DNA sequencing of cytopathologically inconclusive EUS-FNA from solid pancreatic lesions suspicious for malignancy confirms EUS diagnosis

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INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related death in the United States\(^1\) and accounts for 6% of all cancer-related deaths in Denmark. With a 5-year survival rate of 8%, it is one of the most lethal...
malignancies. PDACs develop from pancreatic intraepithelial neoplasia (PanIN) or from pancreatic cystic neoplasms. Surgery is the only curative treatment for patients with localized disease, but unfortunately, nearly 80% of patients present with an unresectable lesion at the time of diagnosis. Therefore, early detection and diagnosis are of great importance. Evaluation of pancreatic lesions suspicious of malignancy is a multidisciplinary task where EUS-FNA plays an important role in the diagnostic process. However, EUS-FNA can be technically challenging and up to 10%–15% of EUS-FNAs have been found to be inconclusive. Therefore, the combination of molecular biomarker detection, such as mutation analysis and microRNA analysis, with clinical information and endoscopic evaluation has emerged as a new approach, which could potentially improve the diagnostic accuracy in these patients. So far, the use of whole-exome sequencing in the field of PDAC diagnostics has been limited to experimental studies on surgically resected solid or cystic lesions. Yet, to our knowledge, it has not been utilized in the differentiation of solid pancreatic lesions (SPLs) with cytology sampled by EUS-FNA.

The aim of this study was to investigate whether mutational analysis, by means of whole-exome sequencing, correlates to a malignant EUS diagnosis in inconclusive EUS-FNA cytology specimens obtained from patients with SPLs.

**PATIENTS AND METHODS**

**Study design and patients**

All patients who underwent primary EUS-FNA at our Endoscopy Unit for SPL suspected of malignancy in 4 years from February 2011 to January 2015 were retrospectively identified by querying the electronic pathology database at Herlev Hospital, Denmark. All EUS-FNA specimens were evaluated and classified according to the Papanicolaou Society International Guidelines for pancreatobiliary cytology and categorized as (I) nondiagnostic, (II) negative (for malignancy), (III) atypical, (IV) neoplastic: benign and other, (V) suspicious (for malignancy), and (VI) positive/malignant. This was a hypothesis-generating feasibility study. Thus, only specimens with a high number of epithelial cells and sufficient DNA extracted were used. All EUS-FNA specimens included had sheets with large numbers (>300) of well-preserved, epithelial cells on the slides. Patients with nondiagnostic samples due to scant cellularity, gastrointestinal contamination only, degeneration, and/or preparation artifact as well as patients below the age of 18 years were excluded. We aimed to include at least 10 patients with specimens with well-preserved, cellular material allowing successful DNA extraction for each of the three patient groups for the feasibility study and ended up with a total of 39 specimens.

Medical records were analyzed and data on demographics and clinical history such as EUS diagnosis, cytological diagnosis, location of the lesion, biomarkers in the blood before EUS-FNA (amylose, carcinoembryonic antigen, and carbohydrate antigen 19–9), postoperative pathology reports, and follow-up imaging were reviewed. To determine the relationship of each patient group (benign, inconclusive, and malignant) with overall survival, patients were followed up until death or the latest medical record entry by means of medical record scrutiny (performed in November 2017), which is linked to the National Death Registry.

**EUS-FNA procedure**

EUS was performed with the patients in propofol sedation using a linear echo-endoscope (EG-3870 UTK, Pentax, Tokyo, Japan/HI Vision Preirus, Hitachi Medical Systems, Tokyo, Japan), by an expert endosonographer (PV). Morphology, size, and location of the lesion were recorded. Next, an FNA needle (Sono-Tip Pro-Control 22G, Medi-Globe, Grassau, Germany) was used to puncture the lesion in that three–four passes were made. Suction with a 20-ml syringe was routinely applied, and the aspirate was smeared onto slides, which were air-dried and sent for cytological evaluation. There was no rapid on-site evaluation of the specimens. Patients were observed for an hour after the procedure and discharged upon uneventful recovery. Adverse events were recorded and classified according to the ASGE guidelines.

