtering method pyAmpli was deployed to withhold only alterations present in more than one amplicon (if more than one amplicon was in the enrichment design) (Beyens et al. 2017). Additionally, all variants that were marked as “somatic” by VarScan2 were withheld. By this combined elimination, genetic alterations that are inherent to the enrichment with the HaloPlex panel and, hence, appear in all amplicons across all samples, are removed as false positive. Additionally, genetic alterations that are present in all three sequenced normal samples will most likely not be oncogenic driver mutations. Hence, these mutations can be discarded as common polymorphisms or artefacts when they are seen in the tumor samples. After this combined filtering, 17 indels and 2270 single nucleotide variations (SNVs) were withheld in the 38 tumors. The resulting median mutation burden of 0.80 per Megabase (Mb, range 0.51-1.54) within the 20 selected genes, with a genomic length of 70.71 Mb, is in line with the previously reported genome-wide mutation burden of 0.82 per Mb in pNEN and is low in comparison to other tumor types (Scarpa et al. 2017). Although caution is needed when comparing mutation burden between the targeted sequencing panel and the previously reported whole-genome data, it seems that the 20 selected genes in the targeted panel show no enrichment for mutations in comparison to other genomic regions. Before further filtering, the median number of genetic alterations per tumor was 56 with the MTOR gene containing the most alterations (figure 1). The most frequent nucleotide substitutions were C <-> T transitions (figure 1). Across all samples, transitions made up 63.4% ± 2.3% of the filtered SNVs, resulting in a ratio of transitions to transversions of 1.74 ± 0.18 in the presented gene panel. In human germline samples, an average transitions to transversion rate of 1.7 is usually seen genome-wide (Lynch 2010). A genome-wide mutation spectrum with a predominant C to T/G to A transition pattern is seen in many adult cancers, including
Identification and validation of somatic and germline mutations

To identify mutations with possible functional impact, RefSeq synonymous as well as intronic SNVs and intronic indels were first removed from further analysis (Pruitt, et al. 2014). To eliminate common single nucleotide polymorphisms (SNPs), only variants with a minor allele frequency (MAF) smaller than 0.05 in the dbSNP v142, ESP65000 and 1000 Genomes databases were withheld (Genomes Project, et al. 2010; Sherry, et al. 2001). Final visual inspection of all remaining variants in Integrative Genomics Viewer (IGV version 2.2.5), led to the identification of 72 mutations with possible functional impact (figure 2) (Thorvaldsdottir, et al. 2012). Of these 72 mutations, 42 alterations were identified in more than 10% of sequencing reads at that genomic position and were considered high-abundance alterations. These 42 alterations were Sanger sequenced in tumor tissue. Additionally, of 27 patients (71%) corresponding normal tissue was available, allowing Sanger sequencing of normal tissue for 22 mutations. Out of the 42 high-abundant variants, 35 were validated through Sanger sequencing, while 6 variants could not be detected in the Sanger electropherogram traces. For one variant, PCR amplification of the tumor DNA region containing the variant was unsuccessful, despite successful amplification in control DNA and use of different primers. Overall, 83.3% of all high-abundant variants could be validated (suppl. table S2). The 35 validated mutations contained three germline RefSeq non-synonymous variants, one in MAPKB1 and two in PIF1 respectively, all unique in three different tumors. None of the mutations were present in the cancer somatic mutation COSMIC v70 database (accession
date: 12th April 2017), while two SNVs were reported in the dbSNP142 database with unknown clinical significance, respectively dbSNP142 rs139868280 (PIF1) and rs201725344 (MAPKB1) (Forbes, et al. 2017; Sherry et al. 2001). The other PIF1 chr15:g.65108822C>T mutation was not found in any of the queried databases, including ExAc (version 03), 1000 Genomes Project (October 2014), dbSNP142 and COSMIC v70 (Forbes et al. 2017; Genomes Project et al. 2010; Lek, et al. 2016; Sherry et al. 2001).

