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Detection of Somatic Mutations in Gastroenteropancreatic Neuroendocrine Tumors Using Targeted Deep Sequencing

SAMUEL BACKMAN[1], OLOV NORLÉN[1], BARBRO ERIKSSON[2], BRITT SKOGSEID[2], PETER STÅLBERG[1] and JOAKIM CRONA[1]

Department of [1]Surgical and [2]Medical Sciences, Uppsala University, Uppsala, Sweden

Abstract. *Mutations affecting the mechanistic target of rapamycin (MTOR) signalling pathway are frequent in human cancer and have been identified in up to 15% of pancreatic neuroendocrine tumours (NETs). Grade A evidence supports the efficacy of MTOR inhibition with everolimus in pancreatic NETs. Although a significant proportion of patients experience disease stabilization, only a minority will show objective tumour responses. It has been proposed that genomic mutations resulting in activation of MTOR signalling could be used to predict sensitivity to everolimus. Patients and Methods: Patients with NETs that underwent treatment with everolimus at our Institution were identified and those with available tumour tissue were selected for further analysis. Targeted next-generation sequencing (NGS) was used to re-sequence 22 genes that were selected on the basis of documented involvement in the MTOR signalling pathway or in the tumourigenesis of gastroenterpancreatic NETs. Radiological responses were documented using Response Evaluation Criteria in Solid Tumours. Results: Six patients were identified, one had a partial response and four had stable disease. Sequencing of tumour tissue resulted in a median sequence depth of 667.1 (range=404-1301) with 1-fold coverage of 95.9-96.5% and 10-fold coverage of 87.6-92.2%. A total of 494 genetic variants were discovered, four of which were identified as pathogenic. All pathogenic variants were validated using Sanger sequencing and were found exclusively in menin 1 (MEN1) and death domain associated protein (DAXX) genes. No mutations in the MTOR pathway-related genes were observed. Conclusion: Targeted NGS is a feasible method with high diagnostic yield for genetic characterization of pancreatic NETs. A potential association between mutations in NETs and response to everolimus should be investigated by future studies.*

Correspondence to: Joakim Crona, MD, Ph.D, Department of Surgical Sciences, Akademiska sjukhuset ing 70, FOA2, 75185, Uppsala, Sweden. Tel: +46 186110000, Fax: +46 186114911, e-mail: joakim.crona@surgsci.uu.se

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Aberrant activation of the mechanistic target of rapamycin (MTOR) pathway has been identified in multiple different cancer types (1) and may occur as a result from both loss of function [phosphatase and tensin homolog (*PTEN*), tuberous sclerosis 1 (*TSC1*) and *TSC2* and gain of function [phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*), *MTOR* mutations (2, 3). Inhibitors of the MTOR signalling complex (rapalogues), such as everolimus, have shown clinical benefit in a variety of cancer types, including renal and breast carcinomas, as well as neuroendocrine tumours (NETs) of pancreatic origin (4-7).

Gastroenteropancreatic NETs are relatively rare tumours, and the majority of patients show advanced disease stage upon diagnosis (8, 9). The treatment arsenal for metastatic disease includes somatostatin analogues, alkylating chemotherapy and signalling pathway inhibitors (4, 10-12). Grade A evidence supports the antitumor effect of the rapalogue everolimus in pancreatic NETs (4, 13). Although a majority of patients with pancreatic NET in the RADIANT 3 trial experienced disease stabilization, only 5% had a significant tumour reduction (4). Everolimus is also approved by the US Food and Drug Administration for treatment of non-functional lung and gastrointestinal NETs following results from the RADIANT 4 trial (14). In order to avoid administration of a less effective treatment associated with significant morbidity, efficient biomarkers capable of predicting NET sensitivity to everolimus are being sought. Recent studies *in vitro* have shown that reduced phosphorylation of the MTOR complex can predict resistance to everolimus in bronchial NETs (15). Furthermore, high-throughput sequencing studies of epithelial cancer have revealed that somatic mutations in genes associated with MTOR signalling can confer sensitivity to everolimus therapy (16-18). Iyer *et al.* sequenced the exomes of bladder carcinomas and identified TSC1 mutations exclusively in responders to everolimus therapy (16). Wagle *et al.* utilized a different approach and

sequenced tumours from exceptional rapalogue responders, revealing an activating *MTOR* mutation in a patient with metastatic urothelial carcinoma (17).

