



## Genetic Analysis of Small Well-differentiated Pancreatic Neuroendocrine Tumors Identifies Subgroups With Differing Risks of Liver Metastases

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### Abstract

**Objective:** The aim of this study was to investigate the key molecular alterations in small primary pancreatic neuroendocrine tumors (PanNETs) associated with the development of liver metastases.

**Background:** Well-differentiated PanNETs with small size are typically indolent; however, a limited subset metastasize to the liver.

**Methods:** A total of 87 small primary PanNETs (<3cm), including 32 metastatic cases and 55 nonmetastatic cases after a 5-year follow-up, were immunolabeled for DAXX/ATRX and analyzed for alternative lengthening of telomeres (ALT) by Fluorescence In Situ Hybridization. A subset of

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these cases, 24 that metastasized and 24 that did not metastasize, were assessed by targeted next-generation sequencing and whole-genome copy number variation.

**Results:** In the entire cohort, high Ki-67 (OR 1.369; 95% CI 1.121–1.673;  $P=0.002$ ), N-stage (OR 4.568; 95% CI 1.458–14.312;  $P=0.009$ ), and ALT-positivity (OR 3.486; 95% CI 1.093–11.115;  $P=0.035$ ) were independently associated with liver metastases. In the subset assessed by next-generation sequencing and copy number variation analysis, 3 molecular subtypes with differing risks of liver metastases were identified. Group 1 ( $n=15$ ; 73% metastasized) was characterized by recurrent chromosomal gains, CN-LOH, DAXX mutations, and ALT-positivity. Group 2 ( $n=19$ ; 42% metastasized, including 5 G1 tumors) was characterized by limited copy number alterations and mutations. Group 3 ( $n=14$ ; 35% metastasized) were defined by chromosome 11 loss.

**Conclusions:** We identified genomic patterns of small PanNETs associated with a different risk for liver metastases. Molecular alterations, such as *DAXX* mutations, chromosomal gains, and ALT, are associated with an increased risk of metastasis in small PanNETs. Therefore, targeted sequencing and/or AsLT analysis may help in the clinical decisions for these small PanNETs.

### Keywords

ALT; genetic subgroups; liver metastases; pancreatic neuroendocrine tumors (PanNETs)

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The incidence of pancreatic neuroendocrine tumors (PanNETs) is 1 per 100,000 individuals,<sup>1</sup> However, autopsy studies highlight a prevalence ranging from 1% to 10% in the general population, suggesting that most PanNETs do not progress to clinical diagnosis.<sup>2,3</sup> In recent years, an increasing number of small and asymptomatic PanNETs have been diagnosed incidentally on routine abdominal imaging.<sup>4</sup> As the risk for metastases has been associated with tumor size and high proliferative index,<sup>5–7</sup> periodic observation without resection has been advocated for small tumors. However, such observational approaches have not been universally adopted, due to a small, but existing risk of liver metastases among small tumors, particularly with the absence of biomarkers predictive for progression.<sup>8,9</sup>

Recent whole genome sequencing and expression profiling suggest that an aggressive phenotype is associated with genetic changes in telomere maintenance and/or mTOR signaling.<sup>10–12</sup> Loss of function of *DAXX* or *ATRX* genes promotes the activation of the alternative lengthening of telomeres (ALT) pathway.<sup>13,14</sup> ALT is associated with chromosomal instability, larger tumor size, higher Ki67 and, in some studies, metastatic spread.<sup>10,15,16</sup> Alterations in mTOR pathway genes are also associated with a poor prognosis and several therapeutic agents targeting the pathway are available for systemic treatment.<sup>10,17–19</sup>

Thus, there is an urgent need to define early biological mechanisms responsible for tumor progression that may serve as clinically useful biomarkers. Here, we analyzed molecular alterations of small sporadic well-differentiated PanNETs (<3 cm in size) to identify alterations associated with metastasis. In the entire cohort of 87 primary PanNETs, we assessed ATRX and DAXX protein expression, and ALT status. In a subset, 24 primary PanNETs that metastasized and 24 primary PanNETs that did not metastasize, matched for

size and proliferation index, were analyzed by targeted next-generation sequencing and for copy number alterations. These genomic analyses identified 3 molecular subtypes with differing risks for liver metastases.

## METHODS

### Study Population

This study was approved by the Institutional Review Board (IRB) of the Johns Hopkins Hospital and tissue samples were retrieved from collaborating institutes with informed consent according to their local Institutional Review Boards. Well-differentiated (G1 and G2) PanNETs < 3 cm in maximum dimension were identified in the pancreatic resection databases of the Johns Hopkins Hospital, University of Verona, Italy; University of Utrecht, The Netherlands; Pederzoli Hospital, Peschiera, Italy. All PanNETs included were sporadic, unifocal, and nonfunctional. The study included patients who developed synchronous or metachronous liver metastases and patients who did not develop distant metastases after a surveillance period of at least 5 years. PanNETs were graded according to 2017 World Health Organization (WHO) classification based on mitotic rate and Ki-67: < 2 mitoses/10 high-power fields and Ki-67 < 3% for grade 1 (G1), and 2 to 20 mitoses/10 high-power fields, and Ki-67 of 3% to 20% for grade 2 (G2).<sup>20</sup>

### Immunohistochemistry (IHC) and Fluorescence In Situ Hybridization (FISH)

IHC for ATRX and DAXX and telomere-specific FISH were performed on formalin-fixed paraffin-embedded (FFPE) tissues. Nuclear labeling was evaluated for DAXX and ATRX and cases were scored as negative if nuclear expression was completely lost (despite retaining cytoplasmic expression) and adequate internal positive controls were present (eg, nuclear labeling of endothelial cells, lymphocytes, islets of Langerhans).<sup>13,21,22</sup> ALT-positive tumors were identified by cell-to-cell telomere length heterogeneity and the presence of large, ultrabright nuclear foci of FISH signals. As previously defined,<sup>13,14,22</sup> cases were classified as ALT-positive when bright nuclear foci occurred in 1% of tumor cells with at least 500 neoplastic cells evaluated.

