Molecular profiling of neuroendocrine malignancies to identify prognostic and therapeutic markers: a Fox Chase Cancer Center Pilot Study

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Background: The rarity of neuroendocrine malignancies limits the ability to develop new therapies and thus a better understanding of the underlying biology is critical.

Methods: Through a prospective, IRB-approved protocol, patients with neuroendocrine malignancies underwent next-generation sequencing of their tumours to detect somatic mutations (SMs) in 50 cancer-related genes. Clinicopathologic correlation was made among poorly differentiated neuroendocrine carcinomas (NECs/poorly differentiated histology and Ki-67 >20%) and pancreatic neuroendocrine tumours (PanNETs/Ki67 <20%) and non-pancreatic neuroendocrine tumours (NP-NETs/Ki67 <20%).

Results: A total of 77 patients were enrolled, with next-generation sequencing results available on 63 patients. Incidence of SMs was 83% (19 out of 23) in poorly differentiated NECs, 45% (5 out of 11) in PanNETs and 14% (4 out of 29) in NP-NETs. TP53 was the most prevalent mutation in poorly differentiated NECs (57%), and KRAS (30%), PIK3CA/PTEN (22%) and BRAF (13%) mutations were also found. Small intestinal neuroendocrine tumours (Ki67 <2%/n=9) did not harbour any mutations. Prevalence of mutations correlated with higher risk of progression within the previous year (32% (low risk) vs 11% (high risk), P = 0.01) and TP53 mutation correlated with worse survival (2-year survival 66% vs 97%, P = 0.003).

Conclusions: Poorly differentiated NECs have a high mutation burden with potentially targetable mutations. The TP53 mutations are associated with poor survival in neuroendocrine malignancies. These findings have clinical trial implications for choice of therapy and prognostic stratification and warrant confirmation.

Gastroenteropancreatic neuroendocrine malignancies are rare, with an annual incidence of 3.65 per 100 000 based on recent SEER data (Lawrence et al, 2011). Less than half of patients (27–46%) with neuroendocrine malignancies present with localised disease and many of these develop recurrent disease after surgical interventions for initially localised disease (Hauso et al, 2008). For unclear reasons, the incidence of these tumours appears to be increasing and, with the long natural history of these tumours, the US prevalence is thought to be in excess of 100 000 (Yao et al, 2008). These tumours can be divided into several subgroups based upon histology and site of origin, with poorly differentiated neuroendocrine carcinomas (NECs) behaving aggressively with short-lived responses to therapy and worse outcomes (Yao et al, 2008).

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Received 2 May 2016; revised 1 July 2016; accepted 6 July 2016; published online 2 August 2016

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Although once treated as a uniform disease in part because of similar histologic appearance, distinctions have been made in recent years between poorly differentiated NECs, neuroendocrine tumours (NETs) of the pancreas (PanNETs) and those originating from other sites in the GI tract. For non-pancreatic NETs (NP-NETs), somatostatin analogues provide symptomatic benefit for patients with neuroendocrine secretory symptoms, and produce a clinically significant static effect on tumoural growth (Rinke et al, 2009). Somatostatin analogues and local therapies represent the mainstay of therapy for non-pancreatic carcinoid tumours and there are recent data supporting the use of mTOR (mammalian target of rapamycin) inhibitors in nonfunctional tumours (Yao et al, 2016). In contrast, PanNETs have demonstrated better response rates than carcinoid tumours to traditional chemotherapy (5-FU/capetecibine, oxaliplatin, temozolomide, streptozosin, and doxorubicin) (Bajetta et al, 2007; Strosberg et al, 2011), and molecularly targeted therapies improve outcomes, with a VEGF-targeted agent (sunitinib) and an mTOR inhibitor (everolimus) demonstrating a progression-free survival (PFS) advantage for patients with advanced PanNETs (Raymond et al, 2011; Yao et al, 2011). However, predictive factors are lacking, and more definitive results for carcinoid tumours are not yet available.

