Peer Review File

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Reviewer A

Comment:

How was the separation NET G3 and NEC carried out?

Answer:

Thank you for your comments.

As we mentioned in the method, pathologic diagnosis was done by the pathologist according to the 2017 WHO classification of NET.

Based on the 2017 WHO classification of NET, Both NET G3 and NEC have > 20 mitoses/10HPF and/or Ki-67 index >20. However, NET G3 was well differentiated neuroendocrine neoplasm and NEC was poorly differentiated neuroendocrine neoplasm.

Thanks.

Comment:

Check spelling and details throughout the manuscript, e.g P-values are described in different ways; P value vs P =

Answer:

Thank you for your comments.

According to your comment, we corrected the error, and unified the term into p-value as follow;

"A statistically significant difference was not observed in the TMB score between well-differentiated NET (grade 1, 2, and 3) and poorly differentiated NEC (median; 3.9 vs. 4.7, p-value = 0.232). In addition, the TMB score did not differ based on the primary tumor site (pancreatic NEN vs. other primary NEN; median; 3.9 vs 4.7, p-value = 0.646)."

Comment:

Is it possible to do more in depth comparisons between NET G3 and NEC, potentially it would be of relevance add more samples for further comparisons between NEC/NET G3 from same primary tumors.

Answer:

Thank you for your comments.

Your comment is very important point. We think that your comment could improve the quality of this manuscript.

However, as you already know, NET and/or NEC are very rare entity among various cancers. Furthermore, this manuscript included only metastatic NET/NEC analyzed for tumor mutational burden (TMB). Therefore, sample size of this study was too small.

Currently, we cannot extend the sample size because of the rarity of study-population. However, we would continue to collect the data for metastatic NET/NEC patients analyzed for molecular characteristics.

Nevertheless, this analysis showed that, despite the very low incidence, there are GEP-NENs with high TMB. For precision medicine, testing for MSI and TMB is needed for this tumor type.

Thanks.

Comment:

Did the authors investigate mismatch repair status of the lesions in all patients, and also examined using immunohistochemistry?

Answer:

Thank you for your comments.

Conventional immunohistochemistry of mismatch repair status was not performed, in this study, we decided the status of microsatelite instability by next-generation sequencing. For this point, we described in the part of method as follows;

"***Next-generation sequencing***

Tumor samples were obtained at the time of initial diagnosis or progression in patients with GEP-NEN. Formalin-fixed paraffin-embedded (FFPE) material was used. Forty (40) ng of DNA were quantified with the Qubit dsDNA HS Assay (Thermo Fisher Scientific) on the Qubit 2.0 Fluorometer (Thermo Fisher Scientific) and then sheared using a Covaris E220 Focused-ultrasonicator (Woburn, MA, USA) and the 8 microTUBE–50 Strip AFA Fiber V2 following the manufacturer's instructions. The treatment time was optimized for FFPE material. The treatment settings were as follows: peak incident power (W): 75; duty factor: 15%; cycles per burst: 500; treatment time (s): 360; temperature (°C): 7; water level: 6. For DNA library preparation and enrichment, the TruSight™ Oncology 500 Kit (Illumina) was used following the manufacturer's instructions. Post-enriched libraries were quantified, pooled, and sequenced on a NextSeq 500 (Illumina Inc., San Diego, CA, USA). The quality of the NextSeq 500 (Illumina) sequencing runs was assessed with the Illumina Sequencing Ana"lysis Viewer (Illumina). Sequencing data were analyzed with the TruSight Oncology 500 Local App Version 1.3.0.39 (Illumina). The TruSight™ Oncology 500 is a comprehensive tumor profiling assay designed to identify known and emerging tumor biomarkers, including small variants, splice variants, and fusions. Importantly, the TruSight™ Oncology 500 measures tumor mutational burden (TMB) and microsatellite instability (MSI), features that are potential key biomarkers for immunotherapy.

TMB was reported as mutations per megabase (Mb) sequenced. There was no consensus of the definition of the high TMB in NET, therefore, we use the cutoff of 10 mutations/Mb as a high TMB [12]. "

Comment:

Please add/compare and discuss results from study doi.org/10.1158/1078-0432.CCR-20-1804

Answer:

Thank you for your comments.