Abundant mutations include a DAXX and CYFIP2 hotspot mutation

Of the 32 validated high-abundant somatic mutations, 21 SNVs were annotated as non-synonymous by RefSeq, 6 as frameshift indels, 3 as stopgain SNVs, 1 as SNV in the 3'-UTR and 1 as nonframeshift indel. The most commonly mutated gene was DAXX with five high-abundance mutations, while four high-abundance mutations were found MEN1, MAP4K2 and PTCH2. ATRX, KANSL1, TSC2 and MAPKB1 each contained three high-abundance mutations. These 8 genes accounted for 80% of all validated high-abundance mutations. No high-abundance mutations were found in PIF1, PIK3CA, PTEN, MAPK9, KRAS and TP53. Using variantDB, all variants were annotated for functional impact prediction using the MutationAssesor, MutationTaster, Provean and PolyPhen algorithms (Vandeweyer et al. 2014). Of all variants, 92.0% was predicted damaging by at least one algorithm, while 36.0% was predicted damaging by at least 3 algorithms. Three mutations were also seen in other tumor types, according the COSMIC database (Forbes et al. 2017). Two recurrent, so-called hotspot, mutations were found, one in CYFIP2 and one in DAXX. The CYFIP2 non-synonymous variant g.156766140G>A (NM_001037333, p.D820N) was found in two pNEN tumors. This variant had previously been identified in skin squamous cell carcinoma, according to COSMIC, and was predicted to be damaging by MutationTaster and PROVEAN.
Within the DAXX gene, one genomic position was altered differently in two tumors, yielding the non-synonymous g.33289247G>A (NM_001141970.1, p.S102L) and the stopgain g.33289247G>T (NM_001141970.1, p.S102X) mutations. Both mutations were predicted damaging by all used prediction algorithms, pointing towards a very likely DAXX loss-of-function in these tumors. The non-synonymous g.33289247G>A DAXX mutations has previously been found as a somatic mutation in one lung squamous cell carcinoma, according to COSMIC.

Low-abundance mutations contain a hotspot mutation in PTCH2

Next to high-abundance mutations, 30 mutations were found in less than 10% of targeted resequencing reads in tumor tissue. These mutations are considered low-abundance mutations. As the detection limit of Sanger sequencing is around 10%, these low-abundant alterations could not be Sanger sequenced (Tsiatis, et al. 2010). However, the Sanger sequencing validation rate of 83% in the high-abundant mutations demonstrates that the used combined filtering strategy (see above) yields mainly true positives. Additionally, only 9% of validated mutations was present in corresponding normal samples, illustrating that the employed filtering strategy selects for somatic variants. Hence, we assume that most of the low-abundance mutations are also both valid and somatic. RefSeq annotated 21 of these 30 low-abundance mutations as non-synonymous SNVs, four as frameshift indels, three as stopgain SNVs, one as splicing SNV and one as SNV in the 3’-UTR. The 3’-UTR PIK3C2A g.17111197_17111198del was found in 3 tumors. In addition, 2 intronic variants were found, the DAXX g.33286734A>C which was seen in 8 pNENs, and the TSC2 g.2124481_2124482insG, identified in 5 tumors. However, functional impact of these intronic mutations remains unclear. When predicting protein impact on the 26 exonic SNVs...
with the MutationAssesor, MutationTaster, Provean and PolyPhen algorithms using variantDB, 84.0% of all SNVs was predicted damaging by at least one algorithm, while 52.0% was predicted damaging by three or more prediction algorithms (Vandeweyer et al. 2014). After inclusion of the four frameshift indels, which can be considered to have a deleterious impact on protein function, 86.6% of all mutations have a likely protein impact. Three mutations were previously found in cancer samples, according to the COSMIC database, including the non-synonymous TP53 g.7578457C>T mutation (NM_001276695.1, p.R158H) which was identified in 36 tumors of various origin (Forbes et al. 2017). Two tumors contained the same stopgain g.45292871C>A mutation (NM_001166292, p.G828X) in PTCH2, predicted to be damaging by MutationTaster, PROVEAN and SIFT. This protein-damaging PTCH2 mutation is hence a novel low-abundance hotspot mutation within pNEN.

Mutational signatures in pNEN

In total, 32 tumors contained mutations while 6 tumors had no mutations that survived filtering (figure 3). Both low-abundance and high-abundance mutations were seen in 11 tumors (29%). High-abundance mutations were seen exclusively in 14 tumors (37%), while 7 tumors (18%) only contained low-abundance mutations (figure 2). MEN1 was mutated most frequently in 8 out of 38 tumors (21%). DAXX and ATRX were both mutated in 5 out of 38 tumors (13%). The PI3K/Akt/mTOR-related gene TSC2 was mutated in 7 out of 38 tumors (18%). Overall, mutations in PI3K/Akt/mTOR-related genes were seen in 29% of the tumors, including a validated non-synonymous g.1308007C>T mutation in MTOR (NM_004958, p.A329T, rs35903812) not previously reported in pNEN. The mitogen-activated kinase and
extracellular signal-regulated kinase (MAPK-ERK pathway) genes MAP4K2 and MAPKBP1 were each mutated in three tumors.