In contrast, much less is known about poorly differentiated NECs, and no prospective studies have evaluated those originating outside of the lung. There is growing recognition that the current WHO grade 3 (G3) category contains two distinct subsets of neuroendocrine neoplasms, one with poorly differentiated histology (poorly differentiated NEC) and the other with well-differentiated histology but discordant Ki67 proliferation index (Basturk et al, 2015; Tang et al, 2016). The poorly differentiated NECs are very aggressive and usually present with advanced stages with dismal prognosis. Treatment strategies for poorly differentiated NECs are often extrapolated from the treatment paradigm for small-cell lung cancer (SCLC) (Walenkamp et al, 2009). These are generally managed with platinum-based chemotherapy with a modest PFS (4 months) and overall survival (11 months) (Moertel et al, 1991; Mitry et al, 1999; Walenkamp et al, 2009; Rindi et al, 2010; Sorbye et al, 2013). After first-line treatment, no further standard therapy has been established for these patients. Recently, several small, retrospective, second-line studies with chemotherapy (temozolomide, oxaliplatin, taxanes, etc.) demonstrate a response rate between 18% and 30% (Sorbye et al, 2014).

Given the large unmet need in this population and the paucity of genetic data specifically for this disease, we developed this prospective study to perform molecular sequencing for patients with advanced neuroendocrine malignancies. The primary objective of this exploratory pilot project was to better elucidate the defining genomic alterations in these tumours. A secondary objective was to identify prognostic and therapeutic targets in order to determine feasibility of a more formalised trial of molecular profiling guiding therapy in this population.

MATERIALS AND METHODS

Patient eligibility and samples. Patients with neuroendocrine malignancies seen at Fox Chase Cancer Center were enrolled onto our prospective study after approval by the Institutional Review Board. Eligibility criteria for this study included consenting adult patients (≥18 years) with histologically confirmed neuroendocrine malignancies of all grades and sites (excluding SCLC and Merkel cell carcinoma). Patients had to have adequate tissue available for sequencing, as determined by pathologist. We excluded small-cell carcinoma and Merkel cell histology from the poorly differentiated NEC cohort to allow for a relatively homogenous patient population and given prior published work on their genomic alterations (Zheng et al, 2015). Patient records/information were anonymised and deidentified before analysis. Tumour samples were obtained from archived formalin-fixed, paraffin-embedded tissue of primary or metastatic site acquired closest to the enrolment date. Patients with insufficient tissue to perform molecular analysis were excluded from the study. At the time of initial enrolment, a peripheral blood sample was also collected to rule out germline mutations and only somatic mutations were reported.

Data collection. Standard demographic data were collected, including gender, age, race, smoking and alcohol use. Clinico-pathologic data were collected on primary tumour location and grade. Date of last follow-up and vital status were collected on all patients. Further assignment to different subgroups was based upon the site of tumour origin as determined by the treating physician: non-pancreatic neuroendocrine tumours or NP-NETs (≤20 mitoses/10 high-power fields (HPFs); Ki67 ≤20%; pancreatic neuroendocrine tumours or PanNETs (≤20 mitoses/10 HPFs; Ki67 ≤20%); or poorly differentiated NECs (poorly differentiated histology; >20 mitoses/10 HPFs; Ki-67 >20%). In order to study the effect of mutational changes on clinical behaviour of neuroendocrine malignancies, treating physicians were also required to classify patients into two arms based on disease characteristics. Arm A consisted of patients with low risk of clinical progression (stable and nonprogressive disease in the prior 12 months) and arm B consisted of patients with high risk of progression (radiographic progression in the prior 12 months, clinical evidence of worsening symptoms, high initial tumour burden requiring chemotherapy or poorly differentiated tumours).

