**Targeted Next Generation Sequencing of Pancreatic Solid Pseudopapillary Neoplasms Show Mutations in Wnt Signaling Pathway Genes.**

Jayson Wang FRCPath PhD1\*, Gareth Gerrard PhD2,3\*, Ben Poskitt BSc 3, Kay Dawson BSc, Pritesh Trivedi BSc, Letizia Foroni FRCPath PhD2, Mona El-Bahrawy FRCPath PhD4

Department of Histopathology, Imperial College London, Hammersmith Hospital, DuCane Road, London W12 0NN.

\*Equal contribution

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**Corresponding author:**

Dr. Mona A. El-Bahrawy

Department of Histopathology

Imperial College London

Hammersmith Hospital

DuCane Road

London W12 0NN

e-mail: [m.elbahrawy@imperial.ac.uk](mailto:m.elbahrawy@imperial.ac.uk)

Tel. +44 20 8383 3442, FAX: +44 20 8383 8141

**Additional affiliations:**

1 Department of Cellular Pathology, St George’s Hospital, London, UK.

2 Centre for Haematology, Department of Medicine, Imperial College London, UK

3 Sarah Cannon Molecular Diagnostics, HCA Healthcare UK, London, UK

4 Department of Pathology, Faculty of Medicine, University of Alexandria, Egypt

**ABSTRACT**

Solid pseudopapillary neoplasms of the pancreas are rare neoplasms that have been shown to harbor recurrent somatic pathogenic variants in the beta-catenin gene, *CTNNB1*. Here, we used targeted next generation sequencing to analyze these tumors for other associated mutations. Six cases of solid pseudopapillary neoplasms were studied. DNA extracted from formalin fixed paraffin embedded tissue blocks was analyzed using the Ion Torrent platform, with the 50-gene Ampliseq Cancer Hotspot Panel v2 (CHPv2), with further variant validation performed by Sanger sequencing. Four tumors (67%) were confirmed to harbor mutations within *CTNNB1*, two with c.109T>G p.(Ser37Ala) and two with c.94G>A p.(Asp32Asn). One case showed a frameshift deletion in the Adenomatous Polyposis Coli gene, *APC* c.3964delG p.(Glu1322Lysfs\*93) with a variant allele frequency of 42.6%. Sanger sequencing on non-tumoral tissue confirmed the variant was somatic. The patient with the *APC* mutation developed metastasis and died. In addition to the four cases harboring *CTNNB1* variants, we found a case characterized by poor outcome, showing a rare frameshift deletion in the *APC* gene. Since the *APC* product interacts with beta-catenin, *APC* variants may, in addition to *CTNNB1*, contribute to the pathogenesis of solid pseudopapillary neoplasms via the Wnt signaling pathway.

**KEYWORDS**

Pancreas, Neoplasm, Mutation**INTRODUCTION**

Solid pseudopapillary neoplasms (SPNs) of the pancreas are rare neoplasms, accounting for only approximately 1-2% of pancreatic neoplasms[[1](#_ENREF_1)]. SPN overwhelmingly affects women and usually has an indolent behavior with generally good prognosis [[2](#_ENREF_2)].

SPNs are usually well-circumscribed lesions, which may occur anywhere in the pancreas [[3](#_ENREF_3)]. The cells are uniform and polygonal or round, with eosinophilic or clear cytoplasm. Small tumors may be primarily arranged in solid sheets, cords or trabeculae. As they grow, degenerative changes lead to the formation of pseudopapillary patterns and cystic zones.

Many investigators support the theory that SPN originates from a multipotential primordial cell, resulting in a lack of a predominant line of differentiation in the tumor or the multidirectional differentiation encountered [[4-6](#_ENREF_4)]. This may explain why some SPNs express neuron-specific enolase, CD56 and occasionally synaptophysin, as well as vimentin, α-1-antitrypsin, progesterone receptors, and CD10 [[7](#_ENREF_7), [8](#_ENREF_8)].

SPNs have previously been shown to have somatic activating mutations in the *CTNNB1* gene involving 83-100% of SPNs [[9-11](#_ENREF_9)]. All are missense mutations leading to loss of binding sites for glycogen synthase kinase-3beta (GSK-3β) phosphorylation, thereby interfering with degradation of the beta-catenin protein. These mutations lead to cytoplasmic and nuclear accumulation of beta-catenin in most SPNs.

Beta-catenin has a dual function (Figure 1). Firstly, it is a major component of the E-cadherin/catenin complex at the adherens junction, which is involved in adhesion between epithelial cells [[12](#_ENREF_12), [13](#_ENREF_13)]. The cytoplasmic domain of E-cadherin is associated with a group of closely related proteins, termed catenins, which mediates the interaction between the E-cadherin/catenin complex and the actin cytoskeleton. We have previously shown that in SPNs, there is abnormal nuclear E-cadherin expression immunohistochemically, in addition to nuclear expression of beta-catenin [[14](#_ENREF_14)].

