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# Targeted next-generation sequencing of FNA-derived DNA in pancreatic cancer

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## ABSTRACT

To improve the diagnostic value of fine-needle aspiration (FNA)-derived material, we perform targeted next-generation sequencing (NGS) in patients with a suspect lesion of the pancreas. The NGS analysis can lead to a change in the treatment plan or supports inconclusive or uncertain cytology results. We describe the advantages of NGS using one particular patient with a recurrent pancreatic lesion 7 years after resection of a pancreatic ductal adenocarcinoma (PDAC). Our NGS analysis revealed the presence of a presumed second primary cancer in the pancreatic remnant, which led to a change in treatment: resection with curative intend instead of palliation. Additionally, NGS identified an unexpected germline CDKN2A 19-base pair deletion, which predisposed the patient to developing PDAC. Preoperative NGS analysis of FNA-derived DNA can help identify patients at risk for developing PDAC and define future therapeutic options.

## BACKGROUND

Cancer-causing genetic variations in human cells often cluster in predictable gene 'hotspots'. In lung cancer and pancreaticobiliary tract cancer, single-gene analysis of endoscopic ultrasound-guided fine-needle aspiration (FNA)-derived DNA samples has yielded valuable diagnostic information.[1] [2] Moreover, performing targeted next-generation sequencing (NGS) on these samples can identify multiple-gene variants in a limited quantity of material.[3] NGS can indeed be reliably performed on FNAs from pulmonary and pancreatic tumours, as the gene variants identified correlated well with matched resected pancreatic tumours.[4] [5] The advantage of using a NGS panel that specifically targets hotspot mutations in 50 cancer genes is that robust ultra-deep sequencing results can be obtained from samples containing extremely low numbers of cancer cells, including DNA obtained from formalin-fixed paraffin-embedded tissue samples of neoplasms of the pancreas.[6]

In the past decade, the mutational landscape of pancreatic ductal adenocarcinoma (PDAC) has been well characterised.[7] Activated pathogenic variants in the proto-oncogene *Kirsten RAS* (*KRAS*) are present in 90% of patients with PDAC. Inactivated variants in the tumour-suppressor genes *TP53*, *CDKN2A* and *SMAD4* have been frequently identified. Recently, published whole-exome and whole-genome sequencing data revealed additional somatic and germline variants in *ARID1A*, *ROBO2*,

*BRCA1*, *BRCA2* and *PALB1*, some of which can direct the optimal choice of adjuvant therapy. Moreover, focal gene amplifications of actionable oncogenes have been identified, including *ERBB2*, *MET*, *FGFR1*, *CDK6*, *PIK3R3* and *PIK3CA*.[7] [8]

As part of an ongoing study, the NGS analysis is performed in preoperative FNA-derived DNA samples obtained from patients with a suspicion of PDAC at our centre. Here, we describe a case of one such patient in which the NGS analysis revealed the presence of a second primary PDAC drastically changing the treatment plan.

## CASE REPORT

A male patient aged 54 years underwent a pancreaticoduodenectomy with en bloc right hemicolectomy 7 years ago, followed by adjuvant gemcitabine therapy. Pathological evaluation revealed a 5 cm PDAC with negative surgical resection margins and 6 out of 21 positive peripancreatic lymph nodes. After 5 years without recurrence of the disease, the patient was discharged from follow-up. Recently, the patient presented with vague abdominal pain. A CT scan revealed a poorly defined mass in the pancreatic remnant close to the pancreatic-jejunal anastomosis suggestive of a local recurrence (figure 1A). An endoscopic ultrasound-guided FNA was performed. Cytological evaluation was not conclusive for carcinoma and was interpreted as 'reactive changes' with a low number of atypical ductal cells (figure 1B). Given the clinical suspicion of recurrent malignancy, palliative chemotherapy was considered as the first therapeutic option during the multidisciplinary team meeting. However, in light of the long interval between the current lesion and the original primary tumour, and despite the limited number of morphologically atypical cells in the FNA sample (estimated at <10% of the cells), we opted to analyse the FNA-derived DNA using targeted NGS with the AmpliSeqCancer Hotspot Panel V.2 (Thermo Fisher Scientific, Cambridge, Massachusetts, USA). The patient provided informed consent for molecular testing.