Specimen analysis. Histologic confirmation of diagnosis and grade, and adequacy of tumour samples, was assessed by trained pathologist with expertise in gastrointestinal and neuroendocrine malignancies. After this, tumour and normal genomic DNA were extracted from a portion of the patient’s tumour tissue and peripheral blood, respectively. Tumour and normal DNA were used for multiplex PCR amplification of targeted regions within the 50 cancer-related genes listed below using the Ion AmpliSeq technology (Life Technologies, Carlsbad, CA, USA). Next-generation sequencing (NGS) was performed using the Ion Torrent Personal Genome Machine (Life Technologies, Guildford, CT, USA) and analysed with Torrent Suite Software (v.3.4.2, Life Technologies). Sequencing results from tumour were compared with normal to identify tumour-specific somatic mutations (substitutions and/or small insertions/deletions) within the targeted regions. For clinical testing purposes, the lower limit of detection of the assay is ~10% mutant allele frequency with variant coverage of at least 250×. Tumour nuclei were required to represent at least 20% of the nuclei in the tested sample to avoid false negative results. Reportable tumour-specific somatic variants were verified using direct sequencing analysis (Sanger sequencing) when indicated based on standard laboratory procedures. The cancer-related genes evaluated included ABL1, AKT1, ALC, APC, ATM, BRAF, CDH1, CDKN2A, CSF1R, CTNNB1, EGFR, ERBB2, ERBB4, EZH2, FBXW7, FGFR1, FGFR2, FGFR3, FLT3, GNA11, GNAQ, GNAS, NF1A, HRAS, IDH1, IDH2, JAK2, JAK3, KDR, KIT, KRAS, MET, MLH1, MPL, NOTCH1, NPM1, NRAS, PDGFRα, PIK3CA, PTEN, PTPN11, RB1, RET, SMAD4, SMARCB1, SMO, SRC, STK11, TP53 and VHL.

The results of genomic testing were issued to the treating physician and further treatment decisions were left to them, and the patients’ responses were followed. Actionable mutations were defined as those with ability to guide therapy using approved or experimental agents. Imaging follow-up was recommended every 3–6 months. Patients were enrolled into three separate groups based upon differentiation and site of origin as described in previous section.
Statistical methods. The patient population was characterised using standard descriptive statistics. Frequency tables were used to describe the distribution of variants identified by sequencing. These tables were created for all patients, and separately by the predefined patient cohorts and arms A and B. Mutations that occurred in $\geq 10\%$ of samples were considered for further analysis. Tumour characteristics and mutation status were compared using Fisher’s exact test. Survival was assessed using log-rank tests and Kaplan–Meier curves. All statistical analyses used Stata (version 12.1, StataCorp, College Station, TX, USA). For each cohort, we defined a future larger study as feasible if at least 10% of patients had an actionable mutation identified by NGS.

RESULTS

Patient characteristics. We enrolled 77 patients onto the study between October 2013 and July 2015. Fourteen patients had insufficient tissue to perform NGS. Patient and tumour characteristics of the remaining 63 patients are summarised in Table 1. Median age was 61 years (range 33–84 years) and male to female ratio was 1 : 1. There were 23 (37%) poorly differentiated NECs, 11 (17%) PanNETs and 29 (46%) NP-NETs.

Mutation analysis. Gene profiling results were available on 63 patients (81%). Mutation frequency differed by tumour type and grade. The incidence of at least one mutation was 83% (19 out of 23) in poorly differentiated NECs, 45% (5 out of 11) in PanNETs and 14% (4 out of 29) in NP-NETs. Thirteen (21%) patients’ tumours harboured more than one mutation (11 poorly differentiated NECs and 2 PanNETs). Incidences of individual mutations in the three defined subsets are shown in Figure 1. The most prevalent mutations in poorly differentiated NECs included TP53 (57%), KRAS (30%), PIK3CA/PTEN (22%) and BRAF (13%). Table 2 lists the mutations identified by grade and location of primary. Poorly differentiated NECs for histology and pancreas for site demonstrated the highest frequency of mutations. Interestingly, well-differentiated NETs of small intestinal origin with Ki67 $\leq 2\%$ did not harbour any mutations in the samples tested ($n = 29$). Potentially actionable mutations, depicted in Figure 2, were found in 35% (8 out of 23) of poorly differentiated NECs (BRAF, PIK3CA, PTEN, WNT and CTNNB1).

Mutations and clinical outcomes. Among the PanNETs and NP-NETs, incidence of mutations was higher in patients with high risk of progression (designated arm B) than those with low risk (designated arm A) (7 out of 22 (32%) vs 2 out of 18 (11%), $P = 0.01$). Only one patient in our cohort was treated with a mutation-guided therapeutic intervention. In this case, everolimus was used in the second line in a 57-year-old female with poorly differentiated NEC, harbouring an inactivating PTEN mutation. She remained on the drug for 5 months when therapy was interrupted and later discontinued because of development of brain metastases.