In addition, beta-catenin acts as a downstream transcription activator of the Wnt signaling pathway [[15](#_ENREF_15)]. In the absence of Wnt ligand, free cytoplasmic beta-catenin accumulates. Phosphorylation of both Adenomatous Polyposis Coli (APC) and Axin by GSK-3β then results in beta-catenin binding to the APC-Axin complex [[16](#_ENREF_16)]. This targets the protein for ubiquitination and proteasomal degradation. When the Wnt ligand binds to the Frizzled receptor on the cell surface, the associated Dishevelled protein is phosphorylated, Axin, APC and GSK-3β are recruited to the receptor complex. Beta-catenin accumulates in the nucleus and forms complexes with the T-cell factor/lymphoid-enhancer factor (Tcf/Lef) family of DNA-binding proteins, resulting in the transcription of target genes. It has been proposed that Wnt signaling activated by *CTNNB1* mutations has an important role for the development of various diseases [[10](#_ENREF_10), [17](#_ENREF_17)].

In this study, we analyzed DNA from six SPN patients using a targeted next generation sequencing platform to determine if other mutations apart from beta-catenin may be found in these tumors.

**MATERIALS AND METHODS**

Patient and sample selection:

The study included resections of six cases of pancreatic SPNs performed between 1993 and 2005, retrieved from the archives of the Hammersmith Hospital. All tumors studied were primary tumors, none of which had metastasis at the time of resection. Ethical approval for use of human tissue has been obtained from the Hammersmith, Queen Charlotte & Chelsea and Acton Hospitals Ethics Committee (Project reference no. 04/Q406/40).

The cases were reviewed by examining the hematoxylin and eosin (H&E) stained sections of each tumor sample and the immunostained slides using Leica Bond III (Nussloch, Germany) staining system, including: CD56 (RTU Leica catalog number PA0191 (Program H1 20) , CD10 (RTU Leica Catalog number PA0270 (H2 20)), alpha-1-antitrypsin (Dako catalog number A0012 (Enzyme 10) dilution 1:3000 (Agilent, Santa Clara, USA)), carcinoembryonic antigen (CEA) (Dako catalog number A0115 (H1 30) dilution 1:1000), beta-catenin (RTU Leica catalog number PA0083 (Program H1 20)), E-cadherin (RTU Leica catalog number PA0387 (Program H2 20)), chromograninA (RTU Leica catalog number PA0430 (Program H1 20)) and synaptophysin (RTU Leica catalog number PA0299 (Program H2 20)) to confirm the diagnosis and select blocks for analysis.

Library Preparation

Genomic DNA was extracted from six SPN samples from formalin-fixed paraffin embedded (FFPE) archive (minimum 10µm sections) using QIAmp FFPE kits and semi-automated using the QIAcube robotic platform using the manufacturer recommended protocol (both Qiagen, Venlo, Netherlands). If the sections showed tumor cellularity of >50% on the hematoxylin and eosin-stained slides, macrodissection was not necessary (published studies have recommended >20% tumor cellularity for this platform) [[18](#_ENREF_18)]. If required, macrodissection would be performed by a qualified pathologist.

An alternative DNA extraction kit was also used for repeat runs of samples 3-6; the Qiagen GeneRead kit was employed as a manual protocol, according to the manufacturer’s recommendations. This included a uracil DNA n-glycosylase (UNG) enzyme incubation step to ameliorate C>T / G>A deamination artefacts associated with formalin cross-linking.

The DNA was quantitated using a Qubit2 fluorometer (Life Technologies, Carlsbad, US) and 10ng of each was used to prepare barcoded Ion Torrent libraries using the 50-gene Ampliseq Cancer Hotspot Panel v2 (CHPv2) primers and Ampliseq Library Kits 2.0-96LV (Life Technologies). The 50 genes and exon regions targeted are listed in Table 1. The libraries were quantitated using the KAPA Library Quant Kit (KK4838; KAPA Biosystems, Wilmington, US), pooled in equimolar quantities using the specified dilution factors with a final volume of 25µl.

Templating and Ion Torrent Sequencing

The library pool was then templated onto Ion Sphere Particles (ISP) using the Ion Torrent One Touch 2 emulsion polymerase chain reaction (PCR) platform (OT2 200 chemistry), quality controlled using the ISP QC kit (Life Technologies) to ensure 10 – 30% templating efficiency and then enriched using the Ion One Touch ES system. The final template library pool was then prepared for sequencing and the Ion Personal Genome Machine (PGM) initialized using the Ion Torrent 200v2 kit. The final library pool was then loaded onto a 316v2 sequencing chip and sequenced on an Ion Torrent PGM using a default 500 flow template with manufacturer supplied CHPv2 region and hotspot BED files.