### DNA Extraction

Five micrometer sections from FFPE tissues were manually macrodissected to enrich for neoplastic cellularity, DNA was extracted using QIAamp DNA FFPE Tissue Kit (Qiagen, German-town) and quantified by Quantifiler Human DNA Quantification kit (Applied Biosystems, Thermo Fisher Scientific, Waltham).

### Targeted Next-generation Sequencing

An Ion AmpliSeq Custom Panel (AmpliSeq Designer version 4.4.7; Life Technologies, Thermo Fisher Scientific, Waltham) was used to perform multiplex PCR and sequencing of 18 genes known to be targeted in pancreatic neuroendocrine or ductal neoplasms (*MEN1*, *DAXX*, *ATRX*, *TSC1*, *TSC2*, *PTEN*, *PIK3CA*, *PHLDA3*, *TP53*, *KRAS*, *GNAS*, *SMAD4*, *CDKN2A*, *RNF43*, *TGFBR2*, *ARID1A*, *BRAF*, *MAP2K4*).<sup>10,12,23,24</sup> Briefly, DNA (4ng) was amplified using AmpliSeq reagents for library preparation, which was then loaded and sequenced onto a 318v2 chip using an Ion Torrent Personal Genome Machine (PGM; Life

Technologies). Postsequencing data analyses, including alignment to the hg19 human reference genome and variant calling, were performed using NextGENe software (v2.4; SoftGenetics, Chicago, IL). Alignments and putative mutations were visually verified using the Integrative Genomics Viewer (IGV, v2.3; Broad Institute, Cambridge) and the NextGENeViewer (v2.4; SoftGenetics).

### SNP Array Analysis

Copy number analysis was performed at the JHU Microarray Core Facility using the Illumina HumanCytoSNP-12 v2.1 BeadChip, according to manufacturer's protocols. Log R ratios (LRRs) and B allele frequencies for each sample were extracted using GenomeStudio software. Prior copy number analysis, LRRs were normalized performing a GC-content correction.<sup>25</sup> We used the Circular Binary Segmentation algorithm to estimate mean LRR for distinct genomic segments, and we applied a mixture model to compute copy number alteration probabilities and to classify each segment accordingly, as implemented in the "CGHcall" and "CGHregion" R-Bioconductor packages.<sup>24,25</sup> Sex chromosomes were not included in this analysis and tumor cellularity was estimated using the "qPure" algorithm.<sup>26</sup> We used the hg19 human reference genome assembly (GRCh37) to annotate segments of copy number alteration with the associated genes. For regions of normal copy number, we further analyzed the B allele frequency distribution, identifying copy neutral loss of heterozygosity (CN-LOH, loss of 1 allele with duplication of the remaining allele). Chromothripsis was defined as the shattering and reassembly of 1 or more chromosomes and was detected using the CTLP Scanner algorithm.<sup>27</sup>

### Statistical Analysis

Continuous variables were reported as median and interquartile range or mean and standard deviation according to the values distribution. Correlation between clinical-pathological characteristics with ALT phenotype and development of liver metastases was assessed by univariate analysis using chi-square test or Fisher exact tests for categorical variables, and Student *t* test or Mann-Whitney *U* for continuous variables. A forward stepwise logistic regression model was used to evaluate independent risk factors associated with development of liver metastasis. A 2-tailed *P* value  $\leq 0.05$  was considered significant. Data were analyzed using SPSS 24.0 for Windows v24.0.

## RESULTS

As illustrated in Figure 1, 87 patients who underwent pancreatectomy for unifocal, well-differentiated primary PanNET  $< 3$  cm in size met the inclusion criteria. These included 32 patients who developed liver metastases (19 synchronous, 13 metachronous), and 55 patients who remained disease-free after at least 5 years of follow-up (mean 117 mo, standard deviation 58 mo).

PanNETs were treated by distal pancreatectomy in 47 patients, pancreatoduodenectomy in 36, and enucleation in 4. There were 54 (62.1%) G1 and 33 (37.9%) G2 tumors (Table 1). Lymphovascular and perineural invasion were present in 19 (21.8%) and 26 (29.9%) tumors,

respectively; AJCC stage<sup>28</sup> was T1 for 36 (41.4%), T2 for 31 (35.6%), and T3 for 20 (23.0%) tumors. Regional lymph node metastases were present in 29 (33.3%) tumors.

### DAXX/ATRX Immunolabeling and ALT Analysis

Immunolabeling for ATRX and DAXX and telomere-specific FISH analysis to detect ALT-positive tumors was performed on 85 of the 87 cases. For 2 cases, the material available for research purposes was limited and only used in the genomic analyses. Loss of DAXX or ATRX was detected respectively in 20/85 (23.5%, 13 metastatic and nonmetastatic) and 6/85 (7.1%, 3 metastatic and 3 nonmetastatic) tumors; the loss of protein expression in either ATRX or DAXX was mutually exclusive. Twenty-seven of the 85 tumors were ALT-positive (31.8%, 18 metastatic and 9 nonmetastatic). By univariate analysis, ALT was strongly associated with loss of DAXX or ATRX nuclear expression ( $P < 0.001$  and  $P = 0.001$ , respectively), Ki-67 index (Fig. 2,  $P = 0.001$ ) and WHO grade ( $P = 0.004$ ). In all but 3 cases, results from IHC and ALT assessment were concordant. Discordant cases included 2 ALT-positive cases with retention of DAXX and ATRX protein expression, and 1 case with loss of nuclear DAXX expression without concurrent ALT.