Survival analysis. Over the 21-month period of study conduct (median follow-up of 17 months), there were 7 deaths and median
survival was not reached in any group. Higher grade was associated with worse survival (median not reached, P = 0.004) with 2-year survival rates of 100%, 94%, and 71% for tumors with Ki67 ≤2%, 3–20%, and >20%, respectively. The NP-NETs demonstrated an improved survival rate compared with PanNETs with 2-year survival of 100% vs 96% (P = 0.04), respectively. The association of individual mutations with survival was also studied. The presence of TP53 mutation correlated with worse survival (2-year survival of 66% vs 97% comparing TP53 mutation positive and wild type, respectively; P = 0.003, see Figure 3) but other mutations were not significantly associated with survival (P > 0.05, data not shown).

**DISCUSSION**

There has been significant progress in the understanding of molecular mechanisms underpinning different gastrointestinal malignancies that has generated interest in identifying predictive and prognostic biomarkers (e.g., *KRAS* and *BRAF* mutations in colon cancer). To date, there have been a relatively small number of efforts to better characterise the molecular abnormalities driving the growth of neuroendocrine malignancies. They are a heterogeneous group of neoplasms with probable varied gene signatures for poorly differentiated NECs, PanNETs and NP-NETs. We found that the majority of the poorly differentiated NECs harbour somatic mutations, some of which are potentially targetable (*BRAF*, *PIK3CA*, *PTEN*, *WNT*, etc.). With an incidence cutoff of 10%, our findings support the feasibility of future clinical trials using molecularly matched therapies in patients with poorly differentiated NECs with mutations in the *BRAF* or *PIK3CA*/*PTEN* pathways. On the other hand, NP-NETs have a relatively stable genome with a very low incidence of mutations, suggesting alternate pathways of proliferation (epigenetic or post-transcriptional modifications). TP53 emerged as a prognostic marker in neuroendocrine malignancies.

In our study, NP-NETs had the lowest rate of somatic mutations (14%) and none of the WHO G1 NETs (Ki67 ≤2%) of small intestinal origin harboured a mutation utilising a 50-gene NGS panel. These are typically more indolent than other epithelial malignancies but can nevertheless metastasise (Anthony et al., 2010). Pathogenic mutations in the ‘classical’ signalling pathways are rare in this subset. Whole exome sequencing of 48 small intestine NETs performed by Banck et al. (2013) represents the first genome-wide sequencing study for this tumour type. This important work revealed a 0.1 somatic single-nucleotide variation per 10⁶ base pairs, suggesting a stable genome for carcinoid tumours. Most genomic alterations were copy number variations and were nonrecurrent. The authors noted genomic alterations in PI3K/AKT/mTOR in 14 patients (29%), suggesting that targeted therapy might be directed at this particular signal transduction pathway. The SRC oncogene was found to be upregulated in 11 (23%) cases without an identifiable mutation. Similarly, Francis et al. (2013) reported frameshift mutations and hemizygous deletions of p27 tumour suppressor (*CDKN1B*) in 11% of small intestine NET, thus implicating cell cycle dysregulation in the aetiology of NET. This particular gene was not a part of our NGS panel.

Contrary to current evidence, one patient with NP-NET in our cohort harboured a TP53 mutation (Yachida et al., 2012; Banck et al., 2013). The primary site was stomach rather than small intestine and there is paucity of data about the genomic makeup of NETs of stomach origin that may explain the new finding.

When considering PanNETs, genes implicated in chromatin remodelling have been found to be altered in the vast majority of cases in other series (Jiao et al., 2011). Among 68 nonfamilial PanNETs, 44% carried somatic inactivating mutations in *MEN1* (the multiple endocrine neoplasia type 1 gene)/*menin*, a component of a histone methyltransferase complex, and 43% had mutations in genes encoding the subunits of a transcription/chromatin remodelling complex consisting of *DAXX* (death-domain–associated protein) and *ATRX* (axin 1/epithelial/mental retardation syndrome X linked). Clinically, mutations in the