**Specimen processing and cytology diagnosis**

The original May–Grünwald–Giemsa-stained FNA direct smears from the three groups were reviewed by a senior cytopathologist (A.T.) nonblinded to diagnosis, and areas on the slides with high concentrations (>90%) of well-preserved, epithelial (benign or malignant) cells were marked. After soaking the slides in xylene, coverslips were removed, and the marked areas were scraped off using a scalpel blade. DNA was subsequently extracted from this cellular material.
Patients were divided into three groups defined by EUS imaging and cytological diagnosis in combination: a benign group (benign cytology, categories II–III, and benign EUS imaging); an inconclusive group (benign cytology or suspicious for malignancy, categories II–V, and malignant EUS findings); a malignant group (malignant cytology, category VI, and malignant EUS imaging). Based on EUS evaluation, patients either had a benign or had a malignant appearing SPL on EUS imaging. Typical EUS features suggestive of a malignant SPL were those of a hypoechoic lesion with irregular outline and with or without upstream dilatation of the main pancreatic duct or the common bile duct. A benign lesion was defined as a focal mass with changes, suggestive of chronic pancreatitis with or without upstream dilatation of the main pancreatic duct or the common bile duct.

**Mutational analysis**

Next-generation sequencing (NGS) was used for whole-exome sequencing of the selected genes. For this purpose, a customized gene panel was used covering the whole exome of the following genes: ARID1A, MSH2, MSH6, CASP8, TGFBR2, MLH1, CTNNB1, PIK3CA, FBXW7, APC, EGFR, MET, BRAF, SMAD2, SMAD4, ATM, KRAS, TP53, and FAM123B. The panel applied was a common cancer panel established in the laboratory suitable for PDAC. This panel was chosen since we wanted to investigate all information in the coding areas of the genes and not just “hot spots” in each gene, thereby generating knowledge of possible new variations. All mutations found were confirmed in ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/), International Agency for Research on Cancer database for TP53 (http://p53.iarc.fr/TP53GeneVariations.aspx), and Leiden Open Variation Database for ATM (https://databases.lovd.nl/) as well as confirmed in the literature and subsequently sorted in pathogenic and nonpathogenic subgroups. Single mutations were designated as nonpathogenic if they were thought not to have clinical or diagnostic relevance, i.e., if they had not been previously reported to be related to cancer. They were also designated nonpathogenic if they had the status uncertain significance in the ClinVar database or were not described in the literature. The opposite was true for pathogenic mutations which were found to be related to cancer. However, a high number of nonpathogenic mutations can be interpreted as compromised DNA repair and may have a diagnostic value. A table of all mutations detected can be seen in Supplementary Tables 1 and 2.

**Table 1. Demographics and characteristics in each patient group**

|                     | Benign (n=10) | Inconclusive (n=13) | Malignant (n=16) | P   |
|---------------------|--------------|---------------------|-----------------|-----|
| Age, median         | 49.9         | 69.4                | 65.5            | 0.059|
| Sex, n (%)          |              |                     |                 |     |
| Male                | 7 (70)       | 10 (77)             | 12 (75)         | 0.929|
| Female              | 3 (30)       | 3 (23)              | 4 (25)          |     |
| Location of lesion, n (%) |         |                     |                 |     |
| Head                | 6 (60)       | 12 (92.3)           | 14 (87.5)       | 0.174|
| Body                | 2 (20)       | 0                   | 2 (12.5)        |     |
| Tail                | 2 (20)       | 1 (7.7)             | 0               |     |
| Increased serum amylase, n (%)* |    |                     |                 |     |
| Yes                 | 2 (20.0)     | 4 (30.8)            | 6 (37.5)        | 0.906|
| No                  | 6 (60.0)     | 9 (69.2)            | 10 (62.5)       |     |
| Data unavailable    | 2 (20.0)     | 0                   | 0               |     |
| Increased serum CEA, n (%)** | |                     |                 |     |
| Yes                 | 1 (10.0)     | 1 (7.7)             | 3 (18.8)        | 0.784|
| No                  | 2 (20.0)     | 5 (38.5)            | 4 (25.0)        |     |
| Data unavailable    | 7 (70.0)     | 7 (53.8)            | 9 (56.3)        |     |
| Increased serum CA-19.9, n (%)*** | |                     |                 |     |
| Yes                 | 0            | 6 (46.2)            | 11 (68.8)       | 0.024|
| No                  | 3 (30.0)     | 2 (15.3)            | 2 (12.5)        |     |
| Data unavailable    | 7 (70.0)     | 5 (38.4)            | 3 (18.7)        |     |