We also analysed the primary PDAC using NGS. Our analysis revealed that the mutational profiles differed between the original lesion and the new lesion. Furthermore, we found a germline pathogenic *CDKN2A/P16* gene variant that predisposed the patient for developing PDAC. The patient had no documented personal history of atypical moles or melanoma. No family history of breast-ovarian carcinoma syndrome or atypical multiple mole

**Figure 1** CT scan and cytological findings in a patient with a suspicion of recurrent pancreatic ductal adenocarcinoma (PDAC). (A) Axial CT scan of the abdomen showed the suspect recurrent tumour (arrow) in the duodenal anastomosis with the remnant pancreatic tail (arrowhead). (B) Giemsa stain of fine-needle aspiration (FNA)-derived cells obtained from the pancreatic lesion. A low number of atypical ductular cells were visible in the complete slide; based on their morphology, these cells were not judged to be unequivocally dysplastic.
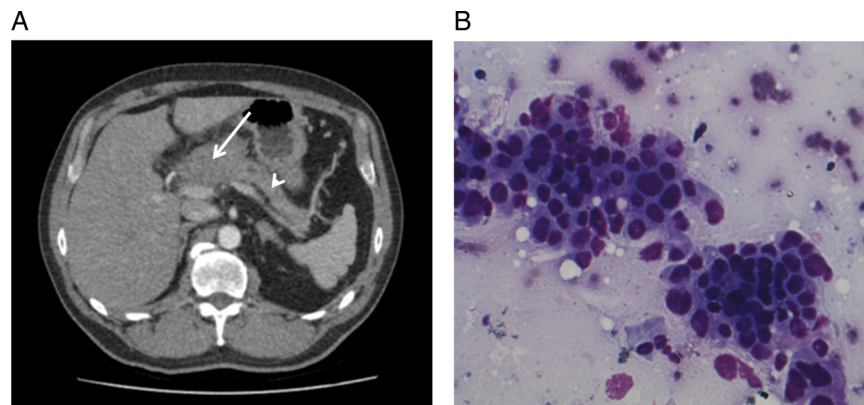


melanoma syndrome was reported. Only after consulting the clinical genetics in a later phase, the patient turned out to have an aunt and nephew who died of melanoma and PDAC, respectively at relatively young ages. At the molecular level, a second primary tumour was considered to be plausible. Based on the NGS data, the treatment plan changed drastically from providing palliative chemotherapy to curative-intended surgical resection of the residual pancreas.

## METHODS
### Selection of tumour cells, DNA isolation and targeted NGS
FNA slides were generated using methanol fixation and Giemsa staining. In general, there are two approaches for molecular analysis of cytology smears, either with or without tumour cell enrichment. The enrichment step is chosen if tumour cells can clearly be distinguished from non-tumour cells. In the case described here, no enrichment step for the FNA sample could be performed. A single FNA slide was used, which was photographed and attached to the patient files. Subsequently, the cover slip was removed by incubation in xylene at room temperature in a separate 50 ml tube to avoid contamination. The incubation period was until the moment the cover slip got loose. Next, the slide was washed in alcohol three times for rehydration of the tissue, once in 100%, once in 70% and once in 50%. The FNA material was scraped from the slide and collected in a microtube for DNA isolation. Because there was no enrichment step, which resulted in a low percentage of atypical cells, bioinformatics thresholds were adopted accordingly. The PDAC resection specimens were examined for regions with the highest tumour percentages. After examination, five 10 μm additional sections were prepared for microdissection and stained with haematoxylin (eosin staining was omitted to preserve the integrity of the DNA). Slides were visualised with an inverted microscope and manually microdissected with a sharp, pointed knife.

