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RESEARCH

Germline pathogenic variants in patients with high-grade gastroenteropancreatic neuroendocrine neoplasms

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Abstract

High-grade gastroenteropancreatic (HG-GEP) neuroendocrine neoplasms (NENs) are highly aggressive cancers. The molecular etiology of these tumors remains unclear, and the prevalence of pathogenic germline variants in patients with HG-GEP NENs is unknown. We assessed sequencing data of 360 cancer genes in normal tissue from 240 patients with HG-GEP NENs; 198 patients with neuroendocrine carcinomas (NECs) and 42 with grade 3 neuroendocrine tumors (NET G3). Applying strict criteria, we identified pathogenic germline variants and compared the frequency with previously reported data from 33 different cancer types. We found a recurrent *MYOC* variant in three patients and a recurrent *MUTYH* variant in two patients, indicating that these genes may be important underlying risk factors for HG-GEP NENs when mutated. Further, germline variants were found in canonical tumor-suppressor genes, such as *TP53*, *RB1*, *BRIP1* and *BAP1*. Overall, we found that 4.5% of patients with NEC and 9.5% of patients with NET G3 carry germline pathogenic or highly likely pathogenic variants. Applying identical criteria for variant classification *in silico* to mined data from 33 other cancer types, the median percentage of

Key Words

- HG-GEP NEN
- etiology
- germline
- pathogenic variants

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patients carrying pathogenic or highly likely pathogenic variants was 3.4% (range: 0–17%). The patients with NEC and pathogenic germline variants had a median overall survival of 9 months, similar to what is generally expected for metastatic GEP NECs. A patient with NET G3 and pathogenic *MUTYH* variant had much shorter overall survival than expected. The fraction of HG-GEP NENs with germline pathogenic variants is relatively high, but still <10%, meaning that that germline mutations cannot be the major underlying cause of HG-GEP NENs.

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Introduction

Neuroendocrine neoplasms (NENs) constitute ~2% of all malignancies and are frequently located in the gastrointestinal (GI) tract and pancreas. High-grade gastroenteropancreatic (HG-GEP) NENs are among the most aggressive cancers; the prognosis is generally poor with frequent metastatic disease at diagnosis and median survival of less than 1 year. These neoplasms are classified based on their morphology and proliferation rate and further subdivided into well-differentiated grade 3 neuroendocrine tumors (NET G3) or poorly differentiated neuroendocrine carcinomas (NECs) (WHO 2019). The molecular features of HG-GEP NENs are not well characterized, but some recent reports have shed new light on the landscape of somatic mutations in these cancers (Tang *et al.* 2016*a*,b, Abel *et al.* 2021, Venizelos *et al.* 2021). Importantly, a pattern emerges where the molecular features of NECs differ from NET G3 (Venizelos *et al.* 2021). While NET G3 resemble other well-differentiated NETs, GEP NECs frequently harbor mutations in major cancer genes, such as *TP53*, *APC*, *KRAS* and *BRAF*.

In addition to the assessment of driver mutations in well-established cancer genes, efforts have been made to pinpoint the mutational processes (Alexandrov *et al.* 2013) molding the molecular characteristics of GEP NECs. Thus, Yachida and colleagues found the mutational signatures in ductal type pancreatic NECs to be dominated by the contribution of single base substitution signature 1 (SBS1), whereas in acinar type pancreatic NECs the dominant contribution of mutations was from SBS5 (Yachida *et al.* 2022). Regarding gastric NECs, signatures SBS17a and SBS17 were more frequent, indicating that these cancers undergo a distinct mutational process as compared to other NECs and therefore may have a separate molecular etiology (Yachida *et al.* 2022). Despite these interesting findings, the mutational signatures observed in NECs have not been linked to specific underlying mechanisms of tumorigenesis or tumor evolution. Thus, the molecular etiology of GEP NEC remains largely unknown.

A proportion of all cancer cases are caused by inherited pathogenic mutations or by soma-wide *de novo* mutations. Among the most classical examples are pathogenic mutations of *RB1, CDKN2A* and *BRCA1* underlying retinoblastoma, melanoma and breast and ovarian cancers, respectively (Meindl *et al.* 2010, Collins & Politopoulos 2011, Benavente & Dyer 2015). In addition, some genes may confer a very high risk of multiple cancer types when mutated in the germline or *de novo*, such as *TP53* underlying the Li–Fraumeni syndrome with a very high risk of sarcomas, breast cancers and so on (Malkin *et al.* 1990). Pan-cancer studies in large sample sets have enabled the assessment of the contribution of germline pathogenic mutations as underlying causes of many cancer types. In a recent comprehensive study (Huang *et al.* 2018), the prevalence of mutations was summarized in a total of 10,389 adult cancer patients across 33 cancer types. In various specific types of adult cancers, pathogenic germline variants in tumor-suppressor genes, including *ATM*, *BRCA1*, *BRCA2*, *BRIP1* and *PALB2,* were reported, while mutations in genes such as *TP53*, *RB1* and *MEN1* were mainly associated with multicancer risk syndromes.