Discussion

This study presents the first ultra-deep targeted resequencing of pNENs in archival tissue. The relative rarity of pNENs has led to only a limited number of large-scale studies on the genetic constitution of pNENs (Jiao et al. 2011; Sadanandam et al. 2015; Scarpa et al. 2017). Additionally, these studies focus on a broad overview of frequent genetic alterations in pNEN and report high-abundance mutations. These high-abundance mutations are present in the majority of tumors cells. However, various studies have demonstrated that the genetic make-up of primary tumors evolves dynamically in time (Burrell, et al. 2013; Stratton, et al. 2009). This time-dependent change of the genetic alterations present in a tumor reflects the appearance and disappearance of subsets of tumor cells, so-called subclones, within one tumor (Burrell et al. 2013; Stratton et al. 2009). In our study, the use of ultra-deep sequencing allowed for the identification of genetic alterations that are present in a low fraction of the tumoral tissue, so-called low-abundance mutations. These low-abundance mutations are indicative for the genetic heterogeneity within a single tumor. Recent genetic studies have identified the insulinoma, MEN-1-like/intermediate and metastasis-like (MLP) RNA expression subtype in pNENs (Sadanandam et al. 2015). Although the MEN-1-like tumors frequently contains MEN1 mutations, the mutational burden of high-abundance mutations in other core pathways seems to be more variable across subtypes (Sadanandam et al. 2015). Additionally, DNA damage repair, chromatin modification, alternated telomere length and the PI3K/Akt/mTOR pathways have been highlighted as core altered pathways in pNEN (Jiao et al. 2011; Scarpa et al. 2017). In this study, we demonstrate that low-
abundance mutations are found in these pathways. Hence, low-abundance mutations might help to better classify tumors. However, validation of these low-abundance mutations remains complex and its lack forms a limitation of our study. The gene with the most high-abundance mutations within our study was the tumor suppressor gene DAXX. ATRX and DAXX form a complex facilitating the incorporation of histone variant H3.3 at the telomeres and, consequently, play a role in alternative lengthening of telomeres (Heaphy et al. 2011; Lewis, et al. 2010). In many cancer types, it has been demonstrated that alternative telomere lengthening (ATL) leads to a prolonged cell survival, which is a hallmark of cancer (Cesare and Reddel 2010). In pNEN, ATL has been associated with a reduced disease-free survival (Singhi, et al. 2017). Additionally, loss of ATRX and DAXX on immunohistochemistry (IHC) staining, caused by inactivating mutations or copy number loss, is associated with increased occurrence of metastasis and reduced disease-free survival (Scarpa et al. 2017; Singhi et al. 2017). Both high- and low-abundance mutations in DAXX and ATRX were identified in this study, including the first-ever validated recurrent, hotspot, mutation in DAXX, with likely loss-of-function on protein level. In line with other studies, exonic (transcript coding or protein encoding) mutations in DAXX and ATRX were mutually exclusive (Jiao et al. 2011; Scarpa et al. 2017). As this is the first study to demonstrate the existence of low-abundance mutations in ATRX and DAXX, conclusions on clinical impact are limited. Nonetheless, detection of low-abundance mutations might lead to the identification of DAXX and ATRX loss before this is apparent on IHC and, thus, an earlier identification of high-risk patients. Given the low number of events in the studied population (both in disease-recurrence and in mortality), this study is not adequately powered to evaluate prognostic relevance of these low-abundance mutations. Hence, further studies are needed. Menin, the nuclear protein encoded by MEN1, impacts ATL by negatively regulating hTERT (Lin and