We added the study that you mentioned in the table 3, and also discussed the results in the discussion as follows;

" *Because NENs are rare tumors, few studies have evaluated TMB in NENs, especially GEP-NENs. Previous studies analyzing TMB in NENs with variable inclusion criteria are summarized in Table 3. Most studies also reported low TMB and low rate of MSI-high in NENs, which is consistent with our analysis (14-16).*

However, in our study, there is no statistically significant differences between TMB of well-differentiated NET and poorly-differentiated NEC. Ine study only analyzed NEC also reported low median TMB (5.68 mutations/Mb) (15). However, other study with large number of patients reported higher TMB in high grade GEP-NEN than in low grade GEP-NEN (17). This study also reported 4% of MSI-high tumors in high grade GEP-NEN, which is relatively high rate comparing other study. Those studies all had different inclusion criteria, therefore, further study would be needed. "

Table 3. Published studies of TMB in NETs

Diagnosis	Total enrollment (n)	Median TMB	TMB IQR	TMB range	MSI-H	PD-L1 (+)	Reference
GEP-NET	31	4.7	3.1 – 6.3	0.8 – 266.4	0	*	Our data
Pancreatic NET	75	(Average) 5.8	*	*	0	2/70 (2.9%)	(16)
GI NEC	29	5.68	*	0.57 – 11.75	0	9/31 (29.0%)	(15)
Metastatic and locally advanced NEN	85	5.45	3.84 – 8.85	*	*	*	(27)
Pulmonary NET	48	0.31	0.22 – 0.67	*	*	*	(28)

NET	164	5.2	2.6 – 10.4	*	*	*	(29)
High grade GEP-NEN	135	(Average) 9.5	*	*	4%	6%	(30)
Low grade GEP-NEN	335	(Average) 5.1	*	*	0%	1%	(30)

* Not available.

Comment:

Did the authors test the performance of TMB to predict MSI status by receiver operating characteristic (ROC) curves? Why/why not? Include.

Answer:

Thank you for your comments.

In this study, we used the TruSightTM Oncology 500 as the platform of sequencing.

As you already know, the TruSightTM Oncology 500 has been widely used as comprehensive tumor profiling assay including TMB and MSI.

Thanks.

Comment:

Prognostic analysis according to TMB in the study population?

Answer:

Thank you for your comments.

The portion of high TMB in GEP-NET/NEC was too small in previous studies.

Consistently, this study included only one high TMB tumor. Therefore, we did not perform the prognostic analysis according to the status of TMB.

Comment:

Did the authors look into genes such as TP53, RB1 KRAS, APC, BRAF, and PI3KCA in high-grade vs low-grade?

Answer:

Thank you for your comments.

We focused the status of TMB and MSI in this manuscript.

A few year ago, our group reported the genomic profiling of metastatic GEP-NET (Journal of Cancer. 2016 May 25;7(9):1044-8. doi: 10.7150/jca.14815. eCollection 2016.)

In that analysis, Of 14 GEP-NET patients available for mutational profiling, 7 (50.0%) patients had one or more aberrations detected. Common aberrations were as follows: SMARCB1 mutation (n=2), TP53 mutation (n=2), STK11 mutation (n=1), RET mutation (n=1), and BRAF mutation (n=1). Gene amplification by nCounter was detected in only 1 patient, showing CCNE1 amplification, and this patient also had a TP53 mutation.

Thanks.

Comment:

Please provide ki-67 levels of each patient. Does Ki-67 correlate to TMB?

Answer:

Thank you for your comments.

We analyzed for this point.

We attached the data of Ki-67 for patients. The Pearson correlation analysis was calculated, and there was no correlation between Ki-67 and TMB (r = 0.366).

No	Ki-67
1	5
2	3
3	3
4	2

5	3
6	10
7	10
8	50
9	5
10	80
11	3
12	20
13	5
14	2
15	55
16	70
17	4
18	25
19	
20	70
21	90
22	
23	5
24	80
25	90
26	5
27	4
28	90
29	15
30	70
31	70

Comment:

Why only 1 grade 2 tumor? These are usually more common. Is it possible to include more?

