Published in final edited form as:

Mod Pathol. 2018 October ; 31(10): 1532–1538. doi:10.1038/s41379-018-0082-y.

Whole exome sequencing of duodenal neuroendocrine tumors in patients with neurofibromatosis type 1

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Abstract

Neurofibromatosis type 1 (NF1) is a hereditary cancer predisposition syndrome characterized by frequent cutaneous and nervous system abnormalities. Patients with NF1 also have an increased prevalence of multiple gastrointestinal and peripancreatic neoplasms – neuroendocrine tumors of the ampulla that express somatostatin are particularly characteristic of NF1. In this study, we characterize the genetic alterations of a clinically well-characterized cohort of six NF1-associated duodenal neuroendocrine tumors using whole exome sequencing. We identified inactivating somatic mutations in the *NF1* gene in three of six tumors; the only other gene altered in more than one tumor was *IFNB1*. Copy number analysis revealed deletion/loss of heterozygosity of chromosome 22 in three of six patients. Analysis of germline variants revealed germline deleterious *NF1* variants in four of six patients, as well as deleterious variants in other tumor suppressor genes in two of four patients with deleterious *NF1* variants. Taken together, these data confirm the importance of somatic inactivation of the wild-type *NF1* allele in the formation of NF1-associated duodenal neuroendocrine tumors and suggest that loss of chromosome 22 is

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Conflict of interest

LDW is a paid consultant for Personal Genome Diagnostics. The other authors report no conflict of interest.

important in at least a subset of cases. However, we did not identify any genes altered in the majority of NF1-associated duodenal neuroendocrine tumors that uniquely characterize the genomic landscape of this tumor. Still, the genetic alterations in these tumors are distinct from sporadic neuroendocrine tumors occurring at these sites, highlighting that unique genetic alterations drive syndromic tumors.

Introduction

Hereditary cancer predisposition syndromes, in which germline alterations lead to an increased risk of neoplasia, represent a unique opportunity to study tumorigenesis, and the results of these studies can have important clinical implications.

Neurofibromatosis type 1 (NF1), also known as von Recklinghausen disease, is an autosomal dominant hereditary cancer predisposition syndrome caused by inactivating germline alterations in the *NF1* gene encoding neurofibromin on chromosome 17q (1). This syndrome affects approximately 1 in 3000 individuals and is characterized by a constellation of clinical findings, including café-au-lait spots, axillary freckling, optic gliomas, and neurofibromas, a small proportion of which can transform into malignant peripheral nerve sheath tumors (1).

Although less common than the cutaneous and nervous system abnormalities, several gastrointestinal and peripancreatic neoplasms also occur at increased prevalence in patients with NF1, including gastrointestinal stromal tumors and neuroendocrine tumors of the duodenum and ampulla (2). Intriguingly, the majority of these neuroendocrine tumors typically have unique morphological features, including prominent psammomatous calcifications, and express the hormone somatostatin (2). Previous genomic analysis of NF1-associated malignant peripheral nerve sheath tumors has revealed critical drivers of malignant transformation in the *SUZ12* gene in these tumors (3). However, little is known about the genetic alterations that drive the formation of gastrointestinal and peripancreatic tumors in NF1 patients.

Multiple lines of evidence suggest that functional and syndromic neuroendocrine tumors will harbor genomic alterations distinct from those in sporadic non-functional neuroendocrine tumors that have previously been characterized in depth (4–6). Whole exome sequencing of a specific clinicopathological group of pancreatic neuroendocrine tumors, sporadic insulinomas, revealed a unique frequently altered hotpsot mutation, suggesting that specific clinical and pathological categories of neuroendocrine tumors are driven by distinct genetic alterations (7). In addition, previous studies have demonstrated that gastrointestinal stromal tumors in NF1 patients are genetically distinct from sporadic gastrointestinal stromal tumors (8). As such, we hypothesize that NF1-associated duodenal neuroendocrine tumors may be a genetically unique category of neuroendocrine tumors. In order to characterize the genomic landscape of these neoplasms in NF1 patients, we performed whole exome sequencing on a clinically well-characterized cohort of six neoplasms.