For low-grade pancreatic NETs, germline pathogenic variants in *MEN1* have been identified as a strong underlying cause, with approximately 60% of patients with such mutations developing pancreatic NETs during their life span (Sakurai *et al.* 2012, Ishida & Lam 2022). In addition, variants in several other genes have also been linked to low-grade pancreatic NETs including *CDKN1B*, *VHL*, *NF1*, *TSC1/2*, *PTEN*, *GCGR, BRCA2* and *MAFA* (Ishida & Lam 2022). For carriers of pathogenic variants in *NF1*, about 10% of these individuals develop NET specifically in the ampulla of Vater (Noe *et al.* 2018), and a pathogenic variant in *MUTYH* has been linked to small intestinal NETs (Dumanski *et al.* 2017). However, the prevalence of germline pathogenic mutations as a potential underlying cause of HG-GEP NENs remains unknown. Here, we assessed targeted sequencing data of 360 cancer-related

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genes in non-neoplastic (normal) tissue, across 198 patients with NECs and 42 patients with NET G3.

Materials and methods

Study design

The aim of this study was to assess the landscape of pathogenic germline mutations in HG-GEP NENs and thereby to provide information on the fraction of tumors that could be explained by such mutations. In total, we assessed sequencing data for 240 patients. All patients were diagnosed with HG-GEP NENs during 2013–2017 and had been prospectively included in a Nordic database. The recruiting centers are the only referral centers for HG NENs in their respective regions, and the cohort should therefore be representative. Inclusion criteria were histopathologically confirmed HG NENs (Ki-67 > 20%) with GEP primary or unknown primary site (CUP) with predominantly GI metastases (defined by radiological CT scans). At the time of protocol development (2014) and study enrollment (2014–2017), all GEP NENs with a Ki-67 > 20% were classified as grade 3 neuroendocrine carcinoma (NEC G3). The current 2019 World Health Organization (WHO) pathology grading system divides HG-GEP NENs into the well-differentiated NET G3 and the poorly differentiated NECs (Nagtegaal *et al.* 2019, WHO 2019). As a result of the new classification, all cases were blinded and reevaluated digitally in 2021–2022 by three experienced NEN pathologists (AP, AC, and IMBL). Out of the 240 patients, 198 patients were reclassified as NECs and 42 patients as NET G3.

For 180 patients, somatic mutations have been reported previously (Venizelos *et al.* 2021). In the previous study, sequencing data on normal tissue were only used for filtering and supporting somatic mutation calling. In that study, 181 patients were included. Upon re-audit of clinical information, for the present study, one patient (11017) was excluded from analysis due to lack of proof of GI-related disease (metastases limited to axilla). In addition, for the present study, we also sequenced normal tissue from an additional 60 patients with HG-GEP NENs, reaching the total number of 240.

DNA isolation

Genomic DNA was isolated from non-neoplastic tissues (blood) using QIAamp DNA Mini Kit (Qiagen).

Microsatellite instability (MSI) status

MSI status was drawn from our previously published somatic data (Venizelos *et al.* 2021) for the 180 patients included in that study. In brief, those analyses were performed using the Promega MSI analysis system (Version 1.2, Promega). For the additional 60 patients included in the present study, only the DNA from normal tissues (blood) was analyzed, precluding any assessment of MSI status.

Library preparation and sequencing

Targeted massive parallel sequencing was performed on DNA from normal peripheral blood leukocyte DNA. Illumina libraries were prepared applying Kapa Hyper Prep kit (Kapa Biosystem, Wilmington, MA, USA) and Agilent SureSelect XT-kit (Agilent). Targeted enrichment was performed using RNA baits (SureSelect, Agilent), targeted against an in-house panel of 360 cancer-related genes (Yates *et al.* 2015). Libraries were sequenced on a MiSeq instrument (Illumina, San Diego, CA, USA) to an average depth of 172× (range 50×–266×).

Data processing and bioinformatics analysis

Raw sequence data were aligned to the human reference genome (Build-UCSC hg19) using Burrows-Wheeler Aligner (BWA) (Li & Durbin 2009). Germline mutations from the vcf files were annotated using ANNOVAR and Ensembl Variant Effect Predictor with default parameters. Furthermore, we applied CharGer (Characterization of Germline Variants) (Scott *et al.* 2019), which queries information from ClinVar database, to classify variants into pathogenic, likely pathogenic, variant of uncertain significance (VUS), as well as likely benign and benign.

In addition to the CharGer software tool, we manually inspected each mutation in the Integrative Genomics Viewer to omit false positives based on coverage as well as read quality. The genome aggregation database (gnomAD) was utilized to examine the population frequency of each variant. Further, variant allele frequencies in the blood samples (and in matched tumors) were used to remove variants that were likely representing clonal hematopoiesis.