While the majority of pancreatic NETs have mutations in chromatin-modulating genes [menin 1 (*MEN1*) and death domain-associated protein (*DAXX*) and chromatin remodeler (*ATRX*) genes], about 15% also harbour mutations in genes involved in MTOR signalling (19-22). This was different from SI NETs, in which the only significantly mutated gene was cyclin-dependent kinase inhibitor 1B (*CDKN1B*), involved in cell-cycle regulation (23). There was no significant association with mutations in genes related to the MTOR pathway in small intestinal (SI) NETs (23-25). We analyzed tumour specimens from patients with NET treated with everolimus for mutations in disease-specific and MTOR pathway genes.

Patients and Methods

Patients and tissues. This was a retrospective study of patients treated at the Department of Endocrine Surgery that included biomaterial from Uppsala Biobank, Endocrine tumour collection (Ethical approval 00-128/3.15.2000). The study was approved by the local Ethical Committee (dnr no. 2011/375 and 2012/160). Written-informed consent was obtained from the individual patients. All patients were above 18 years of age at the time of inclusion. Frozen tumour tissue from patients with histopathologically confirmed NET who had been treated with everolimus (n=6) were identified. Cryosections were obtained and analysed with haematoxylin and eosin stain to confirm a tumour cell content of >50%. Characteristics of the included patients are outlined in Table I.

Response criteria. Responses to therapy were evaluated using Response Evaluation Criteria In Solid Tumours (RECIST) 1.1 Criteria. Briefly, the computed tomographic/magnetic resonance images after therapy were compared to those at baseline and rated as: complete response, with a disappearance of all lesions; partial response if the longest diameter of index lesions decreased by ≥30%; progressive disease if the longest diameter of target lesions increased by ≥20%; and stable disease if the sum of index lesions was between these parameters (26).

DNA extraction. Genomic DNA was extracted from fresh frozen tissue samples using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) as previously described (27). DNA quality and concentrations were assessed using a Nanodrop spectrophotometer (ThermoFischer Scientific, Waltham, MA, USA) as well as a Qubit flourometer (Invitrogen, Carlsbad, CA, USA). Inclusion criteria for library preparation were a 260/280nm spectrum ratio of >1.8 and double-stranded DNA concentration above 5 ng/μl.

Targeted genomic capture. A Truseq Custom Amplicon (Illumina Inc, San Diego, CA, USA) targeted capture and paired end library kit was designed using Illumina Design Studio (Version 2012-12-01; http://designstudio.illumina.com). Targeted genes were selected on the basis of documented involvement in (i) gastroenteropancreatic NET tumourigenesis or (ii) AKT-MTOR signalling pathway. In total 22 genes were included: *MTOR*, GTPase NRas (*NRAS*; exons 2, 3 and 4), endothelial PAS domain protein 1 (*EPAS1*), von Hippel-Lindau disease tumour suppressor (*VHL*), *PIK3CA* (codons 545, 1047), catenin beta 1 (*CTNNB1*; exons 3 and 5), KIT proto-oncogene receptor tyrosine kinase (*KIT*; exons 9, 11, 13, 17), rapamycin-insensitive companion of MTOR (*RICTOR*), fibroblast growth factor receptor 4 (*FGFR4*; codon 388), *DAXX*, serine/threonine-protein kinase B-raf (*BRAF*; codon 600), epidermal growth factor receptor (*EGFR*; codons 18-21), *TSC1, PTEN, MEN1,* GTPase HRAS (*HRAS*; (exons 2-4), GTPase KRAS (*KRAS*; exons 2-4), RAC-alpha serine/threonine-protein kinase (*AKT1*; codon 17), transcriptional repressor protein YY1 (*YY1*; codon 372), *TSC2*, regulatory-associated protein of MTOR complex 1 (*RPTOR*) and cellular tumour antigen p53 (*TP53*). Full details are presented in Table II. In order to be able to detect variants causing alternative splicing in tumour-suppressor genes, coordinates were extended with a padding of 10 base pairs at intron–exon boundaries. Coordinates were obtained from the human reference sequence HG19 and the cumulative target size was 52040 base pairs. The final TruSeq Custom Amplicon design constituted 672 amplicons having a median size of 175 bases. The *in silico* amplicon coverage was >98% with a total gap distance of 827 bases located in regions with homologous sequences: *RPTOR* exon 6, *MEN1* exon 2 and *TP53* exon 6.