Materials and Methods

This study (including the analysis of germline DNA) was approved by the Institutional Review Boards of The Johns Hopkins Hospital and Emory University Hospital. The surgical pathology databases of The Johns Hopkins Hospital and Emory University Hospital were searched for surgically resected neuroendocrine tumors of the ampulla with psammomatous calcifications or immunohistochemical reactivity for somatostatin in patients clinically diagnosed NF1. From tumor samples SOM3-6, formalin-fixed paraffin-embedded tissue blocks of tumor and matched normal were reviewed by a pathologist and macrodissected to enhance neoplastic cellularity. Cores of neoplastic and normal tissue were isolated from formalin-fixed paraffin-embedded blocks using a 0.6mm needle. For tumor samples SOM7-8, fresh-frozen tumor and normal (harvested at the time of surgery and subsequently banked at −70°C) were reviewed by a pathologist and macrodissected after frozen section analysis to enhance neoplastic cellularity. DNA was extracted using the QIAamp DNA FFPE tissue kit (Qiagen) or QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions and quantified using the Qubit 2.0 (Thermo Fisher).

Whole exome sequencing was performed at Personal Genome Diagnostics with capture using the Agilent SureSelect paired end version 4.0 library preparation followed by sequencing on an Illumina HiSeq, as previously described (9). Somatic mutations were identified using the VariantDx pipeline as previously described (9). In order to identify copy number alterations, we utilized the ASCAT software (10). Specifically, we identified approximately 40,000 single nucleotide polymorphisms in the target region with high global minor allele frequency – the normalized number of reads along with the estimated B allele frequency was then used as input for ASCAT. However, due to high dispersion in local sequencing coverage, most of the samples could not be reliably modelled with ASCAT. Therefore, the plots representing normalized read depth and B allele frequency along the chromosomes were visually inspected to identify large chromosomal alterations based on lower number of reads and absence of the band corresponding to heterozygous single nucleotide polymorphisms in the B allele frequency plot. In addition, we performed complimentary copy number analysis with CNVkit to utilize both the on-target and off-target reads (11). Scatterplots were created with CNVkit and visually inspected for each chromosome, and copy number status of tumors were compared to the matched normal sample from the same patient. Deleterious germline variants were identified after alignment of sequence read files to the human genome (hg19) with Burrows-Wheeler Aligner and variant calling with Varscan 2 (12, 13). Classification of variants as either benign, of unknown significance, or deleterious was performed as previously described (14).

Fluorescence in situ hybridization was performed on formalin-fixed paraffin-embedded specimens using a dual color probe (Abbott Molecular). The probe is designed for the detection of chromosomal region 9q34.12 harboring the *ABL1* gene (orange fluorochrome) and the chromosomal region 22q11.23, harboring the *BCR* gene (green fluorochrome). Prior to hybridization, formalin-fixed paraffin-embedded slides were deparaffinized using a VP2000 processor (Abbott Molecular). The slides and the probe were co-denatured at 75°C for 5 minutes and allowed to anneal over night at 37°C, in humidified atmosphere. Following incubation the slides were washed in 2 X SSC/0.3% NP-40 for 2 minutes at 72°C,

and for 2 minutes at room temperature in 2X SSC. The slides were counterstained with DAPI and a cover slip was applied using Vectashield mounting medium (H-1000, Vector Laboratories, Inc.). A fluorescence microscope was used to evaluate signal patterns generated by each probe. In normal interphase cells, the signal pattern showed two orange signals (two copies of *ABL1* gene) and two green signals (two copies of *BCR* gene). Samples showing greater than 10% of the 50 analyzed cells with less than or more than two signals are interpreted as loss or gain, respectively, for the targeted chromosome locus.

Results

Clinical and pathological data

We identified six duodenal neuroendocrine tumors occurring in patients who were clinically diagnosed with NF1. Microscopic evaluation of these tumors showed the typical morphological pattern of homogeneous neoplastic cells with abundant clear or granular eosinophilic cytoplasm and round, smooth nuclei with finely stippled chromatic. The neoplastic cells were organized in nests, trabeculae, and acinar structures, often with prominent interspersed psammomatous calcifications (Figure 1). Clinical and pathological features are summarized in Table 1. All the tumors originated in the duodenum or around the major or minor duodenal papilla. The mean age at time of resection was 52 (range 40–61), and 67% of the patients were male. The mean size of the primary tumor was 2.6 cm, and 50% had lymph node metastases at the time of surgery. When performed, Ki67 proliferation rate was <2% in all cases, consistent with WHO Grade 1 based on current classifications (15, 16). Immunolabeling for somatostatin was positive in four cases, and none had immunolabeling for other commonly expressed pancreatic hormones such as insulin and glucagon. One case was negative for somatostatin labeling, but this case had characteristic glandular psammomatous morphology, as did another tumor in which somatostatin immunohistochemistry was not available. All patients had other histological features suggestive of NF1, including duodenal neurofibromas, duodenal gastrointestinal stromal tumor, and neurofibromatosis-associated vasculopathy (17).