Answer:

Thank you for your comments.

According to your comment, this study included only 1 patient with grade 1.

We don’t know the reason why patient with grade 1 NET was too small in this study.

Maybe, this is retrospective study, therefore, there might be a selection bias.

For this point, we described the part of the discussion as follows;

"There are some limitations to our study. First, due to the rarity of this disease, this study was based on small sample size. Second, this was the retrospective study only included the patients who had NGS. Therefore, there are possibilities that patients with more advanced stage was included. Further study with large and various samples would be needed in the future."

M&M

Comment:

More specific description of the NGS; include sequencing depth of coverage, analytic sensitivity etc.

Reply:

Thank you for your comments. We added more description on the method as follows;

"Next-generation sequencing

Tumor samples were obtained at the time of initial diagnosis or progression in patients with GEP-NEN. Formalin-fixed paraffin-embedded (FFPE) material was used. Forty (40) ng of DNA were quantified with the Qubit dsDNA HS Assay (Thermo Fisher Scientific) on the Qubit 2.0 Fluorometer (Thermo Fisher Scientific) and then sheared using a Covaris E220 Focused-ultrasonicator (Woburn, MA, USA) and the 8 microTUBE–50 Strip AFA Fiber V2 following the manufacturer's instructions. The treatment time was optimized for FFPE material. The treatment settings were as follows: peak incident power (W): 75; duty factor: 15%; cycles per burst: 500; treatment time (s): 360; temperature (°C): 7; water level: 6. For DNA library preparation and enrichment, the TruSight™ Oncology 500 Kit (Illumina) was used following the manufacturer's instructions. Post-enriched libraries were quantified, pooled, and sequenced on a NextSeq 500 (Illumina Inc., San Diego, CA, USA). The quality of the NextSeq 500 (Illumina) sequencing runs was assessed with the Illumina Sequencing Ana"lysis Viewer (Illumina). Sequencing data were analyzed with the TruSight Oncology 500 Local App Version 1.3.0.39 (Illumina). The TruSight™ Oncology 500 is a

comprehensive tumor profiling assay designed to identify known and emerging tumor biomarkers, including small variants, splice variants, and fusions. Importantly, the TruSight™ Oncology 500 measures tumor mutational burden (TMB) and microsatellite instability (MSI), features that are potential key biomarkers for immunotherapy.

TMB was reported as mutations per megabase (Mb) sequenced. There was no consensus of the definition of the high TMB in NET, therefore, we use the cutoff of 10 mutations/Mb as a high TMB [12]."

Comment:

Please add more details on how the MSI and TMB was examined/measured.

Reply:

Thank you for your comments.

In this study, we used the TruSightTM Oncology 500 as the platform of sequencing.

As you already know, the TruSightTM Oncology 500 has been widely used as comprehensive tumor profiling assay including TMB and MSI. We added more details on the method.

Thanks.

Comment:

How was the threshold to define TMB-high (≥ 10 mutations/Mb)/low (< 10 mutations/Mb) established? Please specify.

Reply:

Thank you for your comments. We added reference and more details on the method as follows;

(doi: 10.1016/S1470-2045(20)30445-9.)

"TMB was reported as mutations per megabase (Mb) sequenced. There was no consensus of the definition of the high TMB in NET, therefore, we use the cutoff of 10 mutations/Mb as a high TMB

(12).”

Results

Comment:

Please check number of patients described in the different sections.

Total number of patients: 31

Total number in classified tumors: 1+15+3+15?

Table 1: 1 1+15+3+12

Answer:

Thank you for your comments. We rechecked and corrected the numbers.

We edited the sentence as follows;

“According to the 2017 WHO classification of NENs, 1 (3.2%) patient was diagnosed with a grade 1 tumor, 15 (48.4%) patients with grade 2 tumors, and 3 (9.7%) patients with grade 3 tumors. NEC was diagnosed in 12 (38.7%) patients.”

Comment:

How did the one tumor with high TMB look like histologically? From which part of the tumor was the sample isolated? Would it be possible to also test another distinct histological region within the same tumor, and isolate and sequenced separately?

Answer:

Thank you for your comments.