Mined data set

For comparison of the prevalence of germline variants in HG-GEP NECs to other cancer types, we mined the data available from Huang *et al.* (Huang *et al.* 2018),

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holding germline information for 10,389 patients with 33 different cancer types. In their report, Huang *et al.* applied a variant classification utilizing tiers for variant annotations by adopting the American Association for Molecular Pathology and American College of Medical Genetics and Genomics guidelines and levels of a score of evidence generated by the CharGer algorithm (Scott *et al.* 2019). This approach gave a slightly higher fraction of variants called as likely pathogenic, relative to pathogenic variants. For consistency, we therefore mined the raw data from their report and reclassified according to the identical procedure as we did for our HG-GEP NEN cohort (described under the 'Data processing and bioinformatics analysis' section).

Ethics and consent to participate

The research protocol was approved by the ethics committees in Norway (REK vest 2012/940), Sweden (REC Uppsala Dnr 2012/285) and Denmark (Region Hovedstaden H-4-2012-108). All patients signed informed written consent.

Results

Patient samples

A total of 240 patients diagnosed with HG-GEP NENs were assessed for pathogenic germline variants (198 NECs and 42 NET G3). Demographics are summarized in Table 1. Regarding the primary tumor site, the largest groups were neoplasms in the rectum and colon, followed by pancreas and esophagus.

Prevalence of pathogenic germline variants in GEP NEC

Applying the classification tiers from ClinVar (as described under the 'Materials and methods' section) on sequencing data covering 360 cancer-related genes (Yates *et al.* 2015), we found 9 out of 198 patients (4.5%) with GEP NECs to carry a total of 11 germline pathogenic variants (Fig. 1; Table 2). These nine patients had pathogenic germline variants in major tumor suppressors such as *TP53* and *RB1* but interestingly also in several genes involved in homologous recombination repair (HRR) – *FANCC*, *BRIP1* and *ERCC2*.

Notably, two patients (10017 and 9054) were found to have an identical germline nonsense mutation (c.1174G>A) in the *MYOC* gene, encoding myocilin, which is involved in cytoskeletal functions. Both these patients were males and had large-cell NEC, one with primary tumor site in the rectum and the other in the right colon. The two patients were recruited at two different centers, and there was no known familial relationship between them.

Two patients harbored more than one pathogenic variant: patient 9054 harbored both the *MYOC* variant mentioned above and a nonsense variant in *BRIP*, while patient 5021 harbored both a *TP53* and a *CTNNB1* (β-catenin) variant (Fig. 1; Table 2). The remaining pathogenic variants were found in *MUTYH* (DNA repair / polyposis-related gene), *BAP1* (deubiquitinating enzyme) and *AR* (androgen receptor).

Mining our previously published data on somatic mutations in tumors (Venizelos *et al.* 2021), we found that none of the nine patients with germline pathogenic variants had somatic mutations (single nucleotide variants or indels) as a 'second hit' in their tumors. However, three had somatic copy number loss indicating loss of heterozygosity (LOH) of the affected loci. This was seen for the variants in *TP53*, *RB1* and *FANCC* (Table 2).

One of the nine cases (7032, carrying a pathogenic *ERCC2* variant) was diagnosed with a prostate cancer 10 months prior to the NEN diagnosis. None of the remaining eight patients with pathogenic variants had prior cancers. Neither did any of these nine patients have any family history of NENs.

The average age at diagnosis for the nine patients with germline pathogenic variants did not differ significantly from the remaining patients (64 vs 66 years), but notably, one of the nine patients (5021, carrying both a *TP53* and a *CTNNB1* pathogenic variant) was diagnosed at 39 years of age, placing this patient in the lower 5% of the cohort, with respect to age.

Among the nine carriers of germline pathogenic variants were six males and three females. Thus, the percentage of carriers was very similar between genders (4.5% and 4.7%, respectively).

In addition to the pathogenic germline variants described above, we found four additional variants that fulfilled the criteria for being germline pathogenic variants: two variants in *DNMT3A*, one in *GNAS* and one in *U2AF1*. All these four were detected with low allele frequencies in the blood DNA, and they were absent from the matched tumor samples. Thus, they were considered products of clonal hematopoiesis rather than real germline variants and excluded from the analysis.

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Table 1 Patient characteristics.

Characteristic	Subgroup	*n* patients		
		NECs	NET G3	HG-GEP NENs (total)
Total		198	42	240
Age	<60	44	16	60
	≥60	154	26	180
Gender	Male	134	19	153
	Female	64	23	87
Site	Colon right	38	3	41
	Rectum	47	0	47
	Esophagus	32	1	33
	Gastric	23	1	24
	Unknown	22	7	29
	Pancreas	18	22	40
	Colon left	11	1	12
	Gallbladder/duct	3	0	3
	Other GI	4	7	11
Metastatic site	Liver	120	39	159
	Lung	32	5	37
	Lymph node	66	9	75
	Other	54	13	67
Cell type	Large cell	119	NA	NA
	Small cell	74	NA	NA
	Unknown	5	NA	NA
Ki-67	21–55%	23	35	58
	>55%	171	7	178
	>20% (exact value not specified)	4	0	4
Surgery of primary tumor	Resected (prior to sampling)	69	13	82
	Not resected	129	29	158
Disease	Nonmetastatic (stage I–III)	22	0	22
	Metastatic (stage IV)	176	42	218
Smoking habit	Smoker	43	5	48
	Ex-smoker	65	12	77
	Nonsmoker	77	22	99
	Unknown	13	3	16

Further to the pathogenic variants, we assessed VUS. Among the 198 NEC patients, 84 harbored germline variants were classified as VUS from the ClinVar database (Fig. 1).