Whole exome sequencing

Whole exome sequencing of six NF1-associated duodenal neuroendocrine tumors revealed a range of 2–11 nonsynonymous somatic mutations per tumor (Table 2). Only two genes were mutated in more than one tumor: inactivating mutations (two frameshift and one nonsense) in *NF1* occurred in three tumors, and missense mutations in *IFNB1* occurred in 2 tumors. In addition, somatic mutation in the oncogenic hotspot in codon 600 in *BRAF* was identified in a single tumor.

We also utilized the whole exome sequencing data to identify copy number alterations using two complimentary approaches – ASCAT (10), which analyzed the B allele frequency of germline single nucleotide polymorphisms in the target region, and CNVkit (11), which analyzed copy number based on read depth in the on-target and off-target reads. Both of these approaches revealed deletion/loss of heterozygosity of chromosome 22 in three tumors (Supplementary Figure 1). Key somatic alterations in each tumor are summarized in Table 3.

In addition to the identification of somatic genetic events in NF1-associated duodenal neuroendocrine tumors, we also analyzed the germline sequencing data for deleterious variants in human cancer genes (Table 4). We identified three frameshift germline variants and one nonsense germline variant in *NF1*; four patients had deleterious germline variants in *NF1*. In addition, two of these four patients also had deleterious germline variants in other known cancer genes, including a frameshift deletion in *FANCC* and a splice site mutation in *TSC1*, each in one patient.

Fluorescence in situ hybridization

In order to confirm the result of our copy number analysis utilizing whole exome sequencing data, we performed fluorescence *in situ* hybridization for chromosomes 9 and 22 on neoplastic formalin-fixed paraffin-embedded tissue from all six tumors. We identified loss of chromosome 22 by fluorescence *in situ* hybridization in the three tumors with whole exome sequencing data suggestive of deletion/loss of heterozygosity, while the other three tumors retained two copies of both chromosomes 9 and 22 (Figure 2).

Discussion

We report whole exome sequencing of a clinically well-characterized cohort of duodenal neuroendocrine tumors arising in patients with NF1. In the literature, these tumors are often described as ‘somatostatinomas’ due to their characteristic expression of somatostatin by immunohistochemistry, though clinical symptoms caused by somatostatin expression are exceedingly rare. Other features often seen in neuroendocrine tumors in patients with NF1 are glandular morphology and presence of psammoma bodies. There is substantial overlap between neuroendocrine tumors with somatostatin expression, glandular neuroendocrine tumors with psammoma bodies, and neuroendocrine tumors associated with NF1 (1, 18). As such, these lesions have been previously called “ampullary somatostatinoma” and “glandular duodenal neuroendocrine tumor”. However, in this study, we use the general term “duodenal neuroendocrine tumor”, as not all analyzed lesions expressed somatostatin or showed glandular morphology. The expression of other pancreatic hormones, including gastrin and serotonin, was not analyzed in this study though could be of interest for future studies describing these lesions at the protein level. Of note, sporadic somatostatinomas can show focal expression for gastrin or serotonin (19).

The most common intragenic somatic alteration in this tumor type was inactivating mutation in *NF1* (either by frameshift or nonsense mutation), which occurred in three tumors. In addition to these small somatic mutations, we also identified somatic deletion/loss of heterozygosity of chromosome 22 in three tumors in our whole exome sequencing data and confirmed these deletions by fluorescence *in situ* hybridization. Because the deletion includes the entire chromosome, it is not possible to identify the targeted driver gene. However, multiple well characterized tumor suppressor genes are located on chromosome 22, including *NF2* and *SMARCB1*, which lead to neurofibromatosis type 2 and schwannomatosis, respectively (20–22). Still, the lack of somatic mutations in these tumor suppressor genes raises the alternative possibility that other types of alterations, such as

epigenetic modifications or alterations in non-coding regions, may complement the deletion/loss of heterozygosity of chromosome 22.