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**Table 2. Mutation distribution by location and grade of tumour**

| Site                  | Poorly differentiated NEC (G3) | Differentiated NETs | Total (n/N) |
|-----------------------|--------------------------------|---------------------|-------------|
|                       | n/N                            | Type of mutation    | n/N         | Type of mutation | n/N | Type of mutation | n/N |
| Small intestine       | 17/1                           | TP53                | 2/8         | CTNNB1, PIK3CA   | 0/9 | —                | 3/18 |
| Colon                 | 8/9                            | KRAS, TP53, BRAF, PIK3CA, PTEN, CTNNB1, APC, RB1 | 0/3         | —                | 0/1 | —                | 8/13 |
| Pancreas              | 4/4                            | TP53, PIK3CA, RB1, KRAS | 4/10        | KRAS, TP53, IDH1, RB1 | 1/1 | ATM              | 9/15 |
| Other                 | 7/9                            | PTEN, BRAF, APC, IDH1, TP53, CTNNB1, FBXW7 | 1/2         | TP53             | 1/6 | KRAS             | 9/17 |
| Total                 | 19/23                          |                      | 7/23        |                  | 2/17 |                  | 9/40 |

Abbreviations: Other = G1 – unknown, stomach; G2 = unknown, stomach; G3 = unknown, breast; n = number of patients with mutations; N = total number of patients; NEC = neuroendocrine carcinoma; NET = neuroendocrine tumour.

*a* ≥ 20 Mitoses/10 high-power fields (HPFs) or Ki67 > 20%, poorly differentiated histology.

*b* 1–20 Mitoses/10 HPFs or Ki67 3–20%.

*c* ≤ 2 Mitoses/10 HPFs or Ki67 ≤ 2%.
MEN1 and DAXX/ATRX genes were associated with better prognosis. The same group also found mutations in genes in the mTOR pathway in 14% of the tumours. Another study from Asia found an inverse association of DAXX/ATRX mutations with prognosis (Yuan et al., 2014). The association of DAXX/ATRX and MEN1 with prognosis and their molecular role is an area of active investigation. Our gene panel did not include DAXX/ATRX and MEN1 genes and we did not identify any mutations in the mTOR pathway for PanNETs, perhaps related to the low number of these tumours in our cohort. The PanNETs have paucity of Rb1 and TP53 mutations based on prior literature (Jiao et al., 2011; Yachida et al., 2012) but our analysis reported Rb1 mutation in one and TP53 mutation in two intermediate grade PanNET samples (2–20 mitoses/10 HPFs; Ki67 3–20%).

The molecular mechanisms underlying the aggressive poorly differentiated NEC subtype and determinants of progression are unknown. A few studies describing their genomic landscape have been reported. Abnormal immunolabelling patterns of p53 and Rb were frequent (p53, 95%; Rb, 74%) in poorly differentiated NECs of pancreatic origin (small and large cell histologies), whereas SMAD4/DPC4, DAXX and ATRX labelling were intact in virtually all of these carcinomas (Yachida et al., 2012). Our study provides further insight into the mutational characteristics that define these tumours. Poorly differentiated NECs, despite being considered equivalent to SCLC in terms of management (Strosberg et al., 2010), may have different genomic alterations. Our study detected TP53 inactivating mutations in ~60% (vs >90% in SCLC), Rb1 in 8% (vs 90% in SCLC), KRAS in 30%, BRAF in 13% and PIK3CA/PTEN in 22% of poorly differentiated NECs (Yokomizo et al., 1998; Wistuba et al., 2001; Zheng et al., 2015). Mutations in genes involved in the β-catenin pathway (APC, CTNNB1) were seen in 14% of poorly differentiated NECs. Such mutations are uncommon in SCLC. These striking differences, in part, may be related to variations in the primary site of origin for the poorly differentiated NECs. Thus, sequencing may increase confidence of a GI origin for poorly differentiated NECs presenting with this signature, and the ‘default’ strategy of treating poorly differentiated NECs like SCLC may in fact not be appropriate for some patients. Newly developed therapies and clinical trials may offer opportunities to further assess molecular profiling and targeted agents in these tumours. The ECOG/ACRIN 2142 study is currently comparing first-line platinum-based therapy to a combination of capcitabine and temozolamide for high-grade NETs but therapy is not guided by predictive biomarkers. Snyder et al. (2014) described the importance of tumour genetics in defining the basis of the clinical benefit from checkpoint blockade and immune checkpoint inhibition. In their study, mutational load and expression of neo-antigens correlated with improved overall survival and response to immunotherapy (Snyder et al., 2014). We found a staggering 83% (19 out of 23) incidence of mutations and 47% rate of >1 identified mutations (on the limited 50-gene panel) in poorly differentiated NECs. This suggests that a high level of genomic instability would likely be detected on whole exome or genome