Clinicopathological features associated with the development of liver metastases in univariate analysis were larger tumor size as a continuous ( $P = 0.010$ ) and as a categorical ( $P = 0.023$ ) variable, Ki-67 index ( $P < 0.001$ ), WHO grade ( $P = 0.001$ ), ALT positivity ( $P < 0.001$ ), and DAXX loss ( $P = 0.008$ ). On multivariate analysis, only Ki-67 index (OR 1.369; 95% CI 1.121–1.673;  $P = 0.002$ ), N stage (OR 4.568; 95% CI 1.458–14.312;  $P = 0.009$ ), and ALT positivity (OR 3.486; 95% CI 1.093–11.115;  $P = 0.035$ ) were confirmed to be independent risk factors for liver metastases (Table 2).

### Mutational and Copy Number Analysis

Genetic analyses were performed on a subset of these cases, which included 48 primary PanNETs, of which 24 from the metastatic group, from which the extracted DNA passed the quality controls, and 24 from the control group matched for tumor size and WHO grade (Table 1, Supplemental material, <http://links.lww.com/SLA/B503>).

*Targeted next-generation sequencing* detected a total of 38 somatic mutations in 7 genes among the 48 tumors, with a range of 0 to 3 mutations per tumor (Fig. 3). *MEN1* was mutated in 16 (33%) cases and mTOR pathway genes were mutated in 10 cases, more specifically *TSC2* in 9 cases (19%) and *PTEN* in 1 case (2%). *DAXX* was mutated in 9 cases (19%) and *ATRX* in 1 (2%). *TP53* and *CDKN2A* were mutated in 1 case (2%) each. Comparison of mutations with metastatic status showed that *MEN1* was mutated in 9 metastatic and 7 nonmetastatic cases, *TSC2* in 6 metastatic and 3 nonmetastatic, *ATRX* and *CDKN2A* were mutated in 1 nonmetastatic case each, and *PTEN* and *TP53* were mutated in 1 metastatic case each. Strikingly, *DAXX* was mutated in 8 metastatic cases and 1 nonmetastatic case ( $P = 0.023$ ).

*Genome-wide SNP array analysis* revealed large chromosomal events including recurrent regions of gain and loss. All chromosomal alterations, along with associated genes and noncoding regions, are listed in Supplementary Dataset S1, <http://links.lww.com/SLA/B502>. Tumors that metastasized were characterized by a higher prevalence of chromosomal gains

(1046 regions of gain vs. 661), while nonmetastatic tumors had more chromosomal losses (756 regions of loss vs. 701). Copy number alterations consisted in broad genomic regions generally spanning entire chromosomes (Fig. 4). The most frequent chromosomal gains involved chromosomes 4, 5, 7, 9, 14, 18, and 19 with frequencies ranging from 25% to 50%, while recurrent chromosomal losses were detected in chromosomes 11, 16, and 22 with frequencies ranging from 25% to 75% (Fig. 4). PanNETs with frequent chromosomal gains also exhibited recurrent CN-LOH events, involving the other chromosomes (Supplementary Dataset S2, <http://links.lww.com/SLA/B502>).

Chromosome 11q13.1 containing the *MEN1* locus was lost in 16 cases (33%), while several regions previously described to be gained in PanNETs were also identified in our study: *ERBB2* (chr 17q12) in 12 (25%) cases, *PSPN* (chr 19p13.3) in 10 (21%), and *ULK1* (chr 12q24.33) in 15 (31%). *AKT2* (chr 19q13.2), a serine/threonine-protein kinase that inactivates *TSC2* and therefore upregulates the mTORC1 complex, was amplified in 12 cases (25%), none of which harboring a *TSC2* mutation.

Chromothripsis was detected at chromosome 11 in 4 cases (PanNET2, 11, 27, and 37); at chr 7 in 7 cases (PanNET4, 11, 15, 23, 26, 30, 46); and at chr 9 in PanNET46.

### Genomic Subtypes

Cluster analysis of copy number status stratified the tumors into 3 different molecular subtypes on the basis of recurrent chromosomal alterations (Fig. 5): Group 1, characterized by chromosomal gains; Group 2, with limited number of chromosomal events; Group 3, presenting a recurrent pattern of chromosome loss, mainly affecting chromosomes 11 and 22. Integrating copy number alterations, single nucleotide variants, and ALT status revealed distinct molecular and clinical patterns for each subtype. Overall, Group 1 was strongly associated with metastases compared with the other groups combined (chi-squared test,  $P < 0.03$ ).

Group 1 included 15 PanNETs that, all except one (PanNET8), were ALT-positive. Strikingly, 14 cases lacked DAXX or ATRX protein expression (respectively in 11 and 3 cases), which was due to mutations in 10 cases (*DAXX* in 9, *ATRX* in 1). As expected, all cases that were wild type at the genetic level for *ATRX* and *DAXX* retained nuclear protein expression. *MEN1* was mutated in 5 cases and mTOR pathway genes in 5 cases (*TSC2* in 4, *PTEN* in 1). The majority of Group 1 tumors were G2(11/15) and 73% (11/15) were metastatic.

Group 2 included 19 cases characterized by limited copy number and mutational events. *TSC2* and *MEN1* were mutated in 4 (21%) and 3 (16%) cases, respectively. All cases were ALT-negative, wild-type for the *ATRX/DAXX* genes, and preserved nuclear ATRX/DAXX protein expression. In this group, 42% (8/19) of tumors developed metastases, including 5 G1 and 3 G2 tumors.

Group 3 included 14 cases, all exhibiting recurrent loss of chromosome 11q *MEN1* locus and associated with *MEN1* somatic mutations in 8 (57%) cases. *TSC2* and *CDKN2A* were mutated in 1 case each. Only 1 G2 case that metastasized (PanNET6) was ALT-positive and

displayed loss of nuclear ATRX protein expression. In this group, 35% (5/14) of the tumors were metastatic (4 G2 cases and 1G1).

