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Somatic mutation of *CDKN1B* in small intestine neuroendocrine tumors

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Abstract

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The diagnosed incidence of small intestine neuroendocrine tumors (SI-NETs) is increasing, and the underlying genomic mechanisms have not been defined for these tumors. Using exome/genome sequence analysis of SI-NETs, we identified recurrent somatic mutations and deletions in *CDKN1B*, the cyclin-dependent kinase inhibitor gene, which encodes p27. We observed frameshift mutations of *CDKN1B* in 14 of 180 SI-NETs, and we detected hemizygous deletions encompassing *CDKN1B* in 7 out of 50 SI-NETs, nominating p27 as a tumor suppressor and implicating cell cycle dysregulation in the etiology of SI-NET.

Neuroendocrine tumors (NETs) are rare neoplasms (~1 per 100,000) that are thought to arise from endocrine precursor cells and occur most commonly in the lung, pancreas, and small intestine [1]. Well-differentiated NETs are typically more indolent than other epithelial malignancies but nevertheless can metastasize [1]. Both germline and somatic mutations of the multiple endocrine neoplasia type 1 gene, *MEN1*, are common in lung and pancreatic NETs [1]. Pancreatic NETs are also characterized by recurrent somatic mutations in the *DAXX, ATRX, PTEN* and *TSC2* genes [2]. In SI-NETs, by contrast, evidence for focal events indicative of driver alterations has remained inconclusive; hemizygous loss of chromosome 18 is the most frequent known genomic event, followed by arm level gains of chromosomes 4, 5,14 and 20 [3-5]. Recently, a whole-exome sequencing study of 48 SI-NETs examining somatic single nucleotide variants (SSNVs) identified mutations in several cancer genes although none were recurrently altered [5].

To identify genomic alterations driving tumorigenesis in SI-NETs, we profiled 55 tumors from 50 individuals by a combination of whole-exome and whole-genome sequencing (Fig. 1a and Supplementary Tables 1-5). Mutation analysis of the exome sequencing data with the MuTect algorithm [6,7] revealed a total of 1230 genes with somatic mutations, of which 90% (1113/1230) were mutated in only a single individual. A relatively low non-silent SSNV rate of 0.77/Mb (range 0.13 to 2.51 per Mb) was observed (Figure 1a and Supplementary Fig. 1a and Table 6). Of the 1230 mutated genes in our study, 21 were also found to be mutated in the previous SI-NET study and another 17 in the pancreatic NET study including the cancer census genes *ATRX* and *COL1A1*, each in a single individual (Supplementary Fig. 1b-d) [2,5,8]. The lack of substantial overlap in recurrently altered genes suggests that many of the mutations are passengers. There are potentially therapeutically targetable mutations in genes including *SRC, FYN, KDR* and *IDH1* (R132H), however each are present within a single individual (Supplementary Table 6).

Significantly mutated genes were identified by measuring the nucleotide-specific and sample-specific mutation rates in the SI-NET sequence data, computing an expected gene-specific mutation frequency for the SI-NETs based on the size and nucleotide composition of each gene, and then comparing the actual mutation frequency for each gene to the calculated expected number [9]. This analysis of the 50 SI-NET cases identified statistically significant mutations in only one gene, the cell cycle regulator *CDKN1B* (p=6.5e-10). In total, we found small insertions and deletions within *CDKN1B* in 10% (5/50) of cases (Fig. 1a and Supplementary Table 7), leading to frameshift mutations (Fig. 1b). These mutations were validated by independent PCR and sequencing. Furthermore, copy number analysis identified hemizygous deletions encompassing *CDKN1B* in 7 cases (Fig. 1c). Four out of

these seven SI-NETs with *CDKN1B* deletions retained both copies of chromosome 18 compared to 8 out of 35 SI-NETs without *CDKN1B* deletion (P=0.048, two-tailed Fisher's exact test. The region encompassing *CDKN1B*, 12p13, is frequently hemizygously deleted in ovarian, prostate, non-small cell lung cancer and multiple hematological malignancies [10-16].

To confirm the incidence of *CDKN1B* mutations in SI-NETs, we analyzed two independent cohorts; 48 SI-NETs reported by Banck *et al*. [5] and an extension set of 81 SI-NETs sequenced to a mean 800-fold coverage at *CDKN1B*. Two previously unreported somatic deletions within *CDKN1B* were detected in the Banck *et al*. cohort that was not previously analyzed for indels [5], resulting in frameshift mutations. The extension cohort revealed 7 small indels within *CDKN1B* leading to frameshifts; the extension set did not have paired germline DNA so we cannot exclude the possibility that some of these inactivating alterations are germline. Overall, heterozygous frameshift *CDKN1B* mutations were detected in 8% (14/180) of SI-NETs analyzed.