Informatics

The base calling, alignment (to human genome build hg19/GRCh37) and assembly was by the on-board Torrent Suite v4.2 software and variant calling was by the associated Variant Caller plug-in, using the manufacturer supplied JSON parameter file. Variant and read depth data were visualized with IGV (http://www.broadinstitute.org/software/igv) and the UCSC Genome Browser (University of California, Santa Cruz; <http://www.http://genome.ucsc.edu>); a minimum of 200 read depth was required for wild-type calls (to give limit of detection (LOD) of 5%); key regions were visualized in IGV in the absence of variant calls to check quality. Variant annotation was performed on the cloud-based Ion Reporter v4.2 platform with custom filters and further functional characterization was via MutationTaster (http://www.mutationtaster.org) and Varsome (https://varsome.com). Other variant information and somatic status was from the COSMIC (catalogue of somatic mutations in cancer) database (http://www.cancer.sanger.ac.uk).

Sanger Sequencing Validation of APC Variant

Four 10μm sections were cut from the same FFPE block onto uncoated glass slides; one of which was then stained with H&E stain. This slide was then reviewed microscopically and tumor and normal (non-tumor) tissues marked onto the slide. Using these marked areas as a guide, tissue from the remaining 3 slides were then macrodissected by scraping with a scalpel into two separate 1.5ml tubes. DNA was then extracted from the normal and the tumor tissue using the QIAamp DNA FFPE Tissue kit (Qiagen, Venlo, Netherlands) with a 30μl elution volume. The DNA was then quantitated and quality assessed using a TapeStation 4200 and Genomic DNA ScreenTape (Agilent, CA, USA). Further clean-up and concentrating of the Normal DNA was carried out using a Genomic DNA Clean & Concentrator kit (Zymo Research, CA, USA). For the sequencing PCR and cycle sequencing reactions, 4ng of DNA (Tissue, Normal & kit-provided control) was used in separate reactions using the BigDye Direct Cycle Sequencing Kit and M13-tailed Sanger Sequencing Primers (Hs00532820\_CE) and run on an ABI SimpliAmp thermal cycler (all ThermoFisher, MA, USA) using manufacturer recommended protocol. The post-cycling clean-up was performed using the BigDye XTerminator Purification kit (ThermoFisher) and the capillary sequencing was run on an ABI 3500 Genetic Analyser (ThermoFisher) using POP-7 polymer and the BDX\_ShortReadSeq sequencing profile. The data was exported as AB1 files and visualized in Chromas 2.6.2 (Technelysium, Brisbane, Australia).

**RESULTS**

Specimens

The tumors were from five females and one male. Age ranged between 26 and 64 years of age. The tumor sizes ranged from 6 to 14 cm and all were confined to the pancreas (stage pT2). None of the tumors had lymph node or distant metastasis at time of surgery. Histologically, the tumors showed the typical features of SPN, with solid sheets of polygonal and round cells forming pseudo-papillary structures. The tumor cells strongly expressed CD10, CD56 and alpha-1-antitrypsin and were negative or weak for cytokeratins, CEA and neuroendocrine markers, including chromogranin A and synaptophysin, excluding other diagnoses such as pancreaticoblastoma, acinar cell carcinoma and neuroendocrine neoplasm. All tumors also showed strong nuclear and cytoplasmic staining for beta-catenin, as well nuclear staining for E-cadherin. In 5 cases from female patients, the tumors had well-circumscribed borders, and showed uniform polygonal cells with low mitotic activity.

One case was from a 64 year old male patient. Macroscopically, the tumor was well circumscribed mass with central hemorrhagic cavitation and fragmentation. Microscopically, it was mostly well circumscribed but with focal infiltrative margins (Figure 2). Areas of infarction and necrosis were identified. There was focal nuclear pleomorphism and occasional mitotic activity (1-3 per high power fields), but in other areas, the tumor shows usual morphological features of SPN, with no other histological features to indicate aggressive behavior.

Ion Torrent CHPv2

Cellularity scoring showed all the tissue sections to contain at least 50 to 70% tumor cells. No macrodissection was therefore required for any of the samples. Two samples, (SP1 & SP2), were extracted in one batch by standard Qiagen FFPE protocol and exhibited good sequencing metrics (insert size, read depth, total number of variants and run quality indicators were all within acceptable parameters). Samples SP3 – SP6 were extracted in a second batch using the same protocol, but only SP5 & SP6 showed acceptable sequencing metrics. SP3 & SP4 exhibited many deamination artefacts (C>T and G>A non-hotspot apparent variants, called at <6%). Re-extraction with the GeneRead kit, which included an UNG enzyme step, ameliorated these artefacts, allowing high-confidence variant calling.