After scraping, DNA was isolated from FNA-derived material and formalin-fixed, paraffin-embedded PDAC tissue using a fully automated DNA extraction procedure.[9] The concentration of DNA was measured using a fluorometer (Qubit dsDNA HS, Life Technologies, Carlsbad, California, USA). The AmpliSeq Cancer Hotspot Panel V.2 consists of a single primer pool and is designed to detect somatic cancer hotspot mutations in 200 amplicons covering 50 genes, including genes that are often altered in PDAC (eg, KRAS, TP53, SMAD4 and CDNK2A). Libraries were prepared with 10 ng of genomic DNA, and each sample was uniquely barcoded using IonXpress barcodes (Life Technologies). Ion PGM 318 or Proton P1 chips were prepared using the Ion Chef System and sequenced using the Personal Genome Machine or Proton system, respectively (all from Life Technologies). Variants were analysed using the Geneticist Assistant NGS Interpretative Workbench (V.1.1.8, SoftGenetics, State College, Pennsylvania, USA). The identified variants were classified into five classes, and only class 4 (likely pathogenic) and class 5 (pathogenic) variants were reported.[10]

### Evaluation of genetic variants in the context of morphology
Genetic variations in the KRAS or GNAS gene can occur in precursor lesions of PDAC, including low-grade or high-grade pancreatic intraepithelial neoplasia, intraductal papillary mucinous neoplasm and mucinous cystic neoplasms.[6] Because a stepwise increase in genetic variations occurs during the development of PDAC, we developed a clinical decision scheme in which confirmed genetic variants were placed in the context of the observed morphology and tumour percentage. The sole finding of a pathogenic KRAS variant is molecularly scored as a 'proliferative lesion, at least low-grade dysplasia', keeping in mind that KRAS variants are also present in a low percentage of cases with pancreatitis.[11] A combination of two or more pathogenic variants (eg, a KRAS variant in combination with TP53, SMAD4, CDKN2A and/or other variants) is scored as 'at least high-grade dysplasia'. If genetic variants are absent, it is scored as 'no molecular support of a proliferative lesion'. In the multidisciplinary team meeting, the molecular findings are discussed in the context of the clinical and radiological findings.