Library preparation and MiSEQ sequencing. Library preparation and sequencing was performed at the university core facility (http://molmed.medsci.uu.se/SNP+SEQ+Technology+Platform/) as detailed in the MiSEQ System user guide for Reagent Kit v2. Targeted enrichment and library preparation were performed from 250 ng of double-stranded DNA (Illumina Inc.) according to the manufacturer's instructions. Briefly, upstream and downstream oligonucleotides were hybridized to genomic DNA followed by an extension-ligation process that connected the hybridized upstream and downstream oligonucleotides using a DNA ligase. Extension-ligation fragments were amplified by polymerase chain reaction (PCR) and connected to index adaptor sequences to allow for sample multiplexing using the TruSeq Custom Amplicon Index Kit (Illumina Inc.). The PCR product was purified using AMPure XP beads (Beckman Coulter Inc, Carlsbad, CA, USA). To confirm successful library preparation selected test samples were separated and visualized on a 4% agarose gel. Each library sample underwent quantity normalization and all six samples were pooled together with 88 samples of other origin into a single suspension. The generated paired end libraries were subjected to a single sequencing using an Illumina MiSEQ instrument (Illumina Inc.). Generated sequences were demultiplexed by Illumina MiSEQ reporter 2.1.43 software and the results written to FASTQ files.

Bioinformatic analysis. A manifest file detailing the sequence of the hybridizing oligonucleotides as well as the coordinates of designed amplicons was downloaded from the manufacturer and uploaded into the MiSEQ reporter 2.1.43 software as instructed. Briefly, the sequencing reads were aligned to the reference sequence (human reference sequence HG19) by the Smith-Waterman algorithm using default settings. Variant calling was performed using a variant caller from the Genome Analysis Toolkit using default settings (28). The generated .BAM files were imported into CLC Genomics Workbench 5.51 (Qiagen, Aarhus, Denmark) and quality control analysis was performed. The generated .VCF files were filtered and annotated in CLC Genomics Workbench, synonymous variants without a probable splice site

Table I. *Clinical characteristics of included patients.*

Patient number	Gender	Age at diagnosis, years	Histopatho-logical diagnosis	Ki67+, %	Staging*	Genetic syndrome	Sample localization	Duration of everolimus, months	Discontinued due to	Best response
1	Male	40	PNET	4	IV	No	Primary tumour	14	Side-effects	Stable disease
2	Female	40	PNET	1	IV	No	Lymph node metastasis	8	Progressive disease	Partial response
3	Male	43	PNET		IV	No	Liver metastasis	83 (ongoing)	-	Stable disease
4	Male	46	SINET	2	IV	No	Omental metastasis	3	Side-effects	Stable disease
5	Male	71	SINET	1	IV	No	Primary tumour	10	Side-effects	Stable disease
6	Female	59	SINET	1	IV	No	Liver metastasis	3	Progressive disease	Progressive disease

PNET, Pancreatic neuroendocrine tumour; SINET, small intestinal neuroendocrine tumour. *According to European Neuroendocrine Tumor Society criteria (44, 45).