Overall, the somatic mutations identified in duodenal neuroendocrine tumors in NF1 patients are unique from those identified in sporadic pancreatic neuroendocrine tumors well as sporadic small intestinal neuroendocrine tumors (4–6) – mutations in *NF1* and *IFNB1* do not occur commonly in these other tumor types, nor does loss of chromosome 22. In addition, somatic mutations in genes frequently altered in pancreatic neuroendocrine tumors (*MEN1, ATRX, DAXX*) and small intestinal neuroendocrine tumors (*CDKN1B*) were absent in our cohort. However, to our knowledge, no study has specifically analyzed sporadic duodenal neuroendocrine tumors, which would be the most appropriate comparison for our lesions. Analysis of these sporadic lesions, with subsequent comparison to the genetic alterations in syndromic lesions, remains a future direction.

The genomic landscapes of NF1-associated neoplasms reported in the literature to date is variable. The mutations in the duodenal neuroendocrine tumors in our cohort are distinct from those previously reported in malignant peripheral nerve sheath tumors, which is one of most comprehensively characterized NF1-associated neoplasms. Although the somatic "second hit" in *NF1* has been reported in NF1-associated malignant peripheral nerve sheath tumors, our tumors lacked mutations in *SUZ12, EED,* and *CDKN2A* that frequently occur in this tumor type (3, 23). However, deletions of *IFNB1* (the only gene other than *NF1* with somatic mutations in more than one tumor in our cohort) have been reported in 20% of malignant peripheral nerve sheath tumors, suggesting perhaps a common genetic driver among NF1-associated neoplasms (24). Overall, the alterations in NF1-associated duodenal neuroendocrine tumors are more similar to those reported in plexiform neurofibromas in NF1 patients – whole exome sequencing has previously revealed only a small number of somatic mutations in these benign NF1-associated neoplasms, with somatic mutations in *NF1* as the most common alteration (25). Similarly, whole genome sequencing analysis of a small number of NF1-associated pilocytic astrocytomas revealed that somatic inactivation of *NF1* was the only recurrent genetic alteration (26). Of note, loss of chromosome 22 has not been previously reported in NF1-associated neoplasms.

In three of the six tumors, we did not identify somatic *NF1* mutations. In these cases, deletion/loss of heterozygosity of the wild-type *NF1* allele may not have been accurately identified by our approach. Although our copy number analysis could robustly identify arm-level chromosomal alterations (such as deletion/loss of heterozygosity of chromosome 22), it is possible that our analysis missed focal deletions of the wild-type copy of the gene in these cases due to sequencing approach and coverage depth. Such focal deletions have been previously reported as a mechanism for somatic *NF1* inactivation (3). Alternatively, methylation has been reported as an alternative mechanism for inactivation of the second *NF1* allele, which would not have been identified by our whole exome sequencing approach (26). Although immunohistochemical detection of the neurofibromin protein encoded by *NF1* is another possible assay to interrogate this gene, immunohistochemistry has not been shown to be a reliable predictor of genetic and epigenetic alterations in *NF1* (27, 28). Thus, we did not use neurofibromin protein expression as a surrogate for *NF1* gene status in our study.

Germline deleterious *NF1* variants were identified in four of the six patients. In two of the four patients with a germline *NF1* mutation we were able to demonstrate bi-allelic inactivation of this critical tumor suppressor gene with a second somatic hit to the gene. Of interest, we identified deleterious germline variants in other known familial cancer genes, including a frameshift deletion in *FANCC* and a splice site mutation in *TSC1* – both of these occurred in patients who also had germline deleterious variants in *NF1*. While this could be chance, it could be that the combination of two germline changes increases the penetrance of the phenotype. This phenomenon has been demonstrated on other hereditary diseases: for example, previous data have demonstrated that patients with the clinical finding of familial pancreatitis often have two distinct germline events that together contribute to the phenotype (29, 30). In two of the six cases, we did not identify a disease-causing germline *NF1* variant, as the remainder of the germline variants identified in the coding region of this gene were synonymous. However, the clinical history of NF1 in each patient in our cohort is well documented, and previous studies have highlighted the difficulties in identifying germline *NF1* mutations, as numerous molecular approaches are necessary for comprehensive variant identification (31). Thus, our whole exome sequencing approach was likely not sensitive enough to detect all the disease-causing germline alterations in our cohort.