## RESULTS
### NGS of FNA-derived DNA
The obtained FNA sample of the suspected recurrent PDAC was morphologically scored as atypia with no clear dysplasia or malignancy present. However, the NGS analysis revealed the presence of a class 5 KRAS:c.35G>A p.Gly12Asp pathogenic variant and a class 4 TP53 (intron, splice-site) c.376-1G>T variant in 3.2% and 3.8% of all reads, respectively. The variant calling in our analysis pipeline was confirmed by manual inspection and revealed that the variants were present in both directions. The finding of these gene variants prompted us to change our initial morphological evaluation of atypia to a molecular evaluation of at least high-grade dysplasia. Surprisingly, we also identified a 19-base pair (bp) deletion in the CDKN2A gene (exon 2; c.225_243del19, p.Ala76fs*64) in 47% of all DNA reads. The difference in frequency between this CDKN2A variant and the KRAS and TP53 variants suggested that the CDKN2A deletion was germline in origin. This particular CDKN2A deletion variant is a known germline variant present in Dutch familial atypical multiple mole melanoma families and is known as the p16-Leiden variant.
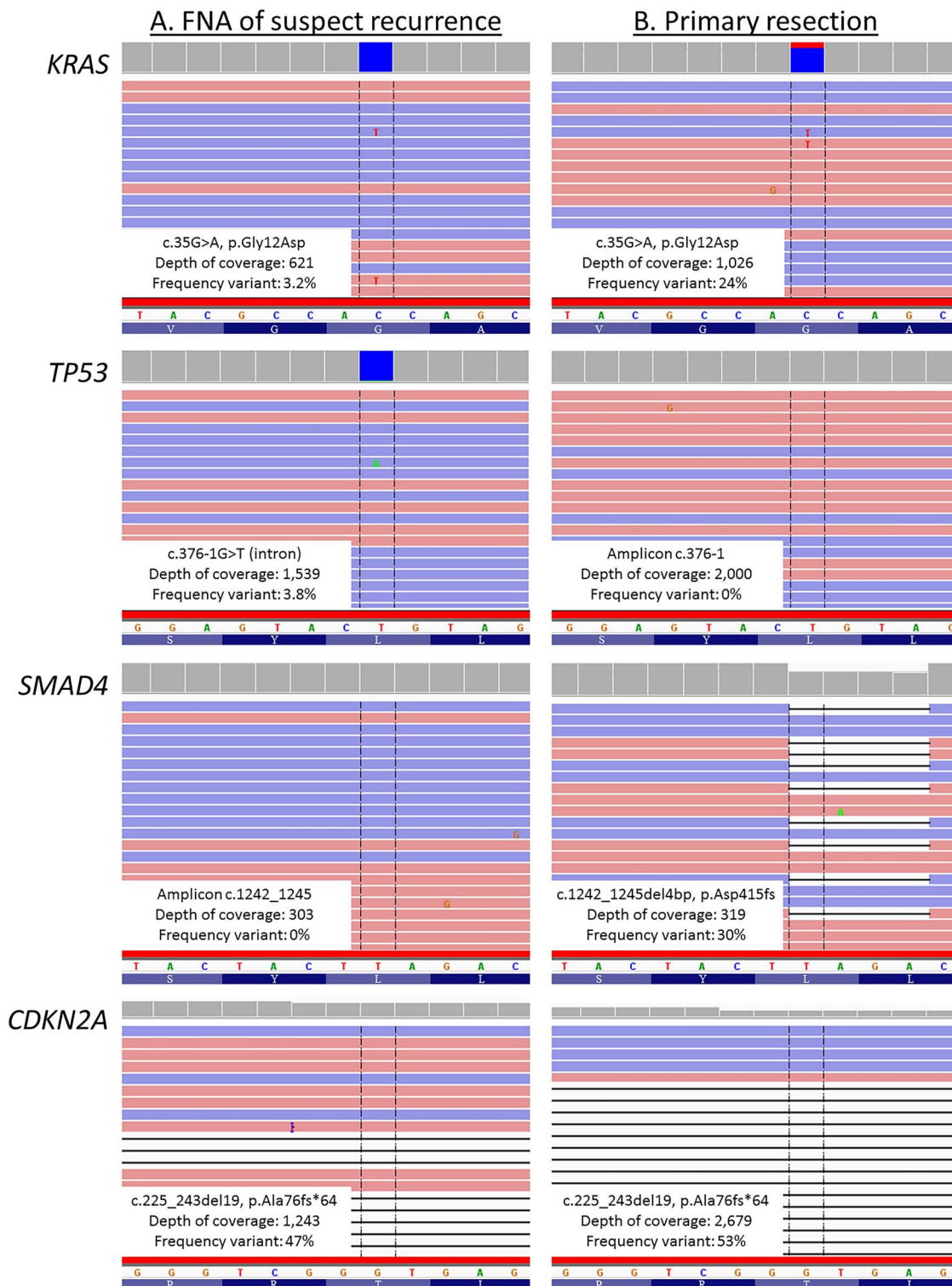
**Figure 2** Overview of the next-generation sequencing (NGS) results. The NGS analysis of DNA obtained from the endoscopic ultrasound-guided fine-needle aspiration (FNA) of the suspect pancreatic cancer recurrence (A) and the primary lesion obtained 7 years ago (B). The analysis revealed the following variants in the suspect recurrent cancer: a class 5 *KRAS*: c.35G>A p.Gly12Asp pathogenic variation was present in 3.2% of DNA reads; a class 4 *TP53* (intron, splice-site) c.376-1G>T mutation was present in 3.8% of DNA reads and a 19-bp deletion (c.225_243del19, p.Ala76fs*64) in *CDKN2A* (also known as the p16-Leiden mutation) was present in 47% of DNA reads. The following variants were identified in the primary lesion: the *KRAS* c.35G>A variant was present in 24% of the DNA reads; a class 5 *SMAD4* c.1242_1245del4bp p.Asp415fs pathogenic variant was present in 30% of DNA reads and the 19-bp *CDKN2A* deletion was present in 53% of DNA reads.

A similar NGS analysis of the primary PDAC revealed the same *KRAS:*c.35G>A pathogenic variant, but not the *TP53* variant with an on target depth of coverage of 2000 DNA reads. Furthermore, a class 5 *SMAD4* c.1242_1245del4bp

(p.Asp415fs) pathogenic variant was found in 30% of all DNA reads in the original PDAC sample, but not in the recent FNA sample with a depth of coverage of 303 DNA reads. Lastly, the presumed *CDKN2A* germline deletion variant was also found in

the original PDAC sample (figure 2). The patient was referred to the department of clinical genetics for analysis of leucocyte DNA, which confirmed the germline origin of the *CDKN2A* deletion.