Of the six samples analyzed, one showed no potentially pathogenic variants (SP6, only single nucleotide variants flagged as common by UCSC) and five exhibited potentially pathogenic variants. Four samples had *CTNNB1* (NM\_001904) missense variants (two carried the c.109T>G p.(Ser37Ala) and two carried the c.94G>A p.(Asp32Asn)). One had a frameshift deletion variant in exon 16 of the *APC* gene (NM\_000038.5), c.3964delG p.(Glu1322Lysfs\*93). All these variants were categorized as pathogenic, with probabilities >0.99 by the *in silico* variant analysis platform, Mutation Taster. The *APC* deletion variant has been previously reported once in a colorectal cancer COSMIC ID (COSM4169380). Mutation Taster labels this disease-related (*P*=1.00) and the deleted base is categorized as evolutionarily conserved, with a PhyloP score of 3.017 and a PhastCons of 1.00; it is also classed as ‘Pathogenic’ by Varsome according to application of the ACMG classification [[19](#_ENREF_19)]. The lack of pathogenic variants in SP6 was unlikely to be due to insufficient coverage as 211 reads over *CTNNB1* exon 3 (LOD = 5%) and 636 reads over *APC* were achieved. Likewise, for SP3, where there were 277 reads covering *CTNNB1* exon 3; the minimum read-depth for automatic wild-type calling in our pipeline (for a LOD of 5%) was 200 reads. The results of the analysis are summarized in Table 2.

Sanger Sequencing Validation

The results of the Sanger sequencing of DNA extracted from macrodissected tumor and normal tissue from the *APC* variant case (SP3) show very clearly that the variant was somatic (present in DNA from the tumor and not the normal tissue) and not germline (which would require the variant to be present in DNA from both tissue sites). Figure 3 shows the Sanger sequencing electropherogram for the normal DNA (A) and tumor DNA (B) and the site of the somatic deletion (NM\_000038.5:c.3964delG; indicated by arrows) and resultant 3’ frameshift that is evident in the tumor and not the normal traces. The equal electropherogram heights of both the variant and reference allele traces is suggestive that loss of heterozygosity was not a factor at this locus.

Patient follow-up

The case with the *APC* frameshift variant was the male patient with SPN. A year later, the patient developed liver metastasis and died. Follow-up data were available for three other patients, who are all alive and well, now more than 7 years after the tumors were removed. No follow up data is available for the remaining two patients.

**DISCUSSION**

SPNs are well recognized to be associated with beta-catenin variants in the majority of cases [[10](#_ENREF_10)]. This is in contrast to other pancreatic neoplasms, which have different genetic changes [[20-22](#_ENREF_20)]. In this study, we used next generation sequencing with the Ion Torrent platform to perform mutational analysis of 50 commonly mutated cancer-associated genes via the AmpliSeq CHPv2 panel. There have been previous studies using next generation sequencing to analyze SPNs of the pancreas. However, in all these reports, the SPNs showed recurrent pathogenic variants only in the *CTNNB1* gene [[22-24](#_ENREF_22)].

Here, we found variants in *CTNNB1* in four (67%) of six cases, confirming that *CTNNB1* variants occur in the majority of SPN [[9-11](#_ENREF_9)] cases. We also detected a separate case of SPN with a frameshift deletion affecting the *APC* gene. This particular variant c.3964delG p.(Glu1322Lysfs\*93) is extremely rare, having only been reported once in a colorectal cancer with COSMIC ID COSM4169380 [[25](#_ENREF_25)]. Our data from the non-tumoral tissue using Sanger sequencing indicate that the variant is somatic, rather than germline. To the best of our knowledge this is the first repo**r**t of a potentially pathogenic APC variant found in SPN. The mutation was a single nucleotide deletion (affecting codon 1322), resulting in frameshift and downstream premature stop codon. Functional impact modeling by Mutation Taster, labeled this event as being disease causing with a probability of *P*=1.00, most probably leading to a strongly truncated protein or even loss of allele function due to nonsense mediated decay, since the loss of 1430 amino acids represents a greater than 10% loss. The deletion also falls within the so-called mutation cluster region (MCR) which is associated with truncating mutations in colorectal cancer. A truncated protein would be consistent with *APC* variants implicated in colorectal cancer, which exert an influence though the loss of the ability of the truncated form of APC to regulate beta-catenin and downstream signaling. Furthermore, our case with the APC variant also showed nuclear accumulation of beta-catenin on immunohistochemistry, which would suggest loss of APC function from both alleles of the gene. There was no evidence of a ‘second-hit’ event in our data: the NGS variant allele frequency and the Sanger data were not suggestive of loss-of-heterozygosity at this locus, nor was any other variant detected that could account for the silencing of the second allele. However, because of the limited size of the NGS panel used (with only 7 amplicons covering exon 16 of *APC*), it is not unreasonable to conclude that the second hit could have been any number of known mutations at a locus not covered by the panel.

No concomitant *CTNNB1* mutation was identified in this tumor and this was verified by ruling out non-calls and insufficient coverage in the sequencing data. Other mutations involving *APC* codon 1322 have been previously reported in colorectal cancers, but also as rare events [[26](#_ENREF_26), [27](#_ENREF_27)]. Our findings of the case with a frameshift deletion in the *APC* gene, in addition to the others with *CTNNB1* missense variants, gives further evidence that the Wnt signaling pathway plays a role in the pathogenesis of SPN, as previously proposed [[11](#_ENREF_11), [28](#_ENREF_28)].