We added more details of this patient as follows;

“There was only one TMB-high tumor in this study. This tumor was grade 3 NET of the pancreas and the liver biopsy was done. This tumor was MSS and TMB was 266.4 mutations/Mb. Ki-67 level

was 55%. Previously, a single case of grade 3 NET of the pancreas with temozolomide-induced high TMB was reported [18]. In the present analysis, 2 patients underwent the NGS test after temozolomide-based therapy and this patient with TMB-high tumor is one of the two. However, the other patient diagnosed with grade 2 NET of the pancreas did not show high TMB (7.8muations/Mb). Although there is the possibility of treatment options for ICIs after certain treatments, further studies are needed."

Comment:

It would be of interest to see an overview of immunotherapy biomarkers (TMB, MSI) with clinically relevant mutations, gene amplifications, transcripts together with immunohistochemistry results.

Answer:

Thank you for your comments.

We focused the status of TMB and MSI in this manuscript.

A few year ago, our group reported the genomic profiling of metastatic GEP-NET (Journal of Cancer. 2016 May 25;7(9):1044-8. doi: 10.7150/jca.14815. eCollection 2016.)

In that analysis, Of 14 GEP-NET patients available for mutational profiling, 7 (50.0%) patients had one or more aberrations detected. Common aberrations were as follows: SMARCB1 mutation (n=2), TP53 mutation (n=2), STK11 mutation (n=1), RET mutation (n=1), and BRAF mutation (n=1). Gene amplification by nCounter was detected in only 1 patient, showing CCNE1 amplification, and this patient also had a TP53 mutation.

Thanks.

Table 1

Comment:

Survival at the time of analysis, 18, is that years or number of patients?

Answer:

Thank you for your comments.

We corrected it in the Table 1 as follows;

"Survived at the time of analysis"

Comment:

Is survival correlated to TMB? Check trend for a better outcome with low/intermediate TMB?

Answer:

Thank you for your comments.

We performed Cox regression, and there was no correlation between TMB and survival (p = 0.652).

Table 3

Comment:

Explain *

Reply:

Thank you for your comments. We added the footnote as follows;

Table 3. Published studies of TMB in NETs

Diagnosis	Total enrollment (n)	Median TMB	TMB IQR	TMB range	MSI-H
GEP-NET	31	4.7	3.1 – 6.3	0.8 – 266.4	0
Pancreatic NET	75	(Average) 5.8	*	*	0
GI NET	29	5.68	*	0.57 – 11.75	0

Metastatic and locally advanced NEN	85	5.45	3.84 – 8.85	*	*
Pulmonary NET	48	0.31	0.22 – 0.67	*	*
NET	164	5.2	2.6 – 10.4	*	*
High grade GEP-NEN	135	(Average) 9.5	*	*	4%
Low grade GEP-NEN	335	(Average) 5.1	*	*	0%

* Not available.

Figure 1

Comment:

Please specify numbers in bold.

Answer:

Thank you for your comments. We added the footnote as follows;