Although a second primary tumour is a possibility, clonal heterogeneity of the first tumour is an important alternative. The *KRAS* c.35G>A variant, which was identified in both lesions would support this option, although it is the most commonly found *KRAS* variant in PDAC. However, due to the long interval between the two lesions (7 years), the patient's genetic predisposition and the different mutation patterns between the two lesions, we concluded that a second primary PDAC was more likely than recurrence of the original primary PDAC with molecular clonal divergence.

As a result of our analysis, the patient underwent surgical resection instead of palliative chemotherapy. Postoperative examination of the lesion revealed a 2 cm sized PDAC without lymph node metastases, extensive inflammation of the residual pancreas and focally a tumour-positive resection margin at the pancreatic-jejunal anastomosis. The presence of all gene variants in *KRAS*, *TP53* and *CDKN2A* identified in the FNA sample was later confirmed in the resected material. Again, the class 5 *SMAD4* c.1242_1245del4bp (p.Asp415fs) pathogenic variant that was identified in the primary PDAC was not found in this sample (on target depth of coverage of 534 DNA reads).

## DISCUSSION

This report illustrates that the application of an NGS panel designed for use in somatic tumour variant analysis can also identify unexpected germline variants. In the described case, a germline *CDKN2A* deletion variant (the p16-Leiden mutation) was detected by our NGS analysis. *CDKN2A* encoding for p16 is completely inactivated in PDAC by a variety of mechanisms.[12] Carriers of a germline variant in *CDKN2A* (ie, p16-Leiden) have an increased risk of developing multiple tumour types at a young age, and a cumulative lifetime risk of developing pancreatic cancer of 15%–20%.[13] Therefore, carriers of the p16-Leiden mutation are offered the opportunity to participate in a screening programme that includes annual MRI.[14] An increased prevalence of second primary tumours has been described among patients with a genetic predisposition for PDAC.[15] Therefore, total pancreatectomy should be considered in PDAC patients with a known germline variant in the *BRCA2*, *PALB2*, *CDKN2A*, *STK11*, *ATM* or *PRSS1* gene, given the significantly increased risk of developing PDAC.[15]

It has to be discussed whether and how patients should be informed about potential results of tumour NGS, because some of these findings can be beneficial for families of these patients, for example, by enrolling in effective surveillance programmes. On the other hand, identification and reporting of germline defects, such as *TP53* pathogenic variants associated with the Li-Fraumeni syndrome, is not always considered to be beneficial for patients and families involved. Molecular tumour boards and independent institutional expert review panels are currently installed in institutions worldwide to discuss such medical ethical and legal dilemmas.[16]

In an ongoing study at our institution, the NGS analysis is performed successfully in all consecutive patients with a suspicion of PDAC. Similar to the described case, this analysis can lead to a change in the treatment plan in some patients. In other patients, clinicians choose to wait for the NGS results due to inconclusive cytology and/or imaging results. In a large majority of patients, the NGS results support the cytology and imaging results (data not shown). We performed the NGS analysis using a focused gene panel targeting the mutation hotspot regions of 50 genes. For other diagnostic or therapeutic purposes, this panel could be expanded to include additional informative gene targets. However, the ability of such an expanded panel to identify low frequency gene variants in limited amounts of material is questionable. Therefore, the use of tumour cell enrichment techniques, such as microfluidic cell sorting, may increase the ability to identify gene variants and to stratify focal gene amplifications and/or deletions.[17]

Multiple pre-analytical factors may influence the success of the NGS analysis of FNA-derived DNA.[18] In our institute, the use of automated nucleic acid extraction decreased the failure rate extensively.[9]

Despite its clear advantages, the current NGS methods are time-consuming and delay the diagnostic process by at least 5 days. However, future advances in the technology will likely decrease this delay considerably. Moreover, NGS-based diagnostics are currently not covered by health insurance in many countries, making the approach potentially impractical from a strictly financial perspective.

In conclusion, we believe that FNA NGS shows great potential to detect germline pathogenic variants in addition to somatic variants in solid tumours. Furthermore, this case shows that the genomic profile of abnormalities might help in distinguishing 'de novo' tumours from metastases or recurrences. Future studies should include large patient series and additional testing of other gene panels.

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