Until now, the role of *APC* variants in the pathogenesis of SPN has only been implicated but no direct link demonstrated. Previously, sequencing of the *APC* gene was attempted by Abraham *et al* in two cases of SPN without *CTNNB1* variants, however, the PCR amplification had failed while the reason for the failure was not specified [[9](#_ENREF_9)]. There have been two case reports of FAP patients, who developed SPN [[29](#_ENREF_29), [30](#_ENREF_30)]. In the one study [[30](#_ENREF_30)], the patient was a male and had presumed germline *APC* gene variant on genetic testing. The SPN was detected incidentally on screening as a FAP patient. However, as this was a germline variant and the beta-catenin positivity was cytoplasmic rather than nuclear, the role of loss-of-function of *APC* in the pathogenesis of SPN was only speculated.

In addition, the tumor from the patient with the *APC* frameshift showed aggressive clinical behavior as the patient developed metastases and died shortly after diagnosis. Metastasis may occur in SPN, but only in 10-15% of cases. Even in metastatic disease, many patients survive for years. However, there have been reports for SPN which were histologically high grade, showing features of undifferentiated or sarcomatoid carcinoma, with nuclear atypia and increased mitotic activity [[31](#_ENREF_31)]. These patients had rapidly progressive disease. We therefore speculate that *APC* variants confer worse tumor behavior than *CTNNB1* in SPN, as has been shown in the colorectal cancer model [[32](#_ENREF_32)], although in fibromatosis, tumors with *APC* variants do not have worse prognosis than those with *CTNNB1* variants [[33](#_ENREF_33)]. It is possible that the severity and prognosis of disease are related to the site of the *APC* variant [[34](#_ENREF_34), [35](#_ENREF_35)].

Secondly, the patient was male. Although less common than in females, male patients with SPN are recognized. Aggressive behavior of SPNs, both histologically and clinically, in male patients has been reported previously in some studies and reviews [[36](#_ENREF_36), [37](#_ENREF_37)], although another study did not arrive at the same conclusion [[38](#_ENREF_38)]. None of these studies comparing male with female patients analyzed either *CTNNB1* or *APC* genes for variants, although Machado *et al* found somatic *KRAS* variants in a male patient [[36](#_ENREF_36)].

One of the six tumors in our study does not harbor detectable variants in *CTNNB1*, *APC* or any of the 50 genes tested, despite having nuclear beta-catenin expression. It may be that other as yet undetected mutations or abnormalities in the other members of Wnt pathway may be involved. Huang *et al* [[11](#_ENREF_11)] examined 27 cases for Axin-1 gene mutations but none were found. Cavard *et al* [[28](#_ENREF_28)] studied gene expression using RT-PCR and ISH and found increased expression of 14 members of the Wnt signaling pathway compared with normal pancreatic tissue (including *FZD7*, *FZD10*, *AXIN2*, *CTNNB1* and *TCF7*). More recently, Park *et al* [[39](#_ENREF_39)] used cDNA microarray and confirmed upregulation of Wnt signaling genes, as well as genes related to the Hedgehog and androgen receptor signaling pathways.

One limitation of the targeted NGS panel used in this study is that it does not have the same breadth of coverage as whole exome sequencing, meaning rare driver events can be missed in some samples; but it does have the advantage of greater sequencing depth, meaning that variants can be detected in low cellularity samples, including potentially sub-clonal variants associated with progression. A recent whole exome sequencing study of nine SPNs showed all had *CTNNB1* driver variants; however, another case-report study of a locally-invasive SPN revealed an *EGFR* exon 21 p.L861Q driver variant, indicating that there may be other pathways associated with SPNs [[40](#_ENREF_40), [41](#_ENREF_41)].

In summary, we demonstrated for the first time a potentially pathogenic variant in the *APC* gene in SPN. We suggest that *APC* variants may be a rarer genetic basis of activation of the Wnt signaling pathway in the development of SPN, and may possibly even result in more aggressive variants. To the author’s knowledge, there are no currently available cell lines derived from SPN or animal models of SPN, which would be helpful in providing experimental evidence to validate this suggestion. We also propose that further studies in a larger cohort of cases should be performed to confirm the incidence of *APC* variant involvement in SPN.

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**DISCLOSURE STATEMENT**

The authors declare that there is no actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations regarding the material in the manuscript that could inappropriately influence (bias) our work. These include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations and grants and any other funding.

**AUTHOR CONTRIBUTIONS**

JW – acquisition and analysis of data; drafting the manuscript and figures.

GG – acquisition and analysis of data; drafting the manuscript and figures.

BP – acquisition and analysis of data.

KD – acquisition and analysis of data.

PT – acquisition and analysis of data.

LF – conception and design of the study.

MEB – conception and design of the study; drafting the manuscript and figures.