## DISCUSSION

PanNETs are characterized by a variable spectrum of clinical behavior.<sup>20</sup> Several lines of evidence suggest that most small primary PanNETs are indolent tumors, supporting the idea that surveillance, rather than surgical resection, could be indicated.<sup>4,8,29,30</sup> However, a limited subset of small primary PanNETs are aggressive and will metastasize, predominantly to the liver. The discrimination of small PanNETs with a high risk of metastasis from those with low risk has important prognostic and therapeutic implications. First, we evaluated clinicopathological features and several tissue-based biomarkers in a cohort of 87 primary well-differentiated PanNETs that were all < 3cm in size. We observed that high Ki-67 (OR 1.369), N-stage (OR 4.568), and ALT-positivity (OR 3.486) were independently associated with liver metastases. While our cohort was limited to small PanNETs, these findings are similar to other recent studies assessing cohorts encompassing the entire range of tumor sizes.<sup>15,16,22</sup>

Next, vailing of a recent whole genome sequencing study,<sup>10</sup> we investigated a series of small primary PanNETs (<3 cm) using whole genome copy number analysis and targeted deep sequencing of 18 relevant genes to assess the genetic alterations associated with metastatic spread. To this end, we compared the genetic alterations in small primary PanNETs that developed liver metastases with those that were metastases-free after a long postresection follow-up period. We identified 3 different molecular subtypes of small primary PanNETs on the basis of the copy number status, matching those recently described in Scarpa et al,<sup>10</sup> which were also characterized by different mutational and clinical features.

In Group 1, a large proportion (11/15, 73%) of tumors were metastatic. The defining molecular features of these tumors were recurrent chromosomal gains, CN-LOH, and a high rate of somatic mutations in the driver genes in our targeted panel. In addition, 14 (of 15) cases were also ALT-positive and all cases displayed loss of either DAXX or ATRX nuclear protein expression and inactivating mutations in either *DAXX* or *ATRX* were observed in 10 of 15 cases, indicating the presence of other possible mechanisms of loss, such as complex karyotype rearrangements in ALT-positive PanNETs.<sup>10</sup> We observed 3 ALT-positive cases, not included in the genetic analysis, which retained DAXX and ATRX nuclear protein expression. These observations may indicate potential additional biological mechanisms, independent of *DAXX* and *ATRX*, that promote ALT in a minority of cases, or by genetic alterations conferring a loss-of-function of either *DAXX* and *ATRX* (eg, missense mutations), but not loss of nuclear protein expression.<sup>13,15,16,22,31</sup> In this study, we observed an almost perfect correlation between recurrent patterns of chromosomal gains and ALT, suggesting that chromosomal gains may represent an early stage of genomic instability associated with ALT. Previous studies that investigated ALT prevalence in large cohorts of resected PanNETs described a direct correlation between ALT and larger size or higher grade.<sup>16,21,22</sup> In our series of small primary PanNETs that metastasized, most of the ALT-positive tumors were G2, suggesting that either ALT increases cell proliferation and metastatic potential or that a high proliferative index in PanNETs increases the probability of

acquiring the requisite mutations that promote ALT. Interestingly, *TSC2* was mutated in 4 cases (27%), however, all the remnant tumors in Group 1 with wild-type *TSC2* showed copy number gains of chromosome 19q (11/15, 73%), suggesting that potential proto-oncogenes functioning in the mTOR pathway may be located in this region. One possible candidate is *AKT2*, which may upregulate the mTORC1 complex when *TSC2* function is retained.<sup>32,33</sup>

In Group 2, a proportion (8/19, 42%) of tumors were metastatic, despite having a low proliferative index in the majority of cases. This group included the majority of G1 metastatic tumors (5 of 9 G1 metastatic PanNETs, 55%). Interestingly, these tumors were characterized by the presence of few mutations and limited chromosomal alterations.

In Group 3, tumors were characterized by frequent biallelic inactivation of *MEN1* and the lowest metastatic potential. In total, PanNETs in our series harbored *MEN1* locus alterations in 69% (33/ 48) of cases, including chromosomal loss, CN-LOH or chromotripsis, and *MEN1* somatic mutations in 33% (16/48) of cases, which were distributed mainly between Groups 1 and 3. Our data do not provide strong evidence supporting an interaction between *MEN1* and the ALT pathway. However, since menin has a critical role in the biology of telomeres by negatively regulating telomerase,<sup>34,35</sup> it is tempting to speculate that tumors at an early disease stage with partial or complete loss of menin may use upregulated telomerase for telomere maintenance, and thus do not require the ALT pathway.

While ALT and ATRX/DAXX alterations play pivotal roles in the progression of a subset of PanNETs, metastatic tumors are present also in the other subtypes and the absence of recurrent genetic changes in Groups 2 and 3 suggests that additional biological mechanisms, or genetic alterations not included in our analysis, may contribute to neoplastic progression in these groups. Limitations of our approach were represented by the relatively limited size of the study cohort and by the absence of confirmation of our results in an independent dataset, which are direct consequences of designing our study around rigorous clinical case selection. Our cohort included patients with either synchronous or metachronous liver metastases; we therefore cannot exclude the possibility that additional genetic alterations occurred after the development of metastatic disease in PanNETs with synchronous metastases. However, in our cohort, no substantial genetic differences were observed between PanNETs with metachronous or synchronous metastases (see Supplementary material, <http://links.lww.com/SLA/B503>), while we observed a high allele frequency for each mutation indicating that these alterations are present in the majority of neoplastic clones of the primary tumor, regardless of the timing of metastasis. This is also consistent with previous reports evaluating ALT in the primary and metastatic lesions from the same patient indicating that molecular alterations that promote ALT occur prior to the development of metastases.<sup>16</sup>