*Serum amylase levels >120 U/L, **Serum CEA >10 µg/L, ***Serum CA-19.9 levels >37 kU/L. Fisher’s exact test was used. CEA: Carcinoembryonic antigen, CA: Carbohydrate antigen
60°C for 4 min, ending with a holding period at 10°C. Polymerase chain reaction (PCR) amplicons were treated with 2 µL FuPa reagent (Thermo Fisher Scientific) to partially digest primer sequences and phosphorylate the amplicons at 50°C for 10 min, followed by 55°C for 10 min and then 60°C for 20 min. Amplicons were ligated to adapters with the diluted barcodes of the Ion Xpress™ Barcode Adapters kit (Life Technologies) for 30 min at 22°C and then 72°C for 10 min. Adaptor-ligated amplicon libraries were purified using Agencourt® AMPure® XP reagents (Beckman Coulter, Tokyo, Japan). The library concentration was adjusted to 100 pM using an Ion Library Quantitation Kit (Life Technologies). Emulsion PCR was performed on the final library out using the Ion OneTouch™ System and Ion OneTouch™ 200 Template Kit v2 (Life Technologies) according to the manufacturer’s instructions. Template-positive Ion Sphere™ Particles were then enriched with Dynabeads® MyOne™ Streptavidin C1 Beads (Life Technologies) using an Ion OneTouch™ ES system (Life Technologies). Purified Ion Sphere particles were loaded on an Ion 318 Chip v. 2. Massively parallel sequencing was carried out on a Personal Genome Machine (PGM) sequencer (Ion Torrent™) using the Ion PGM Sequencing 200 Kit version 2 according to the manufacturer’s instructions.

Sequencing results of the Ion Torrent PGM run were examined by two experienced molecular biologists (T.S.P. and E.H.) to verify that the NGS runs had performed as expected. This includes statistics and quality metrics to evaluate the quality of the actual Ion Torrent run. Coverage statistics using the Ion Torrent Suit Coverage Analysis were used to evaluate the quality of each library in the run. Alignment was performed using the Ion Torrent Suit Variant caller aligning the sequences to the reference genome Hg19. Variants annotated were graphically visualized using the software Integrative Genomics Viewer. Genes were coded as mutated if relevant mutations were identified at a minor allele frequency of 5% and reading depth >×100.

**Follow-up**

The gold standard of a malignant diagnosis was positive histopathology of a subsequent surgical specimen, positive histopathology of sampling at a procedure other than the index EUS-FNA procedure (i.e., percutaneous biopsy, etc.), or progression of disease consistent with malignancy (e.g., development of metastases) during follow-up. The final diagnosis of a benign condition was assumed in cases of histopathology of surgical specimens negative for malignancy and/or uneventful clinical follow-up of at least 12 months.

**Statistical analysis**

Data were presented as median and interquartile range or n (%), as appropriate. The Fisher’s exact test was used for comparison of categorical data. Overall survival in the three groups was determined using Kaplan–Meier statistics and compared using the log-rank test. A two-sided \( P < 0.05 \) was considered statistically significant. For all statistics, IBM SPSS Statistics 22 (SPSS, Chicago, USA) was used.

**RESULTS**

**Patient characteristics and EUS findings**

Thirty-nine patients were identified and constituted the study cohort [Table 1]. The mean age was 66.7 (standard deviation 11). Twenty-nine patients (74.4%) were male, and 32 (82.0%) presented with clinical symptoms of obstructive jaundice, with abdominal pain, or with symptoms suggestive of malignancy such

| Table 2. Distribution of mutations found in the three patient groups |
|---------------------------------------------------------------|
| **Patients with pathogenic mutations, n (%)** | 1 (10) | 13 (100) | 15 (93.5) | <0.001 |
| **Patients with nonpathogenic mutations, n (%)** | 10 (100) | 13 (100) | 16 (100) | 1.000 |
| **Genes with known pathogenic mutations, n (%)** | | | | |
| KRAS | 0 | 7 (53.8) | 13 (81.3) | <0.001 |
| TP53 | 0 | 4 (30.7) | 8 (50.0) | 0.026 |
| BRAF | 0 | 1 (7.8) | 0 | 0.590 |
| CASP8 | 0 | 1 (7.8) | 2 (12.5) | 0.772 |
| PIK3CA | 1 (10) | 6 (46.2) | 1 (6.2) | 0.024 |
| SMAD4 | 0 | 2 (15.4) | 0 | 0.166 |
| MET | 0 | 2 (15.4) | 0 | 0.166 |