**REFERENCES**

1. Papavramidis, T. and S. Papavramidis, *Solid pseudopapillary tumors of the pancreas: review of 718 patients reported in English literature*. J Am Coll Surg, 2005. **200**(6): p. 965-72.

2. Kloppel, G., T. Morohoshi, H.D. John, et al., *Solid and cystic acinar cell tumour of the pancreas. A tumour in young women with favourable prognosis*. Virchows Arch A Pathol Anat Histol, 1981. **392**(2): p. 171-83.

3. Klimstra, D.S., B.M. Wenig, and C.S. Heffess, *Solid-pseudopapillary tumor of the pancreas: a typically cystic carcinoma of low malignant potential*. Semin Diagn Pathol, 2000. **17**(1): p. 66-80.

4. Balercia, G., G. Zamboni, G. Bogina, and G.M. Mariuzzi, *Solid-cystic tumor of the pancreas. An extensive ultrastructural study of fourteen cases*. J Submicrosc Cytol Pathol, 1995. **27**(3): p. 331-40.

5. Miettinen, M., S. Partanen, O. Fraki, and E. Kivilaakso, *Papillary cystic tumor of the pancreas. An analysis of cellular differentiation by electron microscopy and immunohistochemistry*. Am J Surg Pathol, 1987. **11**(11): p. 855-65.

6. Stommer, P., J. Kraus, M. Stolte, and J. Giedl, *Solid and cystic pancreatic tumors. Clinical, histochemical, and electron microscopic features in ten cases*. Cancer, 1991. **67**(6): p. 1635-41.

7. Kosmahl, M., L.S. Seada, U. Janig, D. Harms, and G. Kloppel, *Solid-pseudopapillary tumor of the pancreas: its origin revisited*. Virchows Arch, 2000. **436**(5): p. 473-80.

8. Notohara, K., S. Hamazaki, C. Tsukayama, et al., *Solid-pseudopapillary tumor of the pancreas: immunohistochemical localization of neuroendocrine markers and CD10*. Am J Surg Pathol, 2000. **24**(10): p. 1361-71.

9. Abraham, S.C., D.S. Klimstra, R.E. Wilentz, et al., *Solid-pseudopapillary tumors of the pancreas are genetically distinct from pancreatic ductal adenocarcinomas and almost always harbor beta-catenin mutations*. Am J Pathol, 2002. **160**(4): p. 1361-9.

10. Tanaka, Y., K. Kato, K. Notohara, et al., *Frequent beta-catenin mutation and cytoplasmic/nuclear accumulation in pancreatic solid-pseudopapillary neoplasm*. Cancer Res, 2001. **61**(23): p. 8401-4.

11. Huang, S.C., K.F. Ng, T.S. Yeh, H.C. Chang, C.Y. Su, and T.C. Chen, *Clinicopathological analysis of beta-catenin and Axin-1 in solid pseudopapillary neoplasms of the pancreas*. Ann Surg Oncol, 2012. **19 Suppl 3**: p. S438-46.

12. Takeichi, M., *Cadherin cell adhesion receptors as a morphogenetic regulator*. Science, 1991. **251**(5000): p. 1451-5.

13. Hinck, L., I.S. Nathke, J. Papkoff, and W.J. Nelson, *Dynamics of cadherin/catenin complex formation: novel protein interactions and pathways of complex assembly*. J Cell Biol, 1994. **125**(6): p. 1327-40.

14. El-Bahrawy, M.A., A. Rowan, D. Horncastle, et al., *E-cadherin/catenin complex status in solid pseudopapillary tumor of the pancreas*. Am J Surg Pathol, 2008. **32**(1): p. 1-7.

15. Nelson, W.J. and R. Nusse, *Convergence of Wnt, beta-catenin, and cadherin pathways*. Science, 2004. **303**(5663): p. 1483-7.

16. Behrens, J., B.A. Jerchow, M. Wurtele, et al., *Functional interaction of an axin homolog, conductin, with beta-catenin, APC, and GSK3beta*. Science, 1998. **280**(5363): p. 596-9.

17. Clevers, H., *Wnt/beta-catenin signaling in development and disease*. Cell, 2006. **127**(3): p. 469-80.

18. Goswami, R.S., R. Luthra, R.R. Singh, et al., *Identification of Factors Affecting the Success of Next-Generation Sequencing Testing in Solid Tumors*. Am J Clin Pathol, 2016. **145**(2): p. 222-37.

19. Richards, S., N. Aziz, S. Bale, et al., *Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology*. Genet Med, 2015. **17**(5): p. 405-24.

20. Rishi, A., M. Goggins, L.D. Wood, and R.H. Hruban, *Pathological and molecular evaluation of pancreatic neoplasms*. Semin Oncol, 2015. **42**(1): p. 28-39.