Prevalence of pathogenic germline variants in GEP NEC vs other cancer types

In order to compare the fraction of GEP NECs harboring pathogenic germline mutations to the corresponding fraction in other cancer types, we mined the data from Huang and colleagues (Huang *et al.* 2018). These authors assessed the germline status of 10,389 patients across 33 cancer types. Assessing their raw data of variants within the same 360 gene set and classifying them according to the same criteria as we used for GEP NECs, we found that the fraction of cases with pathogenic or likely pathogenic variants in different cancer types ranged from 0% to 17% (median 3.4%; Fig. 2). Thus, GEP NECs revealed a relatively high fraction of cases harboring germline pathogenic mutations as compared to most other cancer types.

Germline mutations in GEP NEC originating in different tissues

Among the NEC patients with pathogenic variants, three had their primary tumors in the colon, one in the rectum, one in the esophagus, one in the stomach and one tumor was in the gallbladder (the remaining two had unknown primary site; Fig. 3). Comparing these fractions to the corresponding fractions of the tissue's adenocarcinoma counterparts, the fraction of GEP NECs with germline pathogenic mutations seems high, although these data must be interpreted with great caution due to the low number of observations.

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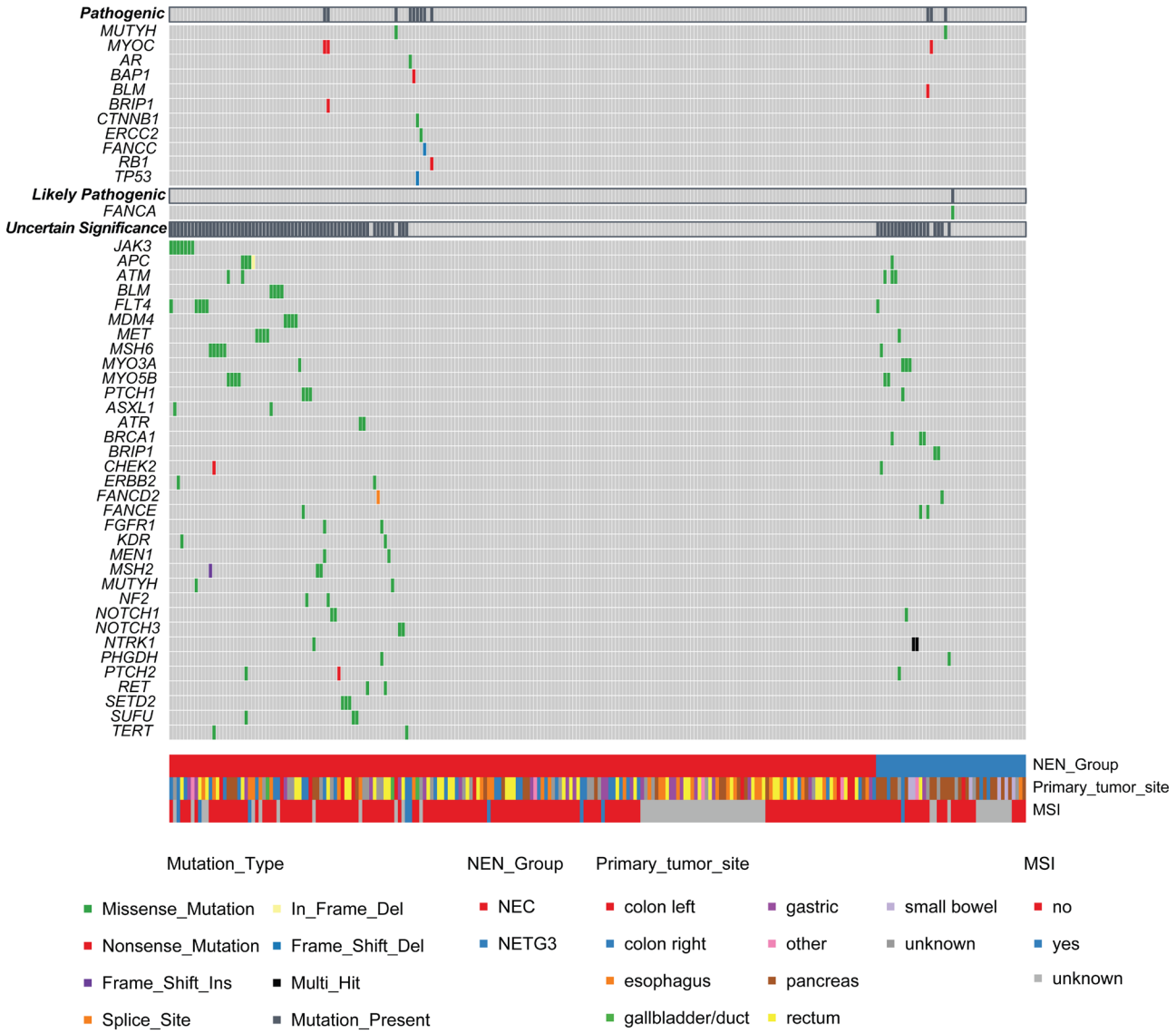