Taken together, our data reveal the importance of somatic *NF1* mutation and chromosome 22 loss in duodenal neuroendocrine tumors in patients with NF1, highlighting the unique genomic landscape of this syndromic neoplasm.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors acknowledge the following sources of support:

NIH/NCI P50 CA62924; NIH/NIDDK K08 DK107781; Sol Goldman Pancreatic Cancer Research Center; Buffone Family Gastrointestinal Cancer Research Fund; Kaya Tuncer Career Development Award in Gastrointestinal Cancer Prevention; AGA-Bernard Lee Schwartz Foundation Research Scholar Award in Pancreatic Cancer; Sidney Kimmel Foundation for Cancer Research Kimmel Scholar Award; AACR-Incyte Corporation Career Development Award for Pancreatic Cancer Research; Rolfe Pancreatic Cancer Foundation; Joseph C Monastra Foundation; The Gerald O Mann Charitable Foundation (Harriet and Allan Wulfstat, Trustees); Lisa Waller Hayes Foundation; Nijbakker Morra Foundation; Dutch Digestive Foundation (CDG 14-02); German Research Foundation (DFG); Associazione Italiana Ricerca Cancro (grant number: 12182); FP7 European Community Grant Cam-Pac (no: 602783); Italian Ministry of University (FIRB RBAP10AHJB); NIH/NCI R00 CA190889.