KRAS: KRAS proto-oncogene, TP53: Tumor suppressor p53, BRAF: B-Raf proto-oncogene, CASP8: Caspase 8, PIK3CA: Phosphatidylinositol-4,5-bisphosphate 3-kinase, SMAD4: Mothers against decapentaplegic homolog 4, MET: Tyrosine-protein kinase Met. Fisher’s exact test was used.
as fever, fatigue, weight loss, and night sweats. Lesions in four patients were the incidental findings on CT or MRI, and three patients had known chronic pancreatitis. Thirty-two of 39 (82%) SPLs were in the head of the pancreas, 4 (10.3%) were in the body, and 3 (7.7%) were in the tail section. No procedural adverse events were observed. No patients presented with cystic lesions.

**Cytology findings**

In total, 16 (40.1%) patients had suspected malignancy on EUS confirmed by malignant cytology, category VI (malignant); 13 (33.3%) patients had suspected malignancy on EUS, but benign cytology or suspicious for malignancy, categories II–V (inconclusive); and 10 patients (26.6%) had a focal mass, which was most likely benign on EUS, and with benign cytology, categories II–III (benign) [Table 1].

**Sequencing findings**

Most mutations found were missense mutations, albeit a few frameshifts and short deletions were also detected [Supplementary Table 1]. Known pathogenic mutations were found in 1 (10%) patient in the benign group, 13 (100%) patients in the inconclusive group, and 15 (93.3%) patients in the malignant group [Table 2 and Figure 1]. We found a statistically significant difference in the frequency of K-RAS ($P < 0.001$), TP53 ($P = 0.026$), and PIK3CA ($P = 0.024$) between the three groups [Table 2]. When analyzed within groups, both the inconclusive and malignant groups differed significantly compared with the benign group with respect to K-RAS. TP53 mutations were found to be more common in the malignant compared with the benign group, but this was also the case for the inconclusive group compared with the benign group, although it did not reach statistical significance [Figure 2]. A majority of the patients in the inconclusive group had pathogenic PIK3CA mutations, which was also significantly different from the malignant group [Figure 2]. Other genes with pathogenic mutations were found not to be significantly different between the three groups. Regarding nonpathogenic mutations, there was no statistical difference between the groups [Table 2].

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**Figure 1.** Graphical presentation of found mutations. K-RAS: K-RAS proto-oncogene, P53: Tumor suppressor p53, BRAF: B-Raf proto-oncogene, CASP8: Caspase 8, PIK3CA: Phosphatidylinositol-4,5-bisphosphate 3-kinase, SMAD4: Mothers against decapentaplegic homolog 4, ARID1A: AT-rich interactive domain-containing protein 1A, MSH2: MutS protein homolog 2, MSH6: MutS homolog 6, TGFBR2: Transforming growth factor, beta receptor II, MLH1: MutL homolog 1, CTNNB1: β-catenin, FBXW7: F-box/WD repeat-containing protein 7, APC: Adenomatous polyposis coli, EGFR: Epidermal growth factor receptor, MET: Tyrosine-protein kinase Met, SMAD2: Mothers against decapentaplegic homolog 2, ATM: Ataxia-Telangiectasia Mutated gene, FAM123B: Family With Sequence Similarity 123B, MAPK: Mitogen-activated protein kinase, TGF-beta: Transforming growth factor beta, Wnt: Wingless integrated, RTK: Receptor tyrosine kinase, UBC: Ubiquitin ligase complex, PI3K: Phosphatidylinositol-3 kinase.
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Final diagnosis and overall survival

The final diagnosis, according to the gold standard, was malignancy in 29 patients (i.e., all patients in the inconclusive and malignant groups). Overall, 10 patients were diagnosed with benign lesions (all patients in the benign group) after a median follow-up of 45.8 months (range 19–78 months).