Figure 1

Germline variants in GEP NENs. Oncoplot showing the germline variants detected in 240 patients (columns), ordered according to affected genes (rows). Pathogenic variants are listed in the top panel, with a framed summary line at the top. Likely pathogenic variants are listed in the middle panel, with a framed summary line at the top. Variants of uncertain significance are listed in the bottom panel, with a framed summary line at the top. Variants are colored according to molecular variant type. 'Multi_hit' indicates that more than one mutation occurs in the same gene in the same patient. The panel under the oncoplot area is composed of three single-row color maps showing in order, from top to bottom, NEN group (NECs or NET G3), primary tumor site and MSI status.

Clinical impact of germline mutations in GEP NEC

Among the nine patients with NECs and detected pathogenic germline variants, one patient (5017, who carried a variant in *AR*) was subject to radical surgery and therefore not comparable to the other patients with respect to clinical outcome. The other eight patients with metastatic disease given palliative chemotherapy had a median overall survival of 9 months, similar to what is generally expected for metastatic GEP NECs. Seven of these eight patients had a partial response to first-line treatment (response rate 88%), whereas median progression-free survival was 6 months. The patients with pathogenic germline variants did not differ from the rest of the cohort with respect to any other available clinical parameters.

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Table 2 Patients with HG-GEP NENs and germline pathogenic variants.

Study ID	Gene	Chr.	Position	Ref.	Alt.	Variant	aa change	ClinVar class	LOH in tumor[a,b]	Cell type	Primary site	Metastases	Gender	OS months
NEC														
5017	*AR*	chrX	66943532	C	T	Missense	p.A871V	Pathogenic	No	Large cell	Colon right	No	Female	68
5021	*TP53*	chr17	7574003	G	–	Frameshift	p.R342X	Pathogenic	LOH	Small cell	Gallbladder /duct	Yes	Male	10
	CTNNB1	chr3	41266113	C	T	Missense	p.S37F	Pathogenic	No					
7032	*ERCC2*	chr19	45856059	C	G	Missense	p.R616P	Pathogenic	–	Large cell	Unknown	Yes	Male	5
7074	*MUTYH*[c]	chr1	45798475	T	C	Missense	p.Y176C	Pathogenic	–	Large cell	Colon left	Yes	Male	9
9037	*RB1*	chr13	48955538	C	T	Stop gain	p.R552X	Pathogenic	LOH	Small cell	Unknown	Yes	Female	14
9054	*MYOC*[d]	chr1	171605478	G	A	Stop gain	p.Q368X	Pathogenic	No	Large cell	Rectum	Yes	Male	26
	BRIP1	chr17	59871059	C	A	Stop gain	p.E458X	Pathogenic	No					
9083	*FANCC*	chr9	98011507	C	–	Frameshift	p.D23X	Pathogenic	LOH	Large cell	Gastric	Yes	Female	8
10017	*MYOC*[d]	chr1	171605478	G	A	Stop gain	p.Q368X	Pathogenic	No	Large cell	Colon right	Yes	Male	8
14027	*BAP1*	chr3	52438516	A	C	Stop gain	p.Y401X	Pathogenic	No	Small cell	Esophagus	Yes	Male	10
NET G3														
5035	*FANCA*	chr16	89813023	G	A	Missense	p.T1161M	Likely pathogenic	LOH		Pancreas	Yes	Female	26
7054	*MYOC*[d]	chr1	171605478	G	A	Stop gain	p.Q368X	Pathogenic	–		Pancreas	Yes	Female	76
14023	*BLM*	chr15	91304245	C	T	Stop gain	p.Q548X	Pathogenic	No		Unknown	Yes	Male	63
14028	*MUTYH*[c]	chr1	45798475	T	C	Missense	p.Y176C	Pathogenic	No		Pancreas	Yes	Male	15

[a]Copy number data for LOH assessment of tumors lacking for cases 7032, 7074 and 7054; [b]no patients had somatic mutations as 'second hit' in tumor cells; [c]identical *MUTYH* variants found in one patient with NEC and one patient with NET G3; [d]identical *MYOC* variants found in two patients with NEC and one patient with NET G3.
LOH, loss of heterozygosity; NEC, neuroendocrine carcinoma; NET G3, grade 3 neuroendocrine tumor; OS, overall survival.