Recent ENETS consensus guidelines for the management of PanNETs suggest that only asymptomatic G1 tumors < 2cm size may be suitable for close observation, due to their very low risk of neoplastic progression.<sup>8,36</sup> In our cohort, biased for the development of liver metastases, 9 metastatic cases were < 2cm. While no specific genetic alterations were observed in these tumors (see Supplementary material, <http://links.lww.com/SLA/B503>), DAXX was mutated in 4 cases (3 with Ki67 < 5%) highlighting its importance as potential



risk-stratification biomarker. We limited our genetic analysis to copy number changes and to targeting driver genes frequently mutated in PanNETs. We did not include analysis of other genes, such as those involved in chromatin remodeling (mutated in approximately 5% of PanNETs), or those containing pathogenic germline variants thought to have a role in the development of up to 17% of sporadic PanNETs.<sup>10</sup> Thus, we cannot exclude that such infrequently altered genes also play some role in metastasis of small PanNETs.

In conclusion, we characterize the biological variability among morphologically similar well-differentiated PanNETs. We identified subtypes of small PanNETs characterized by distinct metastatic potential on the basis of integrated genetic and immuno-staining expression data. ALT-positive tumors have the highest prevalence of metastases suggesting that ALT should be considered a potential biomarker of an aggressive phenotype among small tumors and employed for risk stratification. Importantly, NGS with targeted panels can be performed on small samples<sup>37</sup> and ALT can be accurately be detected on endoscopic ultrasound-guided fine needle aspiration.<sup>31</sup> Thus, we propose that, the concomitant integrated evaluation of targeted sequencing, Ki-67, and ALT on endoscopic ultrasound-guided fine needle aspiration specimens could guide the choice of the surgical treatment, as well as the enrollment into surveillance programs for small and asymptomatic tumors.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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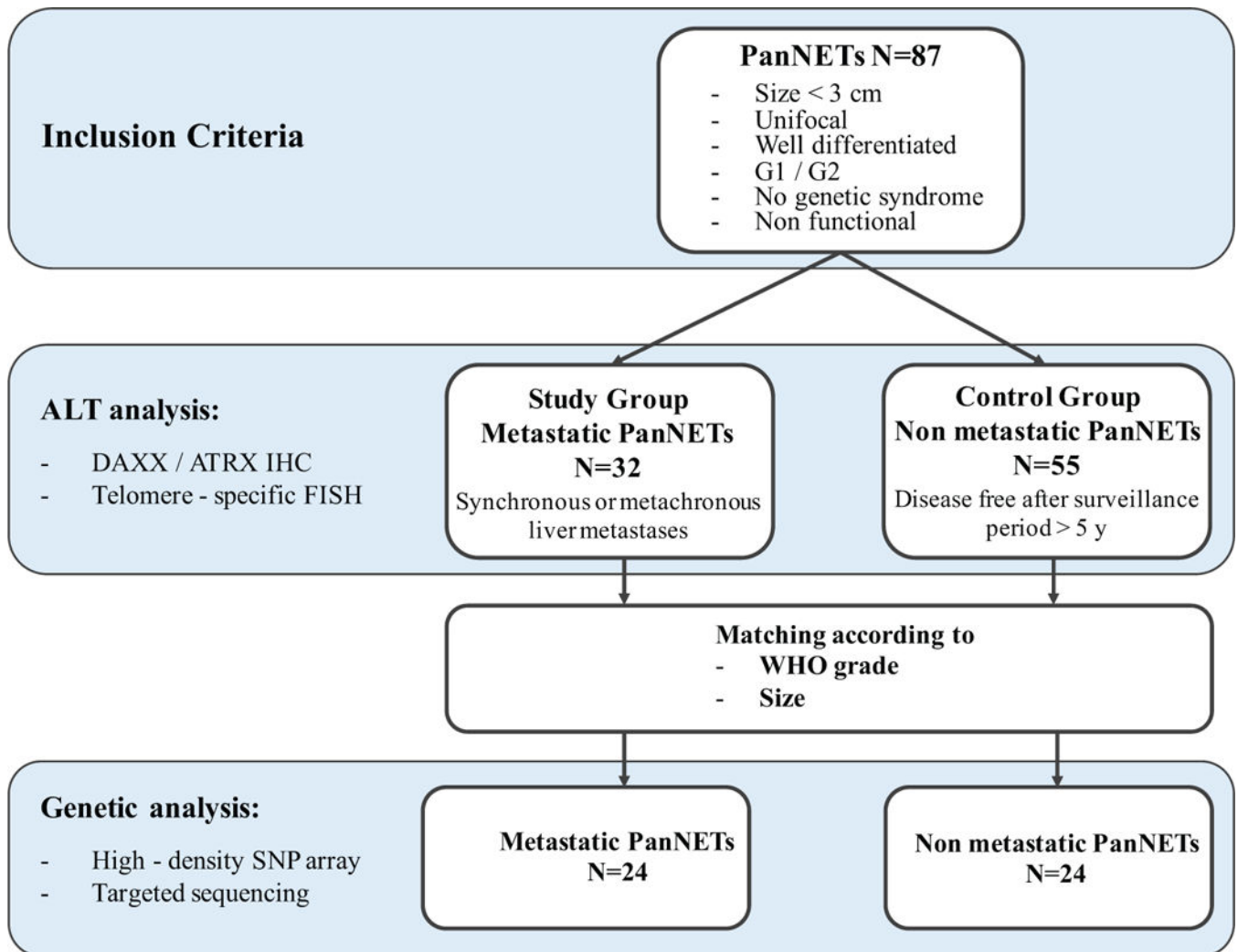
Conceived and designed the study: A.P., J.Y., L.D.W., and C.L.W.; performed the experiments: J.Y., A.P., C.M.H.; acquisition of data: A.P., J.Y., L.M., M.N., C.L., A.P., R.F.d.W., L.A.B., N.R., A.J., S.G., R.G., R.S., C.B., J.H., M.J.W., J.L.C., J.A.O., R.H.H., R.T.L., A.S., C.M.H., L.D.W., and C.L.W.; analysis and interpretation of data: A.P., J.Y., L.M., C.L., A.S., C.M.H., L.D.W., and C.L.W.; statistical analysis: A.P., J.Y., L.M., A.P., and C.M.H.; drafted the manuscript: A.P., J.Y., L.M., A.S., C.M.H., L.D.W., and C.L.W.; revised the manuscript and agreed with the manuscript’s results and conclusions: all the authors; obtained funding: C.L.W., A.P., L.M., A.S., C.M.H., M.N., and L.A.B.; study supervision: C.L.W.