Table II. *Genes included for DNA enrichment. For particular genes with documented hotspot codons, only particular segments were targeted.*

Gene	Encoded protein	Chromosome	Exons, n	Amplicons, n	Selected fragments
MTOR	Serine/threonine-protein kinase MTOR	1	58	123	
NRAS	GTPase NRas	1	3	5	Codons 12, 13, 61
EPAS1	Endothelial PAS domain-containing protein 1	2	16	37	
VHL	von Hippel-Lindau disease tumor suppressor	3	3	10	
PIK3CA	Phosphatidylinositol-4,5-*bis*phosphate 3-kinase catalytic subunit alpha isoform	3	2	5	Codons 545, 1047
CTNNB1	Catenin beta-1	3	2	6	Exons 3 & 5
KIT	KIT proto-oncogene receptor tyrosine kinase	4	2	8	Exons 9, 11, 13, 17
RICTOR	Rapamycin-insensitive companion of mTOR	5	39	82	
FGFR4	Fibroblast growth factor receptor 4	5	1	3	Codon 388
DAXX	Death domain-associated protein 6	6	8	34	
BRAF	Serine/threonine-protein kinase B-raf	7	1	1	Codon 600
EGFR	Epidermal growth factor receptor	7	4	13	Exons 18, 19, 20, 21
TSC1	Tuberous sclerosis 1 (hamartin)	9	23	53	
PTEN	Phosphatidylinositol 3,4,5-*tris*phosphate 3-phosphatase and dual-specificity protein phosphatase PTEN	10	9	61	
MEN1	Menin	11	10	35	
HRAS	GTPase HRas proto-oncogene	11	3	8	Codons 12, 13, 61
KRAS	GTPase KRas proto-oncogene	12	3	5	Codons 12, 13, 61
AKT1	RAC-alpha serine/threonine-protein kinase	14	1	2	Codon 17
YY1	Transcriptional repressor protein YY1	14	1	2	Codon 372
TSC2	Tuberous sclerosis 2 (tuberin)	16	42	91	
RPTOR	Regulatory-associated protein of MTOR complex 1	17	34	63	
TP53	Cellular tumour antigen p53	17	11	25	

effect were removed and the remaining variants were annotated for overlapping information in publicly available databases; Single Nucleotide Polymorphism database (dbSNP) build 137, Catalogue of Somatic Mutations in Cancer (29), database of Human Gene Mutation Data (30) and Leiden Open (source) Variant Database. The impact of non-synonymous amino acid substitution was assessed *in silico* using Polymorphism Phenotyping v2 (31) and Sorting Intolerant from Tolerant (32). Variant annotations were integrated to classify variants as pathogenic, variant of unknown significance (VUS) or polymorphism and selected entries were validated with Sanger sequencing. Primer sequences can be obtained upon request.

Sanger sequencing. Fragments corresponding to pathogenic variants discovered by NGS were amplified by PCR and sequenced using Sanger sequencing. *CDKN1B* had previously been re-sequenced in the included SI NETs without finding any pathogenic variants (33).

Results

Cohort characteristics. Samples from six patients with well-differentiated NETs were included; three originated from the small intestine and three from the pancreas. All patients had NET stage 4 at diagnosis and those with available Ki67

Table III. *Read mapping. Quantity of generated reads as well as general and specific read coverage.*

Patient number	No. of sequence reads	Specificity to targeted regions, %	Mean read coverage	Fold coverage at targeted regions,%			
				1×	10×	40×	80×
1	845998	67.18	667.1	96.54	95.06	93.04	91.93
2	809662	65.2	612.4	96.32	95.09	93.38	91.66
3	848380	68	666.5	96.54	95.46	93.04	90.62
4	950698	72.08	803.4	96.53	94.84	91.6	87.64
5	873216	68.37	697.2	95.93	95.34	92.47	90.03
6	1604126	67.15	1269.7	96.06	95.44	94.82	92.16

Table IV. *Variant calling. Generated variants and results remaining after filtering.*

Patient number	Discovered variants	SNV	INDEL	#1 Targeted genes	Variants remaining after filtering steps			
					#2 Significant mutations	#3 Discard common SNPs	#4 Quality filter	#5 Redundancy filter
1	85	65	20	80	8	5	3	3
2	92	67	25	86	11	7	2	2
3	83	57	26	77	6	2	1	1
4	79	59	20	72	8	3	1	1
5	83	61	22	76	6	2	1	1
6	72	53	19	66	6	2	2	2

SNV: Single nucleotide variants; INDEL: insertion or deletion; SNP: single nucleotide polymorphism.

measurements had been classified as World Health Organisation grade 1 or 2. The included specimens had been sampled from primary tumours (n=2) or distant metastases (n=4).