**Figure 2. Heat map describing somatic mutations identified in each case.** Each column represents one sample, and each row represents one gene. Nonactionable somatic mutations are shown in grey (1) and potentially actionable mutations are shown in yellow (2). Only genes in which a somatic mutation was detected in one or more samples are depicted.

**Figure 3. Adjusted Kaplan–Meier survival curves for patients without and with TP53 mutations (2-year survival 97% vs 66% \(P=0.003\)).**
sequencing of the poorly differentiated NECs, thus making checkpoint inhibition an additional therapeutic strategy worthy of testing. The presence of TP53 mutations correlated with worse survival in our cohort but the results may reflect the higher number of poorly differentiated NECs in the mutation-positive group.

We have previously reported a 16.5% incidence of actionable mutations from a retrospective analysis of 1350 cases of in infradiaphragmatic neuroendocrine malignancies (all grades and sites), but most mutations were not seen more than once (BRAF, CTNNB1, KIT, EGFR, FGFR2, PIK3CA, NRAS and APC) (Astsaturov, 2014). Our group has also reported a near complete response to imatinib seen in a patient with KIT-mutated metastatic NET (Perkins et al, 2014), supporting the theory that modulation of these targets with specific inhibitors carries the potential for beneficial clinical application and highlights the need for molecularly driven studies in neuroendocrine malignancies (Perkins et al, 2014). In our current study, the incidence of potentially actionable mutations was particularly high in the poorly differentiated NEC population (PIK3CA/PTEN (22%) and BRAF (13%)). A future larger study of molecular-guided therapy in this cohort of patients may be feasible as 10% of patients had the mutation identified by NGS. Whether this will result in clinical benefit requires further follow-up and research.

Our study is limited by the number of mutations assessed in the targeted cancer gene panel (n = 50 of cancer-related genes). Banck et al (2013) noted genomic alterations (copy number variations rather than mutations) in the PI3K/AKT/mTOR pathway in 29% of carcinoid samples through whole exome sequencing (compared with the relatively low number of PIK3CA or mTOR mutations found in our study (4%). This in part can be attributed to the lack of gene amplification and copy number variation data in our samples. In addition, mutations that may characterise PanNETs like DAXX/ATRX were not on our panel (Jiao et al, 2011). A minority of the poorly differentiated NECs in our cohort did not harbour any mutations (4 out of 23) and this may be related to the small number of genes tested on the panel. However, our platform incorporated most common driver mutations known to date for which targeted therapies are actively being developed, making the results clinically relevant in the present time. A second potential limitation is that we utilised a archived primary tumour specimen mutation analysis rather than a fresh biopsy. The utility of an present time. A second potential limitation is that we utilised a fresh biopsy remains an important unresolved question. Another limitation of our study is the smaller sample size of cohorts with biopsy.

In summary, we found in this prospective study, clinically significant mutations in poorly differentiated NECs that most commonly included PIK3CA/PTEN and BRAF. Although rare in well-differentiated NETs, the presence of mutations was associated with higher risk of progression and may portend worse survival. Likewise, TP53 mutations are associated with poor survival. These findings have potential implications for neuroendocrine malignancies in terms of choice of therapy, clinical trial enrolment, primary site determination and prognostic stratification, thus supporting the role of molecular sequencing in the setting of clinical trials.

ACKNOWLEDGEMENTS

This study was supported by Cancer Genome Institute Grant-061. This work was also generously supported by Mrs. Jeanne Leinen and the Leinen family, Bonnie and Allen Haldeman and the Deni M. Zodda Trust to Fox Chase Cancer Center.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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