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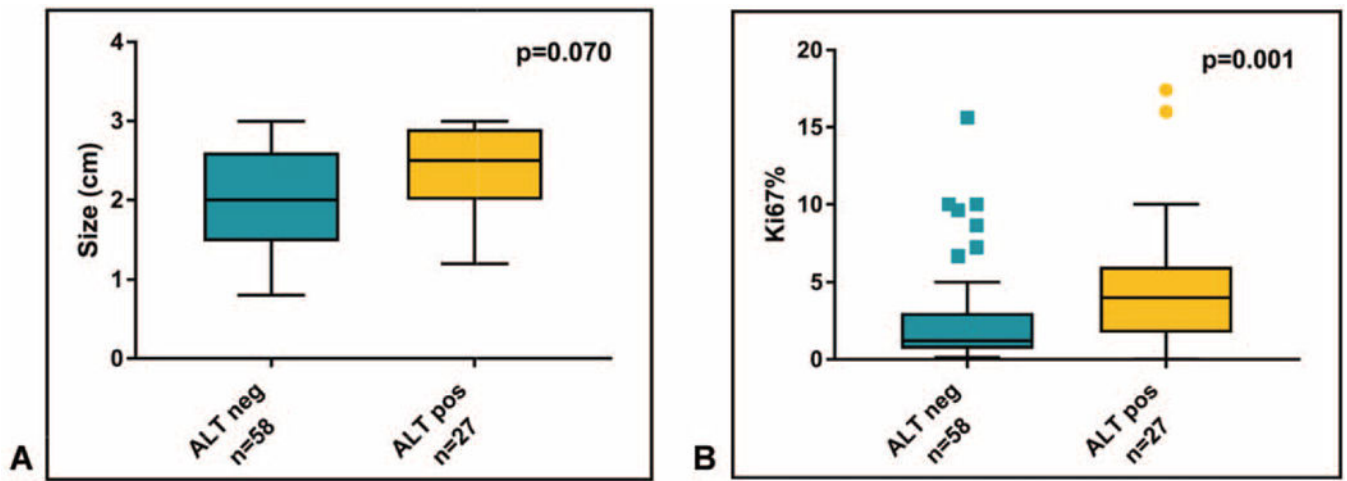
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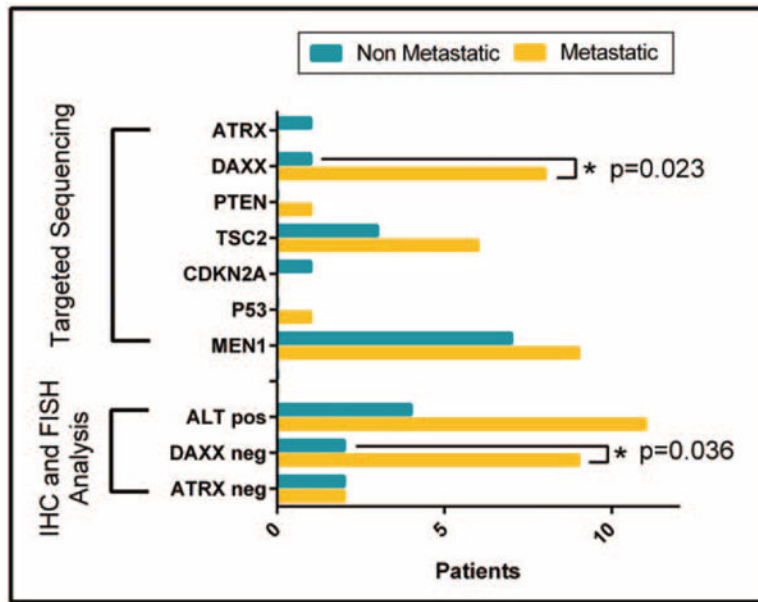