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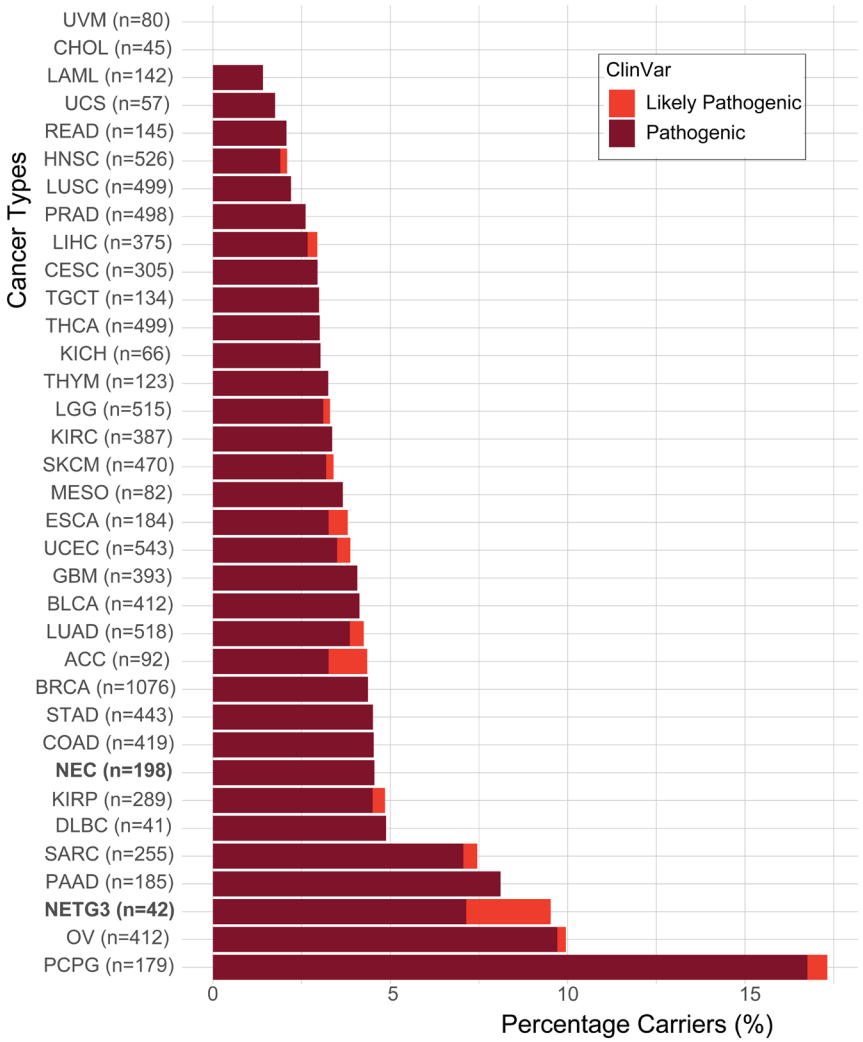

Figure 2
Germline pathogenic variants in different cancer forms. Bars indicate the fraction of patients harboring pathogenic germline variants (dark red) and likely pathogenic variants (bright red) in 35 different cancer forms. Data for GEP NECs and NET G3 (bold) are original data, while the remaining 33 cancer types are reassessment of raw data from Huang *et al*. (2018), applying the same pathogenicity scoring as for NECs and NET G3. ACC, adrenocortical carcinoma; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, cholangiocarcinoma; COAD, colon adenocarcinoma; DLBC, lymphoid neoplasm diffuse large B-cell lymphoma; ESCA; esophageal carcinoma; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LAML, acute myeloid leukemia; LGG, brain lower grade glioma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; MESO, mesothelioma; NEC, neuroendocrine carcinoma; NET G3, grade 3 neuroendocrine tumors; OV, ovarian serous cystadenocarcinoma; PAAD, pancreatic adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; PRAD, prostate adenocarcinoma; READ, rectum adenocarcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; STAD, stomach sdenocarcinoma; TGCT, testicular germ cell tumors; THCA, thyroid carcinoma; THYM, thymoma; UCEC, uterine corpus endometrial carcinoma; UCS, uterine carcinosarcoma; UVM, uveal melanoma.

Prevalence of germline mutations in GEP NET G3

Among the 42 patients diagnosed with NET G3, we found 3 (7.1%) to harbor pathogenic germline variants. These variants were found in the *BLM*, *MUTYH* and *MYOC* genes. Intriguingly, the *MYOC* variant was the same variant as detected in two of the NEC patients and the *MUTYH* variant was the same as detected in one NEC patient. This finding further substantiated the notion that variants in these genes could be particular risk factors for neuroendocrine malignancies. In addition, one patient with NET G3 (2.4%) harbored a germline variant classified as likely pathogenic. This variant was found in the *FANCA* gene, affecting HRR. Again, this finding aligns well with the findings among patients with NEC, where several pathogenic variants were found in genes involved in HRR, further implicating this cellular function in neuroendocrine tumorigenesis.

None of the four patients with germline pathogenic or likely pathogenic variants and NET G3 had somatic mutations as 'second hit' in their tumors, but one patient, carrying a germline variant in *FANCA*, had LOH of the locus (Table 2).

Assessing the overall prevalence of pathogenic and likely pathogenic variants in NET G3, 9.5% of patients harbored such variants, placing NET G3 among those tumor types with the highest prevalence of such variants (Fig. 2). However, this should be interpreted with caution since the number of analyzed patients with NET G3 was low. Three of the NET G3 patients harboring pathogenic or likely pathogenic variants had tumors with the pancreas as the primary site, while the one remaining patient had tumor with unknown primary site. Given the low absolute number of observations, no formal assessment of mutation spectrum across primary sites could be performed.