Out of 29 patients with a malignant diagnosis, 10 were alive 12 months following the index EUS procedure. Out of 10 patients with a benign solid lesion, six were due to chronic pancreatitis, three were due to acute pancreatitis, and one was a thrombosed aneurysm of the hepatic artery.

Overall survival was significantly lower in the malignant (median survival 7.5 months, range <1–25 months) and inconclusive (median survival 9.4 months, range <1–29 months) groups compared to the benign group ($P < 0.001$). Unsurprisingly, we found no significant difference in survival between the inconclusive and malignant groups ($P = 0.247$) [Figure 3].

DISCUSSION

To our knowledge, this is the first study to investigate the usefulness of whole-exome sequencing on macro-dissected cytology specimens from EUS-FNA, in the differential diagnosis of SPLs. Although several studies utilize commercial hot-spot targeted panels, we aimed to examine the whole-exome sequence of potentially relevant genes to elucidate the amount of nonpathogenic mutations in the three groups, in addition to the expected pathological mutations. We have shown that KRAS G12 point mutations, especially KRAS G12D, are commonly present in patients with malignant compared to those with a benign SPL. This was not unexpected since KRAS is mutated in >90% of PDAC cases. In concordance with the literature, 50% of patients in the malignant group had pathogenic TP53 mutations. Only 23% of the patients in the inconclusive group presented with pathogenic TP53 mutations. Since progression of invasive PDAC from its precursors (PanINs) is associated with pathogenic mutations of TP53, one could speculate that the epithelial cells examined in the inconclusive group are less malignant and therefore difficult to classify...
by the pathologist. We found pathogenic PIK3CA mutations mostly in the inconclusive group. The most commonly PIK3CA mutation found was E707K, which has been reported in breast papillary carcinoma.[24] Whether this has an impact on the carcinogenesis in PDAC has not yet been determined. We only found pathogenic SMAD4 mutations in the inconclusive group. The SMAD4 gene is known to be deleted in PDAC.[23] Our method, however, did not include the detection of deletion of chromosome 18q, where SMAD4 is located. Thus, a higher number of cases with SMAD4 alterations would be expected and is possibly hidden in our dataset. KRAS G12 point mutations were present in 53% of the cases with inconclusive diagnosis. Therefore, our data suggest that the presence of KRAS G12 mutations may be used to confirm a malignant EUS diagnosis when the cytopathology diagnosis is inconclusive. An important aspect is that KRAS G12 point mutations have been shown to be prevalent in one-third of patients with chronic pancreatitis.[26] Surprisingly, no patients with a benign diagnosis presented with KRAS G12 mutations even though 90% of these patients in our cohort were diagnosed with chronic pancreatitis. However, several other studies with EUS-FNA have shown low rates of KRAS mutations in patients with pancreatitis.[27-29] KRAS G12 mutation has previously been shown to reliably distinguish malignancy from benign pathology, even in cases where cytology is inconclusive or even benign.[30,31] Based on our results, it would be interesting to test in a larger cohort, whether analysis of a combination of KRAS, TP53, SMAD4, and PIK3CA mutations would increase diagnostic accuracy. This study has several strengths. First, we had well-characterized groups of patients with SPLs and the follow-up period was long, in some cases >6 years, which allowed comparison of overall survival among different groups. Second, the current report is the first to investigate the usefulness of whole-exome sequencing in patients with SPLs and cytopathologically inconclusive EUS-FNA, a relatively frequent clinical dilemma. Third, we have performed NGS on isolated epithelial cells, chosen by an expert pathologist. Fourth, by means of whole-exome sequencing, we have shown that patients with cytopathologically inconclusive EUS-FNA harbor several pathogenic mutations compared to those with a benign EUS-FNA. Their potential role in the diagnosis of PDAC will need to be investigated in larger prospective studies. Our study also has limitations. First, it is a retrospective, hypothesis generating, pilot study, and the cohort is relatively small. Second, CDKN2A was not included in the gene panel used, which is one of the most frequently mutated tumor-suppressor genes in PDACs.[32,33] Third, we did not perform chromosomal deletion analysis on chromosomes 6p, 9p, 13q, 17p, and 18p, which are frequently lost in PDAC.[34,35] Fourth, our study did not include blood samples from the patients for germline mutation assessment which would be useful to compare with whole-exome sequencing data of the pancreatic lesions. This should probably be included in future prospective studies. Fifth, our groups were defined based on both cytology and the endoscopists’ EUS image evaluation, which is subjective. Sixth, our analysis was made on selected FNA specimens, ensuring a high number of tumor cells, which may not be the case for every patient with a pancreatic mass undergoing EUS-FNA. Finally, the study was performed in an expert center, which means that our findings may not be generalized to other settings.