Figure 1

No	Sex	Age	Primary site	Pathology	Metastasis									TMB
					Li	LN	Lu	Pc	Pl	Pe	Ad	Bo	No	
1	M	59	Stomach	NET Gr 2		■							1	3.1
2	F	67	Stomach	NEC	■	■				■			3	3.9
3	M	76	Stomach	NEC	■			■					2	4.7
4	M	45	Stomach	NEC	■	■	■		■				4	1.6
5	F	53	Small bowel	NET Gr 2	■								1	2.3
6	M	60	Small bowel	NEC		■							1	**8.6**
7	F	55	Rectum	NET Gr 1	■	■							2	3.9
8	M	55	Rectum	NET Gr 2	■	■	■	■					4	0.8
9	F	64	Rectum	NET Gr 2	■	■							2	3.1
10	F	65	Rectum	NET Gr 2	■								1	**5.5**
11	F	73	Rectum	NET Gr 2	■	■						■	3	**6.3**
12	M	54	Rectum	NET Gr 3	■								1	2.4
13	M	59	Rectum	NEC		■							1	**4.7**
14	F	25	Pancreas	NET Gr 2	■								1	3.9
15	M	65	Pancreas	NET Gr 2									0	**5.5**
16	M	37	Pancreas	NET Gr 2	■	■							2	1.6
17	M	63	Pancreas	NET Gr 2	■		■						2	2.4
18	M	50	Pancreas	NET Gr 2	■								1	3.1
19	M	38	Pancreas	NET Gr 2							■	■	2	**4.7**
20	M	70	Pancreas	NET Gr 2	■	■							2	**5.5**
21	F	64	Pancreas	NET Gr 2	■								1	**7.8**
22	M	62	Pancreas	NET Gr 3	■								1	**266.4**
23	M	51	Pancreas	NEC	■	■	■			■			4	2.3
24	F	69	Pancreas	NEC		■							1	3.1
25	M	71	Pancreas	NET Gr 3	■		■			■			3	3.9
26	M	70	Bile duct	NEC									0	**6.3**
27	F	69	Gall bladder	NEC	■						■		2	**4.7**
28	M	68	Gall bladder	NEC		■							1	**6.3**
29	F	71	Gall bladder	NEC	■	■							2	**7.0**
30	F	49	Gall bladder	NEC	■	■							2	**8.6**
31	F	54	Liver	NET Gr 2	■								1	**4.7**

"TMB higher than the median (4.7 mutations/Mb) is emphasized in bold letters.

Abbreviations: No, number; Li, liver; LN, lymph node; Lu, lung; Pc, pancreas; Pl, pleura; Pe, peritoneum; Ad, adrenal; Bo, bone; TMB, tumor mutation burden; M, male; F, female; NET, neuroendocrine tumor; Gr, grade; NEC, neuroendocrine carcinoma"

Comment:

Add abbreviations from the figure in figure legend.

Answer:

Thank you for your comments. We added the abbreviations as follows;

"*TMB higher than the median (4.7 mutations/Mb) is emphasized in bold letters.*

Abbreviations: No, number; Li, liver; LN, lymph node; Lu, lung; Pc, pancreas; Pl, pleura; Pe, peritoneum; Ad, adrenal; Bo, bone; TMB, tumor mutation burden; M, male; F, female; NET,

neuroendocrine tumor; Gr, grade; NEC, neuroendocrine carcinoma"

Comment:

Did the authors make any comparisons NET G3 and NEC with regards to tumor primary site?

Answer:

Thank you for your comments.

We only have three NET G3. This study included too small sample size to analyze the comparisons NET G3 and NEC with regards to tumor primary site. This limitation was described as follows;

"There are some limitations to our study. First, due to the rarity of this disease, this study was based on small sample size. Second, this was the retrospective study only included the patients who had NGS. Therefore, there are possibilities that patients with more advanced stage was included. Further study with large and various samples would be needed in the future."

Discussion

Comment:

Please elaborate more about the results and also discuss potential limitations of the study.

Answer:

Thank you for your comments.

According to your comment, we edited sentences as follows;

"Because NENs are rare tumors, few studies have evaluated TMB in NENs, especially GEP-NENs. Previous studies analyzing TMB in NENs with variable inclusion criteria are summarized in Table 3. Most studies also reported low TMB and low rate of MSI-high in NENs, which is consistent with our analysis (14-16).

However, in our study, there is no statistically significant differences between TMB of well-differentiated NET and poorly-differentiated NEC. Ine study only analyzed NEC also reported low median TMB (5.68 mutations/Mb) (15). However, other study with large number of patients reported higher TMB in high grade GEP-NEN than in low grade GEP-NEN (17). This study also reported 4% of MSI-high tumors in high grade GEP-NEN, which is relatively high rate comparing other study. Those studies all had different inclusion criteria, therefore, further study would be needed.

There was only one TMB-high tumor in this study. This tumor was grade 3 NET of the pancreas and the liver biopsy was done. This tumor was MSS and TMB was 266.4 mutations/Mb. Ki-67 level was 55%.

Previously, a single case of grade 3 NET of the pancreas with temozolomide-induced high TMB was reported (18). In the present analysis, 2 patients underwent the NGS test after temozolomide-based therapy and this patient with TMB-high tumor is one of the two. However, the other patient diagnosed with grade 2 NET of the pancreas did not show high TMB (7.8muations/Mb). Although there is the possibility of treatment options for ICIs after certain treatments, further studies are needed.