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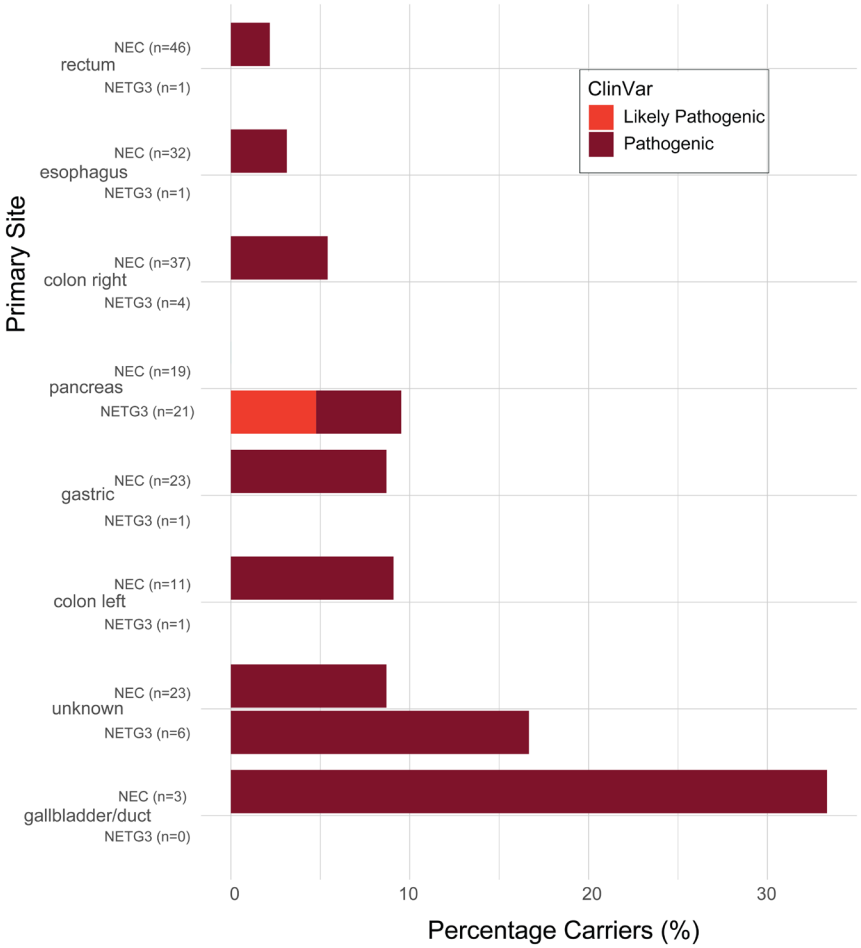

Figure 3
Germline pathogenic variants in GEP NEC and NET G3 with different primary tumor sites. Bars indicate the fraction of patients harboring pathogenic germline variants (dark red) and likely pathogenic variants (bright red). Numbers on the *y*-axis indicate the number of patients in each category. NEC, neuroendocrine carcinoma; NET G3, grade 3 neuroendocrine tumor.

Overall survival for the patient with metastatic NET G3 and germline pathogenic variant in *MUTYH* was only 15 months, much shorter than the 31–42 months generally expected for metastatic NET G3.

Discussion

In the present study, we found that 4.5% of patients with NECs and 9.5% of patients with NET G3 carry germline pathogenic or likely pathogenic variants. This places the prevalence of germline pathogenic variants in HG-GEP NENs in the higher end of the spectrum of different cancer types, as shown in our present comparison.

Regarding the specific genes in which pathogenic variants were detected, we made several interesting observations. Most strikingly, we found two patients with NECs and one patient with NET G3 to harbor the exact same mutation in the *MYOC* gene. This gene is known to be involved in cytoskeletal functions, and germline variants in this gene have been associated with hereditary juvenile-onset open-angle glaucoma (Selvan *et al.* 2022). A multitude of somatic mutations in *MYOC* (including Q368*, presently detected as a germline variant) have previously been reported in several cancer types, with highest frequencies in skin cancers and endometrial cancers but also with a relatively high frequency (2.5%) in colon cancers (Forbes *et al.* 2017). In our previous report on somatic variants in HG-GEP NEN, we found *MYOC* mutations in 2 patients out of 152 GEP NECs. Notably, among the 29 NET G3, none of the patients harbored somatic *MYOC* mutations (Venizelos *et al.* 2021). Although further studies are warranted, and the mechanism(s) underlying the potential increased risk, our data indicate a role for *MYOC* variants in the tumorigenesis of HG-GEP NEN.