21. Hosoda, W., E. Sasaki, Y. Murakami, K. Yamao, Y. Shimizu, and Y. Yatabe, *GNAS mutation is a frequent event in pancreatic intraductal papillary mucinous neoplasms and associated adenocarcinomas*. Virchows Arch, 2015. **466**(6): p. 665-74.

22. Wu, J., Y. Jiao, M. Dal Molin, et al., *Whole-exome sequencing of neoplastic cysts of the pancreas reveals recurrent mutations in components of ubiquitin-dependent pathways*. Proc Natl Acad Sci U S A, 2011. **108**(52): p. 21188-93.

23. Kubota, Y., H. Kawakami, M. Natsuizaka, et al., *CTNNB1 mutational analysis of solid-pseudopapillary neoplasms of the pancreas using endoscopic ultrasound-guided fine-needle aspiration and next-generation deep sequencing*. J Gastroenterol, 2015. **50**(2): p. 203-10.

24. Mafficini, A., E. Amato, M. Fassan, et al., *Reporting tumor molecular heterogeneity in histopathological diagnosis*. PLoS One, 2014. **9**(8): p. e104979.

25. Christie, M., R.N. Jorissen, D. Mouradov, et al., *Different APC genotypes in proximal and distal sporadic colorectal cancers suggest distinct WNT/beta-catenin signalling thresholds for tumourigenesis*. Oncogene, 2013. **32**(39): p. 4675-82.

26. Miyaki, M., M. Konishi, R. Kikuchi-Yanoshita, et al., *Characteristics of somatic mutation of the adenomatous polyposis coli gene in colorectal tumors*. Cancer Res, 1994. **54**(11): p. 3011-20.

27. Ishioka, C., T. Suzuki, M. FitzGerald, et al., *Detection of heterozygous truncating mutations in the BRCA1 and APC genes by using a rapid screening assay in yeast*. Proc Natl Acad Sci U S A, 1997. **94**(6): p. 2449-53.

28. Cavard, C., A. Audebourg, F. Letourneur, et al., *Gene expression profiling provides insights into the pathways involved in solid pseudopapillary neoplasm of the pancreas*. J Pathol, 2009. **218**(2): p. 201-9.

29. Ruo, L., D.G. Coit, M.F. Brennan, and J.G. Guillem, *Long-term follow-up of patients with familial adenomatous polyposis undergoing pancreaticoduodenal surgery*. J Gastrointest Surg, 2002. **6**(5): p. 671-5.

30. Inoue, T., Y. Nishi, F. Okumura, et al., *Solid pseudopapillary neoplasm of the pancreas associated with familial adenomatous polyposis*. Intern Med, 2015. **54**(11): p. 1349-55.

31. Tang, L.H., H. Aydin, M.F. Brennan, and D.S. Klimstra, *Clinically aggressive solid pseudopapillary tumors of the pancreas: a report of two cases with components of undifferentiated carcinoma and a comparative clinicopathologic analysis of 34 conventional cases*. Am J Surg Pathol, 2005. **29**(4): p. 512-9.

32. Huels, D.J., R.A. Ridgway, S. Radulescu, et al., *E-cadherin can limit the transforming properties of activating beta-catenin mutations*. EMBO J, 2015.

33. Heinrich, M.C., G.A. McArthur, G.D. Demetri, et al., *Clinical and molecular studies of the effect of imatinib on advanced aggressive fibromatosis (desmoid tumor)*. J Clin Oncol, 2006. **24**(7): p. 1195-203.

34. Church, J., X. Xhaja, L. LaGuardia, M. O'Malley, C. Burke, and M. Kalady, *Desmoids and genotype in familial adenomatous polyposis*. Dis Colon Rectum, 2015. **58**(4): p. 444-8.

35. Couture, J., A. Mitri, R. Lagace, et al., *A germline mutation at the extreme 3' end of the APC gene results in a severe desmoid phenotype and is associated with overexpression of beta-catenin in the desmoid tumor*. Clin Genet, 2000. **57**(3): p. 205-12.

36. Machado, M.C., M.A. Machado, T. Bacchella, J. Jukemura, J.L. Almeida, and J.E. Cunha, *Solid pseudopapillary neoplasm of the pancreas: distinct patterns of onset, diagnosis, and prognosis for male versus female patients*. Surgery, 2008. **143**(1): p. 29-34.

37. Ansari, D., J. Elebro, B. Tingstedt, et al., *Single-institution experience with solid pseudopapillary neoplasm of the pancreas*. Scand J Gastroenterol, 2011. **46**(12): p. 1492-7.

38. Cai, Y.Q., S.M. Xie, X. Ran, X. Wang, G. Mai, and X.B. Liu, *Solid pseudopapillary tumor of the pancreas in male patients: report of 16 cases*. World J Gastroenterol, 2014. **20**(22): p. 6939-45.

39. Park, M., M. Kim, D. Hwang, et al., *Characterization of gene expression and activated signaling pathways in solid-pseudopapillary neoplasm of pancreas*. Mod Pathol, 2014. **27**(4): p. 580-93.