Response to everolimus. Time on therapy ranged from 3 to 83 months. Three of the patients discontinued their therapy due to side-effects and two due to disease progression (Table I). One patient showed a partial response and four had stable disease. Patient 3 experienced durable disease stabilization that is ongoing at 83 months.

Sequence coverage. A median of 860798 (range 809662 to 1604126) paired end reads were generated. Sequence read mapping showed a median specificity of 67.6% (range 65.2-72,1%) to the bases targeted by enrichment. The median coverage at the targeted bases was 667.1 (range=404-1301). A total of 95.9-96.6% of bases had a coverage of at least 1-fold, while 94.8-95.5% had a coverage of at least 10-fold and 87.6-92.2% of bases had a coverage of at least 80-fold. Detailed sequencing statistics are presented in Table III.

Discovered genetic variants. Variant calling generated a total of 494 genetic variants; there were 362 single nucleotide variants and 132 insertions/deletions (Table IV). In order to identify possibly pathogenic variants, a series of stringent filtering steps were applied. After filtering a total of four variants were identified as pathogenic and six were classified as VUS. All pathogenic variants were successfully validated by Sanger sequencing (Figure 1). Pathogenic variants were detected exclusively in NETs of pancreatic origin: *MEN1* c.1724_1735delTCAACTCGAGCG, p.Ile575_Ala579delinsThr in patient 1, *MEN1* c.1486G>T, p.Glu496* in patient 2 (Figure 2). In the tumour of patient 3, there were two pathogenic mutations detected: *MEN1* c.1189G>T, p.Glu397* and *DAXX* c.1622_1623insT, p.Ser541fs. Due to the absence of DNA from non-tumoural tissues, we were unable to determine whether these variants were somatic or occurred in the germline.

Performance and throughput time. Following optimization of the bioinformatic workflow, the theoretical throughput time for the executed assays was 7 days. This was divided to 1 day for sample preparation and quality assessment, 2 days for sample enrichment and multiplexing, 1 day for MiSEQ sequencing, as well as 1 day for bioinformatics processing and interpretation. Validation of the generated results with Sanger sequencing may be estimated to require an additional 7-14 days depending on the complexity of the

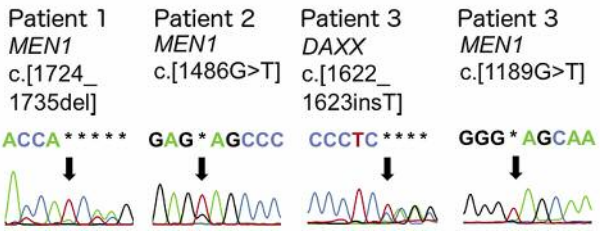

Figure 1. *Chromatograms from validation of the detected pathogenic mutations.*

specific fragment. The total reagent cost of the executed assays including quality control, library preparation, multiplexing and sequencing was 84 USD per sample. This excluded the non-recurring costs of hardware and software.

Discussion

We report successful validation of a targeted deep sequencing workflow for the sequencing of genes associated with pancreatic NET pathogenesis and mTOR signalling. The method showed a high diagnostic yield and was able to validate previous mutational findings observed in PNETs. Since we were unable to detect any clinically actionable mutations, the limited material does not allow us to draw any conclusion regarding the potential impact of such screening in clinical practice.

Theoretically, a relatively high sequence depth should improve detection of genetic variants with low allelic frequency. Our samples had 92-95% of the targeted regions covered by at least 80 sequencing reads, theoretically this should allow for near-perfect sensitivity for genetic variants with low allelic frequency (0.2) (34). A similar pipeline for library preparation, sequencing and bioinformatic processing was previously validated as having near-perfect sensitivity in NETs of other origin, further supporting the robustness of the results here (35). This is in line with the current literature maintaining that targeted NGS has a higher yield and lower cost than traditional methods (35-37). Although targeted NGS has been shown to be an effective method for re-sequencing of formalin-fixed paraffin-embedded tissues, no such conclusions can be drawn from this study as it exclusively analysed frozen tumour material.