The presence of heterozygous inactivating mutations in *CDKN1B* is consistent with the possibility that *CDKN1B* acts as a haploinsufficient tumor suppressor gene in SI-NETs. One possible explanation is that some p27 expression is necessary for cell proliferation, as has been described in certain oncogenic models [17,18], thus making bi-allelic deletion disfavored. Several recurrently cancer-mutated genes, including *FBXW7*, *PTEN*, *DICER1* and *CREBBP* have recently been reported to be haploinsufficient tumor suppressors in mouse genetic models of cancer [19-22]. The increased susceptibility to tumors following DNA damage observed in *Cdkn1b* heterozygous knockout mouse models along with elevated cellular proliferation [23-26] is consistent with the hypothesis that *CDKN1B* is haploinsufficient for tumor suppression.

Hemizygous loss of chromosome 18 (log2 (copy number/2)<-0.1) was found in ~78% (43/55) of SI-NETs, but was associated with only a slight increase in mutation rate genome wide (Supplementary Fig. 2 and 3). Two genes, including *BCL10*, a gene mutated in colorectal cancer [27], were found to be altered exclusively in the 12 cases with diploid chr. 18 (Supplementary Fig. 3d). Because of the high frequency of hemizygous deletion of chr.18, we examined the cohort for somatic mutations to growth inhibitory or "STOP" [28] genes within the three frequently deleted regions of this chromosome. While we observed no somatic mutations, it is possible that hemizygous loss of these genes may contribute to SI-NET tumorigenesis through altered gene dosage (Supplementary Table 8). In addition, comparison with genes mutated in small cell lung cancer (SCLC) [29,30], a tumor type that shares neuroendocrine characteristics with SI-NETs, showed 199 genes with mutation in common in both studies; however, this overlap may be due to the high overall mutation rate in SCLC rather than a shared mechanism of tumorigenesis (Supplementary Fig. 4 and Tables 9 and 10).

To survey for genomic rearrangements, we performed whole genome sequencing on 24 tumor/normal sample pairs. The number of somatic rearrangements detected by paired-end and split-read mapping [31,32] ranged from 0 to 45 per case, with a median of 7 (Supplementary Fig. 5 and Table 11). Of those, 20% (33/163) of rearrangements involved genes or promoter regions, leading to five potential fusion proteins and 2 in-frame deletions,

however none were recurrent (Supplementary Table 12). The concordance of SSNVs identified in whole-genome and whole-exome was on average ~95% when sufficient coverage was available [6].

Tumor heterogeneity within epithelial tumors can be exceptionally complex and cells shed from the primary can form distant metastases [33]. Approximately 25% of SI-NETs are multifocal tumors at time of resection and 10-15% of neuroendocrine metastases are diagnosed as being of unknown primary origin [34,35]. When we compared exomic mutations and copy number data for the paired primary and metastatic tumors, we observed no overlap in SSNVs or SCNAs between the primary and metastasis in 2 out of 5 primary/metastatic/normal trios (Fig. 2 and Supplementary Table 13). We confirmed that germline SNPs were concordant in the trios to exclude sample mix-up. In one particular case (A16), the primary tumor contained a *CDKN1B* frameshift mutation while the metastasis did not, a phenomenon also reported for *PIK3CA* and *EGFR* in breast and non-small cell lung cancer, respectively [36,37]. It is hypothesized that the metastases in these two cases may have been derived from either an undiagnosed independent primary lesion, a subclonal population that was not detected by sequencing, or a clone that was shed from the primary tumor early in progression prior to the acquisition of major genomic events. In contrast, Banck *et al.*, assessed the overlap of 35 gene mutations in paired primary and metastasis samples and observed an 83% concordance in 5 cases [5]. Given the small number of primary/metastatic/normal SI-NET trios (five cases each) in our two cohorts, the difference between these datasets is consistent with statistical fluctuation. A Fisher's exact test comparing a case series with two primary/metastatic discordances and three concordances, to a case series with five primary/metastatic somatic mutation concordances and no discordances, yields $p = 0.22$, consistent with the null hypothesis that the two datasets are identical. This suggests that SI-NETs are observed in a population wherein a subset of patients may harbor multifocal tumors and a subset may harbor unifocal tumors. The discordance amongst primary and metastasis along with the multifocal nature of SI-NETs highlights a challenge in identifying underlying driving events in these tumors.