CONCLUSION

This is the first study using whole-exome sequencing of selected genes to investigate samples from patients with SPLs and pathologically inconclusive EUS-FNA. We found pathogenic mutations in KRAS, TP53, BRAF, CASP8, PIK3CA, SMAD4, and MET in both the inconclusive and malignant cohorts. The frequency of pathogenic mutations in KRAS, TP53, and PIK3CA differed significantly compared with the benign cohort.

Our findings suggest that detection of genetic mutations, in general, may be an important adjunct to cytopathologically inconclusive EUS-FNA, which is in line with previous studies.[27,28,30] Whole-exome sequencing of a combination of KRAS, TP53, SMAD4, and PIK3CA mutations may potentially aid further in the workup of these patients.

Supplementary Materials

Supplementary information is linked to the online version of the paper on the Endoscopic Ultrasound website.

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Conflicts of interest

There are no conflicts of interest.
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**Supplementary Table 1. Mutations found in the screen**

|                     | Benign diagnosis | Inconclusive diagnosis | Malignant diagnosis |
|---------------------|------------------|------------------------|---------------------|
| **FAM123B mutations** | p.A1118V        |                        |                     |
| **TP53 mutations**  | p.P72R           | p.P72R                 | p.P72R              |
|                     | p.S240N          | p.R136H                | p.S149P             |
|                     | p.V147DEL        | p.T125M                | p.C277X             |
|                     | p.T125M          | p.R273L                | p.P273C             |
|                     | p.Q105fs         | p.E198*                | p.S241F             |
|                     |                  |                        | p.E246K             |
|                     |                  |                        | p.P209_p.P211del    |
|                     |                  |                        | p.V197G             |
|                     |                  |                        | p.H179R             |
|                     |                  |                        | p.R136G             |
| **KRAS mutations**  | p.G12D           | p.G12D                 |                     |
|                     | p.T20M           | p.G12R                 |                     |
|                     | p.G12R           | p.Q61K                 |                     |
| **ATM mutations**   | p.S49C           | p.S49C                 | p.N1983S            |
|                     | p.D1853N         | p.S49C                 | p.E365K             |
|                     | p.S99G           | p.E365K                | p.F858L             |
|                     | p.Y2437C         | p.A1512S               | p.A1309T            |
|                     | p.A1512S         | p.D1853N               | p.D1853N            |
|                     | p.K2966R         |                        |                     |
| **SMAD4 mutations** | p.D537G          |                        | p.S357P             |
|                     | p.R135X          |                        |                     |
| **SMAD2 mutations** |                  |                        |                     |
| H441Y               |                  |                        |                     |
| E389F               |                  |                        |                     |
| **BRAF mutations**  |                  |                        |                     |
| A749T               | p.E533K          |                        |                     |
|                     | p.V600E          |                        |                     |
| **MET mutations**   | p.D1020N         | p.R988C                | p.T992I             |
|                     | p.N375S          |                        |                     |
| **EGFR mutations**  | p.R521K          | p.R521K                | p.R521K             |
|                     | p.E513fs         | p.E513fs               | p.A155fs            |
| **APC mutations**   | p.D491Y          | p.V1822D               | p.A1246T            |
|                     | p.E1145K         | p.A2247V               | p.V1822D            |