Another recurrent variant was observed in *MUTYH*. This variant was found in one patient with NEC and another with NET G3. The protein product of *MUTYH* is a glycosylase involved in base excision repair, and germline variants have been implicated in high somatic mutation rates and risk of colorectal cancer (Robinson *et al.* 2022). Interestingly, a pathogenic variant in *MUTYH* has previously been linked to small intestine NET in two

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different families (Dumanski *et al.* 2017), and different *MUTYH* variants have been detected in cases of pancreatic NETs (Scarpa *et al.* 2017). Although the variant as detected in our present study was a different one, taken together, these observations support a role for *MUTYH* variants in NEN development. The molecular and cellular etiology of NECs is debated, and the possible potential evolution from a well-differentiated NET to a poorly differentiated NEC is controversial (Tang *et al.* 2016*b*, Botling *et al.* 2020, Pelosi *et al.* 2021). Although our current data are no evidence for linear evolution, it is interesting to note that the recurrent pathogenic mutations both in *MYOC* and in *MUTYH* are found both in NEC and in NET G3 cases.

Another striking observation is that a substantial fraction of the pathogenic and likely pathogenic variants we observed was in genes related to HRR other than *BRCA1/2*. Such variants are well established as underlying causes of the breast, prostate and ovarian cancers (Kohlhase *et al.* 2014, Ramus *et al.* 2015, Li *et al.* 2019). In addition to providing insight into the underlying causes of HG-GEP NEN tumorigenesis, it should be noted that the presence of HRR deficiency could be a lead forward in the exploration of new treatment options for patients with HG-GEP NENs: although sensitivity to PARP inhibition was originally linked to *BRCA1* mutations, it has recently become evident that such sensitivity may also be caused by defects in other genes involved in HRR (Eikesdal *et al.* 2021).

However, we also found several pathogenic variants in genes not directly involved in HRR or any other kind of DNA repair. Thus, taken together, the diversities in affected genes and their functions strongly indicate that there is no single unifying mechanistic cause underlying a majority of those cases of HG-GEP NENs caused by germline pathogenic variants.

Regarding the clinical trajectory of the GEP-NEC disease, these did not differ between patients carrying germline pathogenic mutation and those who did not. Median survival for the eight metastatic NEC cases was 9 months, not far from the 11–12 months generally expected for metastatic GEP NECs (Walter *et al.* 2017, Elvebakken *et al.* 2021, Morizane *et al.* 2022). Although the number of patients may be too limited to draw firm conclusions, our data do not suggest that any specific adaptations within the current treatment strategies should be implemented for those patients carrying pathogenic germline variants. Instead, as mentioned above, the findings reported here should rather be used to point forward to potential exploration of alternative treatments outside of current standards. The patient with metastatic NET G3 and germline pathogenic variant in *MUTYH* had an overall survival of only 15 months. This is substantially shorter than the 31–42 months expected for metastatic NET G3 in general (Chan *et al.* 2021, Liu *et al.* 2021, Spada *et al.* 2021), but given the single observation, no firm conclusions can be drawn.

From a technical point of view, our approach for identification of pathogenic germline variants was somewhat conservative. We identified a slightly lower fraction of variants than, for example, Huang and colleagues (Huang *et al.* 2018), from whose data we mined for reassessment. The differences were mainly that we detected a lower fraction of variants in the category 'likely pathogenic', while the fraction of variants in the category 'pathogenic' was similar. In general, the stringency in the definition of 'likely pathogenic' differs between studies and potentially precludes the direct comparison of frequencies. For future studies assessing variants in GEP NENs, such potential technical differences in the annotation of the detected variants should be taken into account. Further, all normal tissue samples in the present study were sequenced by a targeted panel of 360 cancer-related genes. Although perhaps unlikely, it may be that some patients harbor mutations in genes not included in the panel, which could contribute to the development of GEP NECs. Further, our analysis was restricted to SNVs and indels, which may be a limitation to our data: it may be that some few patients have germline copy number alterations that could be an underlying cause of cancer. Notably, in recent reports both in prospective population-based cohorts and in hospital-based cohorts, we have shown embryonic (constitutional) methylation of *BRCA1* to cause a significantly increased risk of breast and ovarian cancer later in life (Lonning *et al.* 2018, 2022). Such epigenetic events have not been assessed for the HG-GEP NEN cohort. As such, our present data may be an underestimate of the real fraction of HG-GEP NEN that have germline variants and/ or constitutional molecular features as a lead cause of disease development.

Although our data show that the fraction of HG-GEP NENs with germline pathogenic variants is relatively high as compared to other cancer types, the fraction is still only <10%. Even with the precautions discussed above and the fact that our results may slightly underestimate the fraction, this means that the presence of such mutations cannot be the major underlying cause of HG-GEP NENs. As such, the molecular etiology of the majority of these neoplasms is still largely unknown.

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Declaration of interest

AV is currently an employee at Oxford Nanopore. HS has received research support from Novartis, Amgen and Ipsen and honoraria from Novartis, Ipsen, Pfizer, Keocyt, AstraZeneca, BMS, Roche, Amgen, Merck, Shire, Hutchinson and Celgene. HE has received honoraria from Bayer. AP has received honoraria from Roche. GOH has received grants or honoraria from Ipsen, Amgen, BMS, MSD, Roche and Bayer. SK has received research support, grants, or honoraria from AstraZeneca, Pfizer, Illumina, Pierre-Fabre, Novartis, Sobi, Amgen, Sanofi Aventis and Roche.

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