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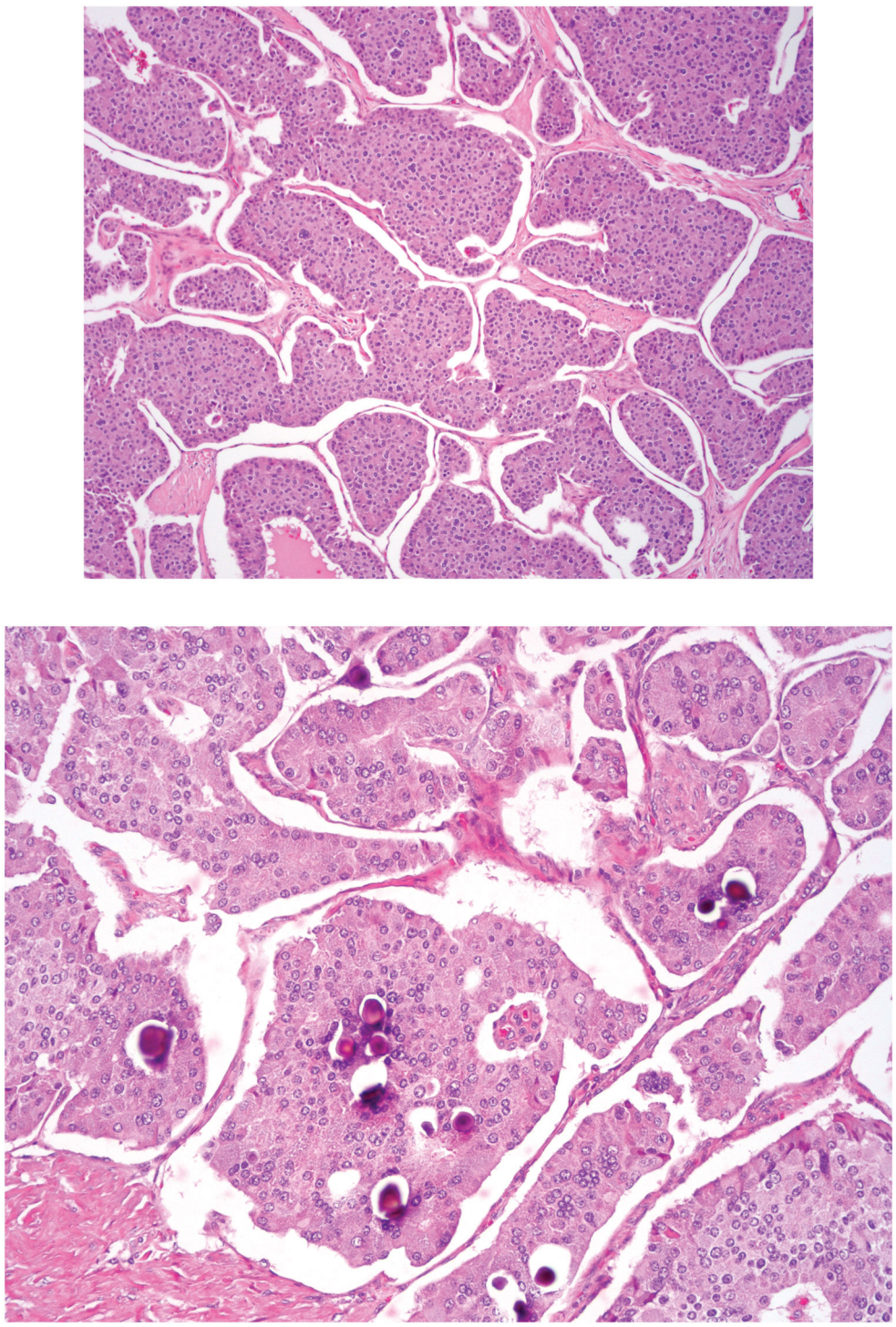
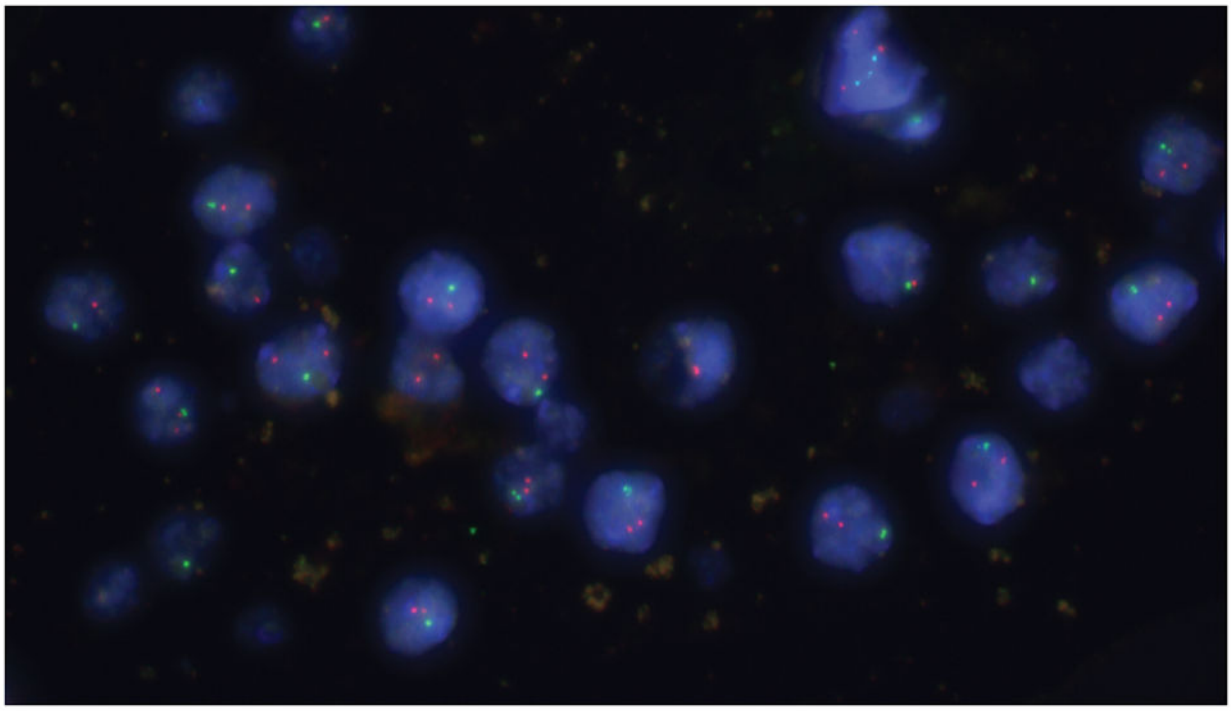


Figure 1.

Histopathology of duodenal neuroendocrine tumors in NF1 patients. A. A low-power view of duodenal neuroendocrine tumor shows characteristic nested architecture. Hematoxylin-and-eosin, 10X. B. A high-power view shows characteristic cytologic features, including

monotonous round nuclei with finely stippled chromatin, amphophilic granular cytoplasm, and psammomatous calcifications. Hematoxylin-and-eosin, 20X.