*Substitute mutation in TP53. The */X indicates a stop codon.*
| Nonpathogenic mutations | Unknown significance | Pathogenic mutations |
|-------------------------|----------------------|----------------------|
| **TP53 mutations**      |                      |                      |
| p.P72R                  |                      | p.R136H (ClinVar pathogenic) |
| p.V147DEL (IARC no cancer hotspot) | | p.T125M (IARC cancer hotspot)(ClinVar likely pathogenic) |
| p.P209_p.P211del (R110L nonpathogenic, IARC no cancer hotspot) | | p.G105fs (IARC cancer hotspot) |
| p.V147fs                |                      | p.R273L (IARC cancer hotspot) (ClinVar pathogenic) |
| p.S149P (IARC no cancer hotspot) | | p.P273C (one of the six most prevalent hotspot mutations) |
| p.S240N (small reduction in p53 activity, IARC no cancer hotspot) | | p.G105fs |
| **KRAS mutations**      |                      |                      |
| p.T20M                  |                      | p.G12D (ClinVar pathogenic) |
| **ATM mutations**       |                      |                      |
| p.F858L (ClinVar benign) | p.E365K              |                      |
| p.A1309T (ClinVar benign) | p.Y2437C           |                      |
| p.D1853N (ClinVar benign) | p.K2966R           |                      |
| p.S49C (ClinVar benign) |                      |                      |
| p.Y2437C (ClinVar uncertain significance) | |                      |
| p.D1853N (ClinVar benign) | |                      |
| p.S99G (ClinVar likely benign) | |                      |
| p.N1983S (LOVD benign) | |                      |
| **SMAD4 mutations**     | p.R135X              | p.D537G (ClinVar likely pathogenic) |
| p.R135X                 |                      | p.D537G (ClinVar likely pathogenic) |
| p.S357P                 |                      |                      |
| **SMAD2 mutations**     | p.H441Y              |                      |
| p.H441Y                 |                      |                      |
| **BRAF mutations**      | p.E533K              | p.V600E (ClinVar pathogenic) |
| p.E389F                 |                      |                      |
| **MET mutations**       | p.A749T              |                      |
| p.N375S                 | p.D1020N             | p.R988C              |
| p.T992I                 |                      |                      |
| **EGFR mutations**      | p.R521K              |                      |
| p.R521K                 | p.E513fs             |                      |
| p.A155fs                |                      |                      |
| **APC mutations**       | p.D401Y              |                      |
| p.V1822D (ClinVar benign) | p.E1145K           |                      |
| p.G1836R (ClinVar likely benign) | p.S2352I   |                      |
| p.V2630I (ClinVar likely benign) | p.A2247V |                      |
| p.R1589C (ClinVar likely benign) | p.A1246T |                      |
| **FBXW7 mutations**     | p.A2603C             |                      |
| p.S282L                 |                      |                      |
| p.D27Y                  |                      |                      |
| **PIK3CA mutations**    | p.N1072S             | p.E707K (likely pathogenic) |
| p.I391M (ClinVar benign) | p.E259K             | p.R88Q (ClinVar likely pathogenic) |
| **CTNNB1 mutations**    | p.R212C              | p.T411 (ClinVar pathogenic) |
| **MLH1 mutations**      |                      |                      |
| **Contd...**
### Supplementary Table 2. Contd...

| Nonpathogenic mutations | Unknown significance | Pathogenic mutations |
|-------------------------|----------------------|----------------------|
| p.I219V (ClinVar benign) | p.R217C (ClinVar uncertain significance) |
| **TGFBR2 mutations**    |                      |                      |
| p.V412M (ClinVar likely benign) | p.N303S | p.D302H (ClinVar benign, conflicting results in literature but for certain types of cancer, this mutation is a risk factor) |
| **CASP8 mutations**     |                      |                      |
|                          | p.D344H |                      |
| **MSH6 mutations**      |                      |                      |
| p.G39E (ClinVar benign) | p.S677N (ClinVar uncertain significance) |                      |
|                          | p.R976H (ClinVar uncertain significance) |                      |
|                          | p.E1281G |                      |
|                          | p.P199fs |                      |
| **MSH2 mutations**      |                      |                      |
| p.G322D (ClinVar benign) | p.N186S (ClinVar uncertain significance) |                      |
|                          | p.S323F (ClinVar uncertain significance) |                      |
|                          | p.E132X |                      |
|                          | p.L213V |                      |
| **ARID1A mutations**    | p.G1340V |                      |
| **FAM123B mutations**   | p.A1118V |                      |