**FIGURE 1.**  
Study workflow.

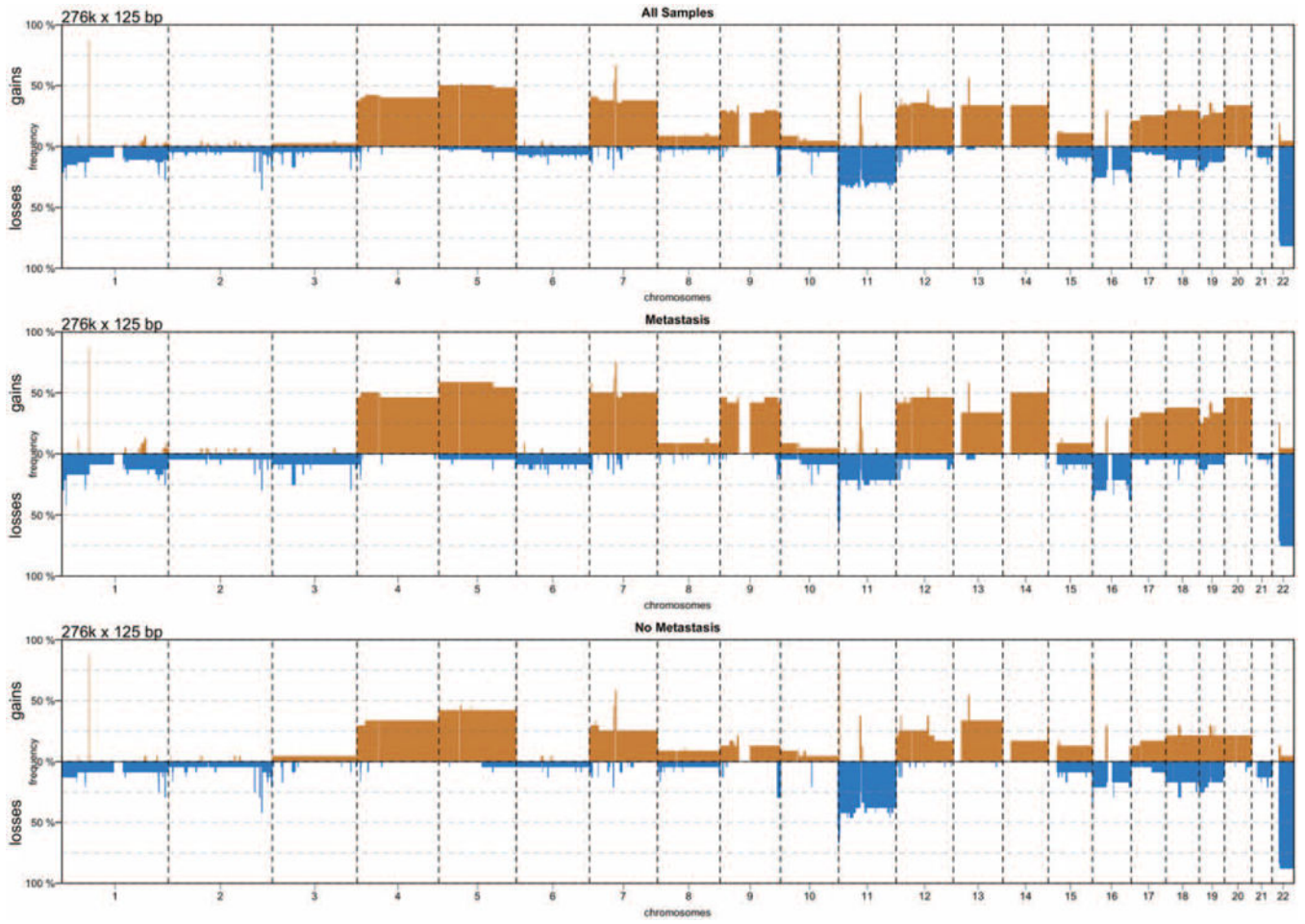


**FIGURE 2.**

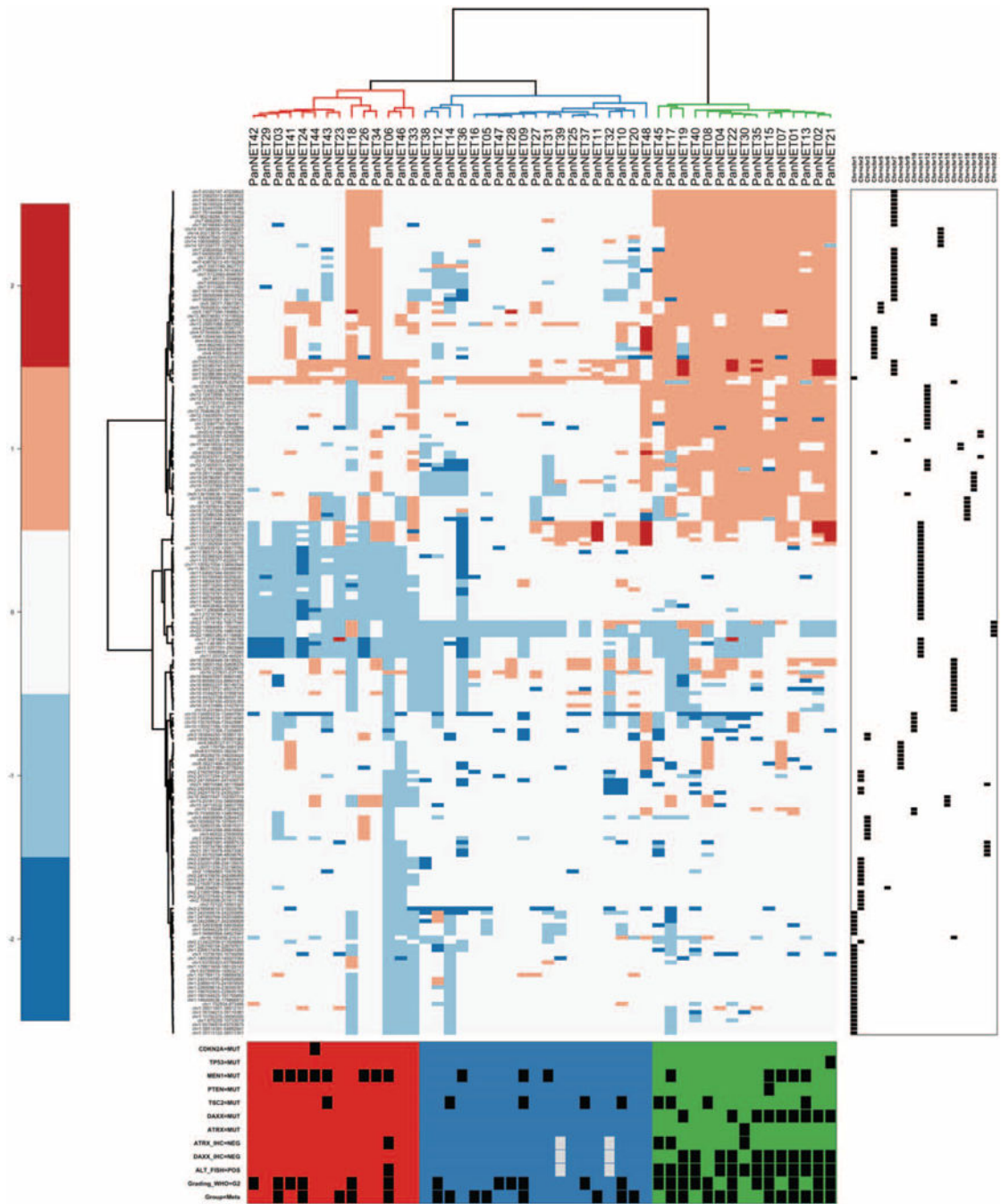
Tumor size (A) and Ki67% (B) stratified by ALT status. Box and whisker plots depict median and 10 to 90 percentile ranges.