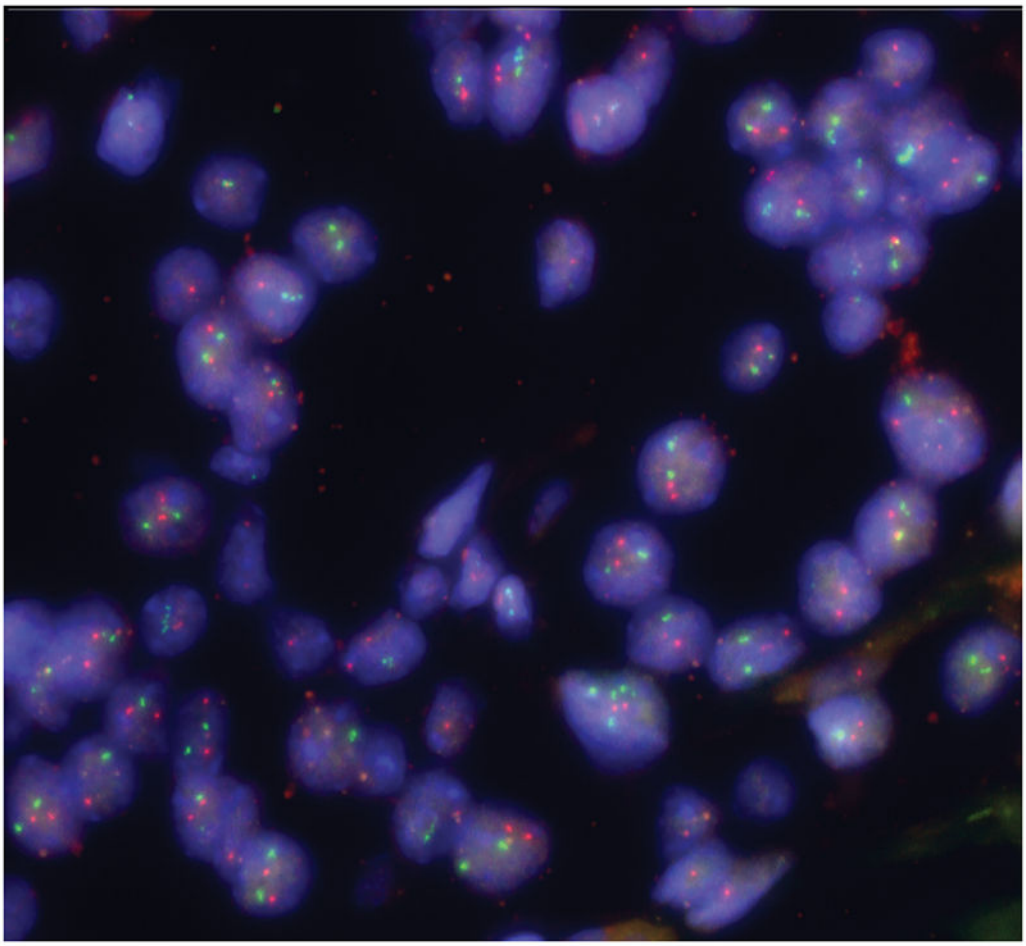


Figure 2.

Fluorescence *in situ* hybridization of chromosomes 9 and 22 in NF1-associated duodenal neuroendocrine tumors. A. Fluorescence *in situ* hybridization demonstrates loss of chromosome 22 in neoplastic cells, as they contain two red signals (chromosome 9) but only one green signal (chromosome 22). B. Fluorescence *in situ* hybridization on a tumor without chromosome 22 deletion shows two signals in both the red and green probes.