There are some limitations to our study. First, due to the rarity of this disease, this study was based on small sample size. Second, this was the retrospective study only included the patients who had NGS. Therefore, there are possibilities that patients with more advanced stage was included. Further study with large and various samples would be needed in the future. "

Reviewer B

This article looks at MSI and tumor mutation burden status in both NETs and NECs of the gastrointestinal tract, demonstrating only a rare case that is TMB-high and no MSI-high cases. I have two major concerns that should be clearly addressed before publication:

Comment:

1. The numbers are very very small, especially considering that NET and NEC are biologically distinct entities. There are only 12 cases of NEC included in the study and other publications have show that up to 20% of GEP NEC can be MSI-high so publishing a study saying MSI-high is rare in NEC would be a problematic message to send. i would recommend looking at separate larger cohorts of either NET or NEC and not combining in one group since these have different precursors, different genomics and different clinical behavior.

Answer:

Thank you for your comments.

Your comment is very important point. We think that your comment could improve the quality of this manuscript.

However, as you already know, NET and/or NEC are very rare entity among various cancers. Furthermore, this manuscript included only metastatic NET/NEC analyzed for tumor mutational burden (TMB). Therefore, sample size of this study was too small.

Currently, we can not extend the sample size because of rarity of study-population. However, we continue to collect the data for metastatic NET/NEC patients analyzed for molecular characteristics. Nevertheless, this analysis showed that, despite very low incidence, there are GEP-NENs with high TMB. For precision medicine, testing for MSI and TMB is needed for this tumor type.

Also, we added sentences as follows;

" *Because NENs are rare tumors, few studies have evaluated TMB in NENs, especially GEP-NENs.*

Previous studies analyzing TMB in NENs with variable inclusion criteria are summarized in Table 3. Most studies also reported low TMB and low rate of MSI-high in NENs, which is consistent with our analysis (14-16).

However, in our study, there is no statistically significant differences between TMB of well-differentiated NET and poorly-differentiated NEC. Ine study only analyzed NEC also reported low median TMB (5.68 mutations/Mb) (15). However, other study with large number of patients reported higher TMB in high grade GEP-NEN than in low grade GEP-NEN (17). This study also reported 4% of MSI-high tumors in high grade GEP-NEN, which is relatively high rate comparing other study. Those studies all had different inclusion criteria, therefore, further study would be needed "

Thanks.

Comment:

2. There is no data described here demonstrating a pathologic re-review of the specimens and WHO criteria for diagnosing GEP NET versus NEC drastically changed in 2017 so a re-review of all specimens should be performed using uniform criteria. For example, case 6 is said to be a NEC from the small bowel which is extremely rare if this is from the ileum, but could be possible if this were from the duodenum. Most high-grade NEN from the ileum are actually grade 3 NET.

Answer:

Thank you for your comments.

According to your comment, we re-analyzed all specimens based on 2017 WHO classification.

We edited sentences as follows;

"Patients selection

Among patients diagnosed with GEP-NEN between 2013 and 2022, we selected patients based on MSI status and TMB evaluated using next-generation sequencing (NGS). The following patient

clinicopathologic characteristics were analyzed: age, gender, pathologic diagnosis, site of metastasis, number of metastases, TMB, and MSI status. Pathologic diagnosis was re-reviewed by pathologist according to the 2017 WHO classification of NENs (11).”

Comment:

I favor rejecting unless both of the above issues can be adequately resolved.

Answer:

Your comment is very important point. We think that your comment could improve the quality of this manuscript.

However, as you already know, NET and/or NEC are very rare entity among various cancers. Furthermore, this manuscript included only metastatic NET/NEC analyzed for tumor mutational burden (TMB). Therefore, sample size of this study was too small.

Currently, we cannot extend the sample size because of rarity of study-population. However, we continue to collect the data for metastatic NET/NEC patients analyzed for molecular characteristics.

Nevertheless, this analysis showed that, despite very low incidence, there are GEP-NENs with high TMB. For precision medicine, testing for MSI and TMB is needed for this tumor type.

Thanks.