**FIGURE 3.** Somatic mutations, ATRX/DAXX protein expression, and ALT status in metastatic and nonmetastatic PanNETs.



**FIGURE 4.** Rate of chromosomal gains (red) and losses (blue) per chromosome in metastatic and nonmetastatic PanNETs.



**FIGURE 5.** Cluster analysis of the copy number state stratified the tumors into 3 different subtypes characterized by different mutational and clinical patterns (as shown at the bottom). Hierarchical clustering was based on copy number alterations, using the Manhattan distance and the Ward clustering method.



**TABLE 1.**

Univariate Analysis of Clinical, Pathological, and Biomarker Data Associated With Liver Metastases

Variables	All Patients n = 87	ALT n = 85		P	Liver Metastases n = 87		P
		Negative n = 58	Positive n = 27		No n = 55	Yes n = 32	
Male, no. (%)	44 (50.6)	32 (55.2)	12 (44.4)	0.485	27 (49.1)	17 (53.1)	0.825
Age, mean (SD), yr	57 (12)	57 (17)	57 (19)	0.884	58 (12)	56 (13)	0.483
Location of tumor				0.643			0.115
Head and uncinate, no. (%)	36 (41.4)	25 (43.1)	10 (37.0)		19 (34.5)	17 (53.1)	
Body and tail, no. (%)	51 (58.6)	33 (56.9)	17 (63)		15 (46.9)	36 (65.5)	
Tumor size, median (IQR), cm	2.2 (1.1)	2.0 (1.1)	2.5 (0.9)	0.070	2.0 (1.1)	2.5 (0.8)	<b>0.010</b>
Size				0.098			<b>0.023</b>
cm	6 (6.9)	6 (10.3)	0 (0.0)		6 (10.9)	0 (0)	
1–2 cm	36 (41.4)	25 (43.1)	9 (33.3)		26 (47.3)	10 (31.3)	
>2 cm	45 (51.7)	27 (46.6)	18 (66.7)		23 (41.8)	22 (68.8)	
Ki67 index %, median (IQR)	2.0 (3.3)	1.2 (2.3)	4.0 (4.3)	<b>0.001</b>	1.0 (2.1)	4.2 (6.7)	<b>0.001</b>
WHO grade				<b>0.004</b>			<b>0.001</b>
G1, no. (%)	54 (62.1)	42 (72.4)	10 (37.0)		42 (76.4)	12(37.5)	
G2, no. (%)	33 (37.9)	16 (27.6)	17 (63.0)		13 (23.6)	20 (62.5)	
Lymphovascular invasion, no. (%)	19 (21.8)	8 (13.8)	11 (40.7)	<b>0.010</b>	8 (14.5)	11 (34.4)	0.057
Perineural invasion, no. (%)	26 (29.9)	10 (17.2)	15 (55.6)	<b>0.001</b>	10 (18.2)	16 (50)	<b>0.003</b>
T stage, no. (%)				0.210			0.215
T1 stage, no. (%)	36 (41.4)	26 (44.8)	10 (37.0)		26 (47.3)	10 (31.3)	
T2 stage, no. (%)	31 (35.6)	17 (29.3)	13 (48.1)		16 (29.1)	15 (46.9)	
T3 stage, no. (%)	20 (23.0)	15 (25.9)	4 (14.8)		13 (23.6)	7 (21.9)	
N stage, no. (%)				<b>0.007</b>			<b>0.001</b>
Nx-0 stage, no. (%)	58 (66.7)	44 (75.9)	12 (44.4)		45 (81.8)	13 (40.6)	
N1 stage, no. (%)	29 (33.3)	14 (24.1)	15 (55.6)		10 (18.2)	19 (59.4)	
Liver metastases, no. (%)	32 (36.8)	14 (24.1)	18 (66.7)	<b>0.001</b>			
ALT pos, no. (%)	27/85 (31.8)				9/53 (17)	18/32 (56.3)	<b>0.001</b>
DAXX neg, no. (%)	20/84 (23.8)	1 (1.8)	19 (70.4)	<b>0.001</b>	7/52 (13.5)	13/32 (40.6)	<b>0.008</b>
ATRX neg, no. (%)	6/85 (7.1)	0 (0)	6 (22.2)	<b>0.001</b>	3/53 (5.7)	3/32 (9.4)	0.668

*P* values in bold represent significant statistical differences.

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**TABLE 2.**

Multivariate Analysis of Clinical, Pathological, and Biomarker Data Associated With Liver Metastases

<b>Variables</b>	<b>OR</b>	<b>IC 95%</b>	<b>P</b>
Ki-67	1.369	1.121 to 1.673	0.002
N stage (N1)	4.568	1.458 to 14.312	0.009
ALT positivity	3.486	1.093 to 11.115	0.035

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