Table 1

Clinical and pathological features of duodenal neuroendocrine tumors in NF1 patients

Case	Age	Sex	Size (cm)	Lymph node metastasis	Somatostatin IHC	Ki67	Psammomatous calcifications	Other pathological features
SOM3	59	M	2.3	no	positive	<2%	yes	neurofibromas in surrounding soft tissue
SOM4	52	F	1.5	yes	positive	<2%	yes	duodenal GIST
SOM5	61	F	3.4	yes	negative	<2%	yes	neurofibromatosis-associated vasculopathy
SOM6	46	M	3.4	yes	not available	<2%	yes	neurofibromatosis-associated vasculopathy
SOM7	40	M	3	no	positive	not available	no	duodenal and cutaneous neurofibromas
SOM8	55	M	2.1	no	positive	<2%	yes	neurofibromatosis-associated vasculopathy

IHC: immunohistochemistry

Table 2

Somatic mutations identified by whole exome sequencing in NF1-associated duodenal neuroendocrine tumors

Tumor	Gene symbol	Mutation type	Amino acid change
SOM3	*BRAF*	Nonsynonymous coding	p.V600E
SOM3	*CEACAM20*	Nonsynonymous coding	p.R332W
SOM3	*DCTN1*	Nonsynonymous coding	p.R997L
SOM3	*FAM135B*	Nonsynonymous coding	p.S105N
SOM3	*LRRC39*	Frameshift	p.T240fs
SOM3	*MSN*	In-frame deletion	p.344delE
SOM3	*MTRNR2L2*	Nonsynonymous coding	p.S12L
SOM3	*NEB*	Nonsynonymous coding	p.L560V
SOM3	*NELL2*	Nonsynonymous coding	p.G841V
SOM3	*NF1*	Frameshift	p.F150fs
SOM3	*USP34*	Nonsynonymous coding	p.V609L
SOM4	*PCNT*	Nonsynonymous coding	p.G136S
SOM4	*SMC3*	Splice site acceptor	splice site
SOM5	*CAMKK2*	Nonsynonymous coding	p.R104Q
SOM5	*DACT1*	Nonsynonymous coding	p.S759L
SOM5	*JAKMIP1*	Nonsynonymous coding	p.G108D
SOM5	*PPIP5K1*	Nonsynonymous coding	p.F1144V
SOM5	*RNASE7*	Nonsynonymous coding	p.P48T
SOM5	*SP4*	Nonsynonymous coding	p.K705N
SOM6	*AIM1L*	Nonsynonymous coding	p.R25W
SOM6	*ANO10*	Frameshift	p.D45fs
SOM6	*ATXN10*	Nonsynonymous coding	p.E312K
SOM6	*FETUB*	Frameshift	p.I147fs
SOM6	*FGA*	Splice site acceptor	splice site
SOM6	*IFNB1*	Nonsynonymous coding	p.A76S
SOM6	*RDM1*	Nonsynonymous coding	p.R143K
SOM6	*VWA8*	Nonsynonymous coding	p.I709K
SOM6	*WBSCR17*	Nonsynonymous coding	p.G314R
SOM7	*CDH23*	Nonsynonymous coding	p.G540S
SOM7	*EZH2*	Nonsynonymous coding	p.D185H
SOM7	*IFNB1*	Nonsynonymous coding	p.G135R
SOM7	*MYO1B*	Nonsynonymous coding	p.M493L
SOM7	*NF1*	Frameshift	p.W267fs
SOM8	*AHCYL2*	Nonsynonymous coding	p.R468H
SOM8	*IL5*	Nonsynonymous coding	p.V17M
SOM8	*NF1*	Nonsense	p.Q1822X
SOM8	*PLS3*	Nonsynonymous coding	p.K300N

See Supplementary Table 2 for complete data on all identified somatic mutations

Table 3

Summary of key genetic alterations in NF1-associated duodenal neuroendocrine tumors

Tumor	Somatic *NF1* mutation	Germline *NF1* variant	Chromosome 22 copy number
SOM3	p.F150fs	none identified	deletion/LOH
SOM4	none identified	p.S436X	wild-type
SOM5	none identified	p.Q514fs	deletion/LOH
SOM6	none identified	none identified	deletion/LOH
SOM7	p.W267fs	p.T1951fs	wild-type
SOM8	p.Q1822X	p.L1152fs	wild-type

Table 4

Deleterious germline variants identified in NF1 patients with duodenal neuroendocrine tumors

Patient	Gene	Genomic DNA Alteration	Amino Acid Alteration
SOM4	*NF1*	chr17:29533304C>A	p.S436X
SOM5	*TSC1*	chr9:135773001insA	splice site
SOM5	*NF1*	chr17:29546036delAG	p.Q514fs
SOM7	*NF1*	chr17:29661957insA	p.T1951fs
SOM8	*FANCC*	chr9:98011507delC	p.D23fs
SOM8	*NF1*	chr17:29559859delACTC	p.L1152fs