Additionally, it plays a key role in chromatin remodeling and gene expression through histone acetylation and deacetylation (Kim, et al. 2003). MEN1 mutations have been implicated in pNEN oncogenesis, both as part of the familial MEN1 syndrome, and in sporadic tumors (Corbo, et al. 2010; Crona and Skogseid 2016; Jiao et al. 2011; Scarpa et al. 2017). In 12-14% of all pNEN patients, a mutation in PI3K/Akt/mTOR-related genes is reported, including mutations in PTEN, MTOR, DEPDC5, TSC1, TSC2 and PIK3CA (Chou, et al. 2016; Jiao et al. 2011; Scarpa et al. 2017). In this study, PI3K/Akt/mTOR-related mutations could be identified in 29% of all tumors, including a novel p.A329T mutation in MTOR in pNEN. This increased frequency of mutations in the PI3K/Akt/mTOR-related genes is due to the detection of low-abundance mutations in these tumor samples. The relation of everolimus efficacy and PI3K/Akt/mTOR pathway-related mutations remains the subject of study in pNEN. However, in other cancer types, mutations of the PI3K/Akt/mTOR pathways seems to confer everolimus sensitivity (Grabiner et al. 2014; Wagle, et al. 2014). In neuroendocrine neoplasms and other cancer types, cross-talk activation between the PI3K/Akt/mTOR pathway and the mitogen-activated kinase and extracellular signal-regulated kinase (MAPK-ERK pathway) through PI3K has been described (Carracedo, et al. 2008; Valentino et al. 2014; Zitzmann, et al. 2010). Until now, no mutations in MAPK-ERK pathway-related genes have been reported in pNEN. In this study, mutations in MAP4K2 and MAPKBP1 were identified, further implicating this pathway in pNEN and providing additional rationale for the use of MEK-inhibitors in pNEN, either in combination with mTOR inhibitors or alone. Although TP53 is frequently mutated in other cancers, TP53 mutation frequency is low in pNEN, as demonstrated by only two low-abundance TP53 mutations being present in our cohort (Jiao et al. 2011; Scarpa et al. 2017). Additionally, the pro-apoptotic gene CYFIP2 was mutated in four tumors. In two tumors, the exact same mutation was present, a so-
called hotspot mutation. This validated mutation has never been reported before in pNEN and is predicted to be protein-damaging, warranting further studies into the role of CYFIP2 in pNEN. Three mutations (one high-abundance and two low-abundance) were found in SMAD4. Unlike in small intestinal neuroendocrine neoplasms, where SMAD4 loss is relatively common, the frequency of SMAD4 mutations in pNEN has been the matter of debate (Banck, et al. 2013; Bartsch et al. 1999; Perren, et al. 2003). Multiple low-abundance recurrent mutations were identified, including a hotspot mutation in PTCH2. PTCH2 encodes the Patched 2 protein, which is involved in hedgehog signaling (Rahnama, et al. 2004; Smyth, et al. 1999). PTCH2 mutations haven been found in basal cell carcinoma, medulloblastoma and rhabdomyosarcoma and myeloproliferative neoplasms (Klein, et al. 2016; Smyth et al. 1999; Taeubner, et al. 2018). However, this is the first time that hedgehog signaling has been implicated in pNENs. As the technical limitations of Sanger sequencing didn’t allow validation of this low-abundance mutation, further studies in replication cohorts are needed to confirm hedgehog signaling as a novel pathway in pNENs. Given that various hedgehog signaling inhibitors have been approved for use in basal cell carcinoma, confirmatory studies on the role of hedgehog signaling could open new therapeutic options for pNENs, harboring PTCH2 mutations (Lacouture, et al. 2016). Finally, the limited availability of fresh-frozen tissue in pNEN could hinder the implementation of next-generation sequencing technology in pNEN diagnostics. In contrast, archival tissue, necessary for histological diagnosis of pNEN, is frequently available and can be easily manipulated and stored in a cost-effective manner. A possible limitation of the use of FFPE, is the induction of false-positive mutations by formalin fixation (Williams, et al. 1999). These mutations often follow a deamination pattern, resulting in transitions (C>T or G>A). Hence, when formalin fixation would result in a considerable number of additional induced mutations, there would be an increase in the
number of transitions (C>T or G>A) in comparison to transversions (A>C or G>T). However, the ratio of transitions to transversion in this study is similar to transition-transversion ratios seen in normal human (germline) samples and in pancreatic neuroendocrine neoplasm samples from fresh-frozen tissue (Jiao et al. 2011). Therefore, the impact of formalin-fixation can be considered to be relatively limited. To further reduce any potential impact, the in-house developed pyAmpli tool filters specifically for mutations present in more than one amplicon and, thus, amplified DNA fragment (Beyens et al. 2017). As fixation-induced “deamination” is stochastic and happens randomly in all DNA strands, the chance that two different DNA molecules have exactly the same formalin fixation-induced is very limited. Hence, our study demonstrates that by using this approach archival FFPE tissue can be used reliably in genetic analysis of pNENs.