40. Guo, M., G. Luo, K. Jin, et al., *Somatic Genetic Variation in Solid Pseudopapillary Tumor of the Pancreas by Whole Exome Sequencing*. Int J Mol Sci, 2017. **18**(1).

41. Neill, K.G., J. Saller, S. Al Diffalha, B.A. Centeno, M.P. Malafa, and D. Coppola, *EGFR L861Q Mutation in a Metastatic Solid-pseudopapillary Neoplasm of the Pancreas*. Cancer Genomics Proteomics, 2018. **15**(3): p. 201-205.

**TABLES**

Table 1: The summary of genes targeted by the CHPv2 panel.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Gene** | **Exons Covered** |  | **Gene** | **Exons Covered** |  | **Gene** | **Exons Covered** |
| *ABL1* | 4 - 7 |  | *FGFR3* | 7,9,14,16,18 |  | *NOTCH1* | 26,27,34 |
| *AKT1* | 3 & 6 |  | *FLT3* | 11,14,16,20 |  | *NPM1* | 10 |
| *ALK* | 23 & 25 |  | *GNA11* | 5 |  | *NRAS* | 2 - 4 |
| *APC* | 16 |  | *GNAQ* | 5 |  | *PDGFRA* | 12,14,15,18 |
| *ATM* | 8 - 63 |  | *GNAS* | 7,8 |  | *PIK3CA* | 2,5,7,8,10,14,19,21 |
| *BRAF* | 11 & 15 |  | *HNF1A* | 3,4 |  | *PTEN* | 1,3,5-8 |
| *CDH1* | 3,8,9 |  | *HRAS* | 2,3 |  | *PTPN11* | 3,13 |
| *CDKN2A* | 2 |  | *IDH1* | 4 |  | *RB1* | 4,6,10,11,14,17-22 |
| *CSF1R* | 7,22 |  | *IDH2* | 4 |  | *RET* | 10,11,13,15,16 |
| *CTNNB1* | 3 |  | *JAK2* | 12 |  | *SMAD4* | 3-12 |
| *EGFR* | 3,7,15, 18-21 |  | *JAK3* | 13,16 |  | *SMARCB1* | 2,4,5,9 |
| *ERBB2* | 19-21 |  | *KDR* | 6,7,19,21,26,27,30 |  | *SMO* | 3,5,6,9,11 |
| *ERBB4* | 3-23 |  | *KIT* | 2,9-18 |  | *SRC* | 14 |
| *EZH2* | 16 |  | *KRAS* | 2 - 4 |  | *STK11* | 1,4,6,8 |
| *FBXW7* | 4-10 |  | *MET* | 2,11,14,16,19 |  | *TP53* | 2,4-8,10 |
| *FGFR1* | 4 & 7 |  | *MLH1* | 12 |  | *VHL* | 1-3 |
| *FGFR2* | 4,6,9 |  | *MPL* | 10 |  |  |  |

Table 2: The summary of potentially pathogenic mutations detected in samples of Solid Pseudopapillary Tumors of the Pancreas.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Sample number** | **Gene** | **Base Change** | **Amino Acid Change** | **Variant Frequency** | **COSMIC** | **Pathogenic Likelyhood\*** |
| SP1 | *CTNNB1* | c.109T>G | p.(Ser37Ala) | 31.4% | COSM5675 | *P*>0.99 |
| SP2 | *CTNNB1* | c.109T>G | p.(Ser37Ala) | 46.5% | COSM5675 | *P*>0.99 |
| SP3 | *APC* | c.3964delG | p.(Glu1322Lysfs\*93) | 42.6% | COSM4169380 | *P*=1.00 |
| SP4 | *CTNNB1* | c.94G>A | p.(Asp32Asn) | 41.9% | COSM5672 | *P*>0.99 |
| SP5 | *CTNNB1* | c.94G>A | p.(Asp32Asn) | 51.2% | COSM5672 | *P*>0.99 |
| SP6 | ND | - | - | - | - | - |

\* Mutation Taster Disease Causing probability score; ND = not detected

**FIGURE LEGENDS**

Figure 1: The E-cadherin/catenin complex and Wnt signaling pathway.

Figure 2: Microphotographs of the SPN with APC mutation in a male patient with aggressive histological and clinical features. (a) Immunohistochemistry of the tumor showing nuclear beta-catenin staining (200x), (b) Tumor showing area of necrosis/infarction (20x), (c) Tumor with area of infiltrative margin (200x), (d) Area of tumor with increased nuclear pleomorphism (400x).

Figure 3: Sanger Sequencing validation of APC c.3964delG (NM\_000038.5:c.3964delG), comparing DNA extracted from normal (A) and tumour (B) tissue, macrodissected from unstained slides prepared from the same FFPE sample block (patient SP3). The red arrow indicates the G that was deleted in the tumour (resulting in frameshift, indicated by double-peaks) but not the normal derived DNA.