Accumulating evidence supports the use of mutations within MTOR pathway genes in order to predict response to MTOR inhibitors (17, 18, 38-40). Everolimus is an oral allosteric inhibitor of MTOR approved for use in patients with pancreatic NETs. Although disease stabilization was achieved in a high proportion of patients with progressive disease in the RADIANT 3 trial, radiological response was seen only in a minority of patients (4). Furthermore, a large proportion of

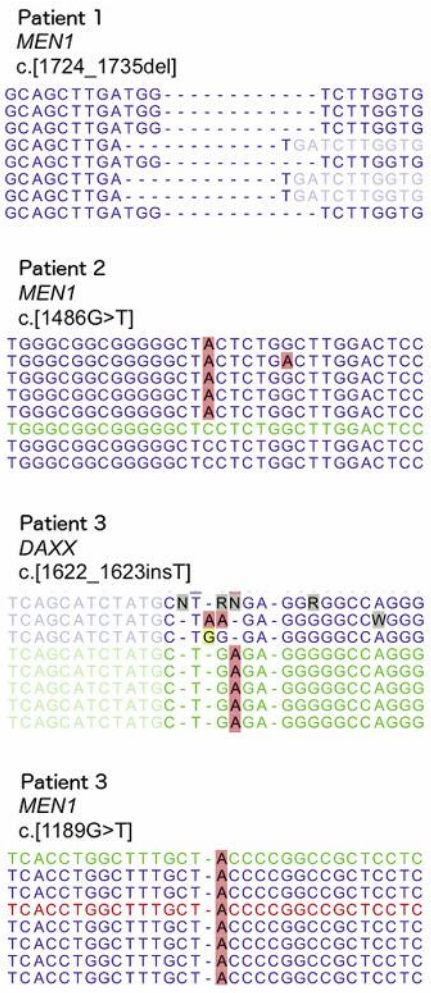

Figure 2. *Representative screenshots from CLC Genomics Workbench 5.01 revealing read mappings at the corresponding locations of the pathogenic mutations.*

patients experienced severe toxicities. In order to identify those with a high likelihood of clinical response to rapalogue treatment, clinical and molecular biomarkers are needed.

We were able to detect pathogenic *DAXX* and *MEN1* mutations in NETs of pancreatic origin. There were no pathogenic mutations discovered in the analyζed SI NET tumours. Subsequent to the design of this study, large genome-wide sequencing studies have revealed that SI NETs lack mutations in the loci that were targeted by this study (23, 25).

Only two patients in this study showed a satisfactory response, with one partial response and one durable disease stabilization, similar to the real-life experience of everolimus treatment in NETs. There were no mutations in MTOR pathway genes. Larger studies such as SEQTOR (NCT02246127) may provide more definitive answers to whether mutations in MTOR pathway genes could have a predictive role in patients with

pancreatic NETs. In patient 3, who has ongoing disease stabilization of 83 months, as well as a decrease in glucagon secretion with consequent relief of related symptoms, targeted NGS detected mutations within the *MEN1* and *DAXX* genes. Mutations in *DAXX* have been shown to cause an alternative lengthening of telomeres (ALT) phenotype (19, 20) enabling replicative immortality. It has been shown that ALT mutations render sensitivity to ATR inhibitors (41). Whether ALT-deficient tumours are particularly sensitive to MTOR inhibition would of interest to investigate in further studies.

It has been demonstrated that NETs may exhibit both inter- and intratumoural genetic heterogeneity, at both the level of single nucleotide variations (33) and copy number variations (42). Similarly to the current clinical practice, we investigated one sample per patient. Consequently genetic heterogeneity could be a source of error for this study, as is the case for clinical testing in general. One approach to overcoming this may be to sequence DNA derived from plasma in order to find variants of prognostic importance in circulating tumour DNA (43).

To conclude, this study shows that targeted NGS is a feasible method for genomic characterization of pancreatic NETs. There were no mutations in mTOR pathway-related genes, neither in the patient with a partial response, nor in those with stable disease. Given the low patient number (n=6) with mixed radiological responses, our material does not allow us to draw any further conclusions regarding a potential association between everolimus response and somatic mutations in NETs.

Conflicts of Interest

The Authors declare no conflicts of interest

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