In conclusion, this study adds to the growing body of evidence on the broad genetic constitution of pNENs by demonstrating the presence of low-abundance mutations and genetic heterogeneity in pNENs. We highlight the importance of the ATRX/DAXX pathway by reporting the first-ever pNEN-specific protein-damaging hotspot mutation in DAXX, and uncover novel genes and pathways involved in pNENs, including pro-apoptotic CYFIP2, hedgehog signaling and the MAPK-ERK pathway.

Declaration of interest

Timon Vandamme: advisory role and speakers’ fees for Ipsen and Novartis.

Wouter de Herder: advisory role and speakers’ fees for Ipsen and Novartis.

Marc Peeters: advisory role and speakers’ fees for Ipsen and Novartis.
Funding

This work was supported by the Flemish Agency of Scientific Research (FWO grant G.0327.13N) and the ENETS-Ipsen 2013 Translational Research Fellowship.

Author contributions

TV, MB, GB, AS performed the sequencing experiments. TV, MB, KOdB processed the experimental data, performed the analysis, drafted the manuscript and designed the figures. PP, KB and LH performed pathological review of all tumor and normal samples and aided in patient selection. KK and WW aided in patient selection and collected clinicopathological data. LH, WW, MP and GVC were involved in planning and supervised the work. All authors discussed the results and commented on the manuscript.

Acknowledgements

We would like to thank Lesley De Backer from the Multidisciplinair Oncologisch Centrum Antwerpen (MOCA) and NETwerk for her support in collecting clinicopathological data of all patients included at the University Hospital Antwerp.
References


Table legends

Table 1. List of genes (including encoded protein, chromosomal positions and number of exons) and the calculated coverage of the custom HaloPlex enrichment panel. * Multifunctional protein also associated with FAS signaling pathway.

Figure legends

Figure 1. Summary plot of SNVs (N=2270), insertions (N=9) and deletions (N=8) of all 38 samples, after amplicon-based filtering.

Figure 2. Filter strategy and abundancy of mutations across tumors. Filtered variants are SNVs or indels that are not RefSeq synonymous or intronic and have a minor allele frequency (MAF) ≤ 0.05 in dbSNP v142, ESP65000 and 1000 Genomes. Abundance is considered low for alterations found in <10% of targeted resequencing reads in tumor tissue and high when an alteration is present in ≥10% of targeted resequencing reads. Mutation are validated somatic when mutation is present in Sanger sequencing of tumor but not in normal (if available). Germline mutations are present in Sanger sequencing traces of both tumor and normal tissue. Not validated SNVs are those SNVs with absent Sanger traces or inconclusive results. All low-abundance SNVs or indels are assumed to be somatic. Pie-chart shows the percentage of patients whose tumors contained only low-abundance mutations (grey), only high-abundance mutations (yellow), both (red) or no mutations (blue).

Figure 3. Mutation plot showing frequency, type of mutations, abundancy and mutational distribution of genetic alterations across 38 included tumors, after filtering and validation (if
executed), including both high-abundance (N=32) and low-abundance (N=30) mutations.

Low-abundance mutations are marked with red, in addition to color-coded mutation type.

When a tumor contains multiple mutations within one gene, this is annotated as multi-hit (dark green).
<table>
<thead>
<tr>
<th>RefSeq gene id</th>
<th>Encoded protein</th>
<th>Chromosomal position</th>
<th>Exons</th>
<th>Size (bp)</th>
<th>Coverage (%)</th>
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<td>MTOR</td>
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High abundance SNVs
- \( n = 42 \)
- \( \geq 10\% \) of reads

Low-abundance SNVs
- \( n = 72 \)
- < 10\% of reads

Germline
- \( n = 32 \)

Assumed somatic
- \( n = 30 \)

Not validated
- \( n = 7 \)

Possible protein impact

Abundance

High abundance SNVs
- \( n = 42 \)
- \( \geq 10\% \) of reads

Low-abundance SNVs
- \( n = 30 \)
- < 10\% of reads

Abundancy in % patients
- High: 37\%
- Both: 16\%
- Low: 18\%
- None: 29\%

Validation

Somatic
- \( n = 32 \)

Germline
- \( n = 3 \)

Not validated
- \( n = 7 \)

Assumed somatic
- \( n = 30 \)
Gene mutation plot after filtering

- MEN1
- PIK3C2A
- TSC2
- ATRX
- DAXX
- PTCH2
- CYFIP2
- KANSL1
- MAP4K2
- MAPKBP1
- MTOR
- PIF1
- PTEN
- SMAD4
- TP53
- WNT1

Legend:
- Frame shift deletion
- Multi-hit
- Non-synonymous
- Splice site
- 3’ UTR
- Frame shift insertion
- Stopgain
- Low-abundance