Somatic mutations targeting the cell-cycle regulatory gene *CDKN1B* were the most frequent gene-specific events in SI-NETs. *CDKN1B* encodes a cyclin-dependent kinase inhibitor that binds to and inhibits Cdk2 and Cdk4 [38,39]. Mouse models of *Cdkn1b* haploinsufficiency have larger body and organ size and enhanced sensitivity to mitogenic stimulation owing to greater Cdk2 activity [23-25]. In contrast to *CDKN2A* and related genes encoding Cdk4/Cdk6 inhibitors, somatic mutations in *CDKN1B* have been recently reported at low frequency in breast and prostate cancers [16,40,41]. *CDKN1B* is also known as *MEN4*, a gene mutated in the germline of families with a phenotype of MEN-1 syndrome without an identifiable *MEN1* gene mutation [42]. Furthermore, menin, the MEN1 gene product, associates with promoter regions to mediate expression of *CDKN1B* and *CDKN2C* through epigenetic regulation [43,44].

In summary, this study presents a comprehensive genomic analysis of somatic variants and whole-genomes of SI-NETs. SI-NETs are dominated by large, arm-level copy number gains and losses but there were strikingly few recurrent somatic gene alterations. The discovery of recurrent *CDKN1B* mutations raises the possibility that the p21/p27/p57 family may

represent haploinsufficient tumor suppressors, and suggests a focus on cell cycle regulation in understanding the pathogenesis of SI-NETs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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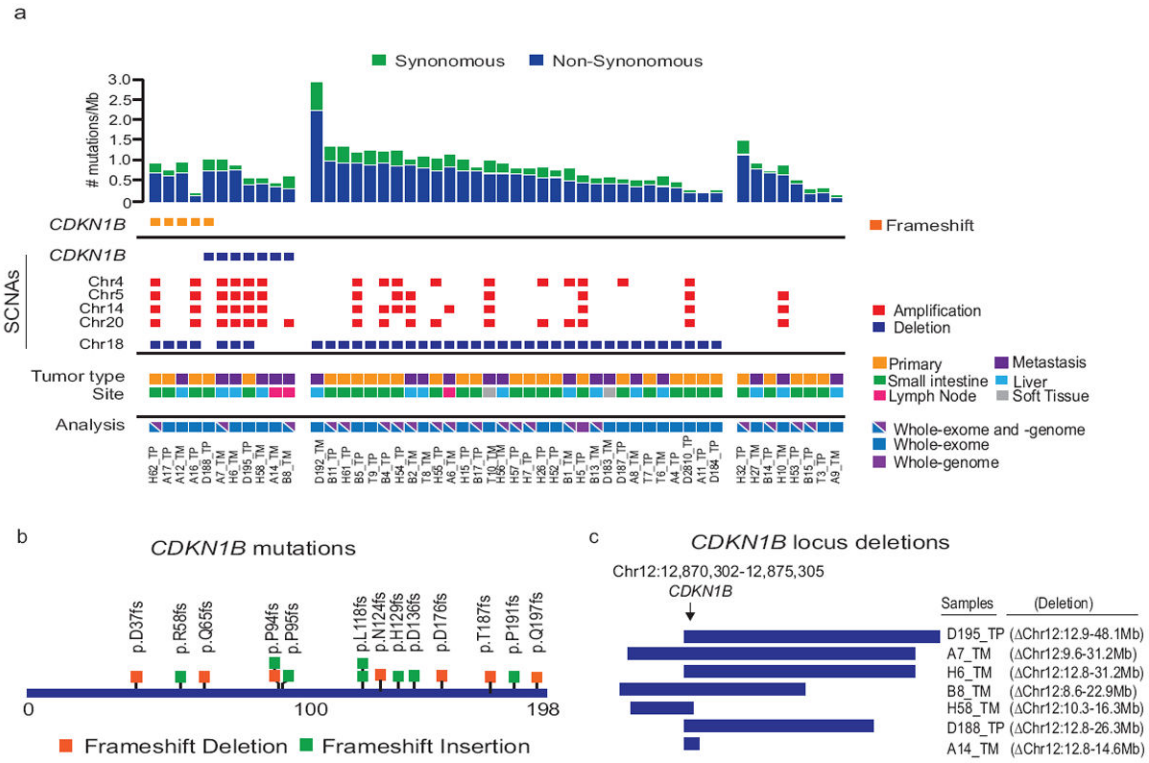

Figure 1.

Mutational analysis of 31 small bowel and 19 metastatic SI-NETs.

a) Top panel shows the somatic mutation rate per megabase (Mb) of covered target sequence in the 50 cases. Middle panel shows the recurrent somatic mutation of *CDKN1B* and prominent somatic copy number alterations found in each tumor. Primary (TP) and metastatic (TM) tumors, sites and type of sequencing performed are indicated by colored boxes. b) Schematic representation of frameshift mutations identified in *CDKN1B*. c) Schematic of the hemizygous deletions identified targeting *CDKN1B*.

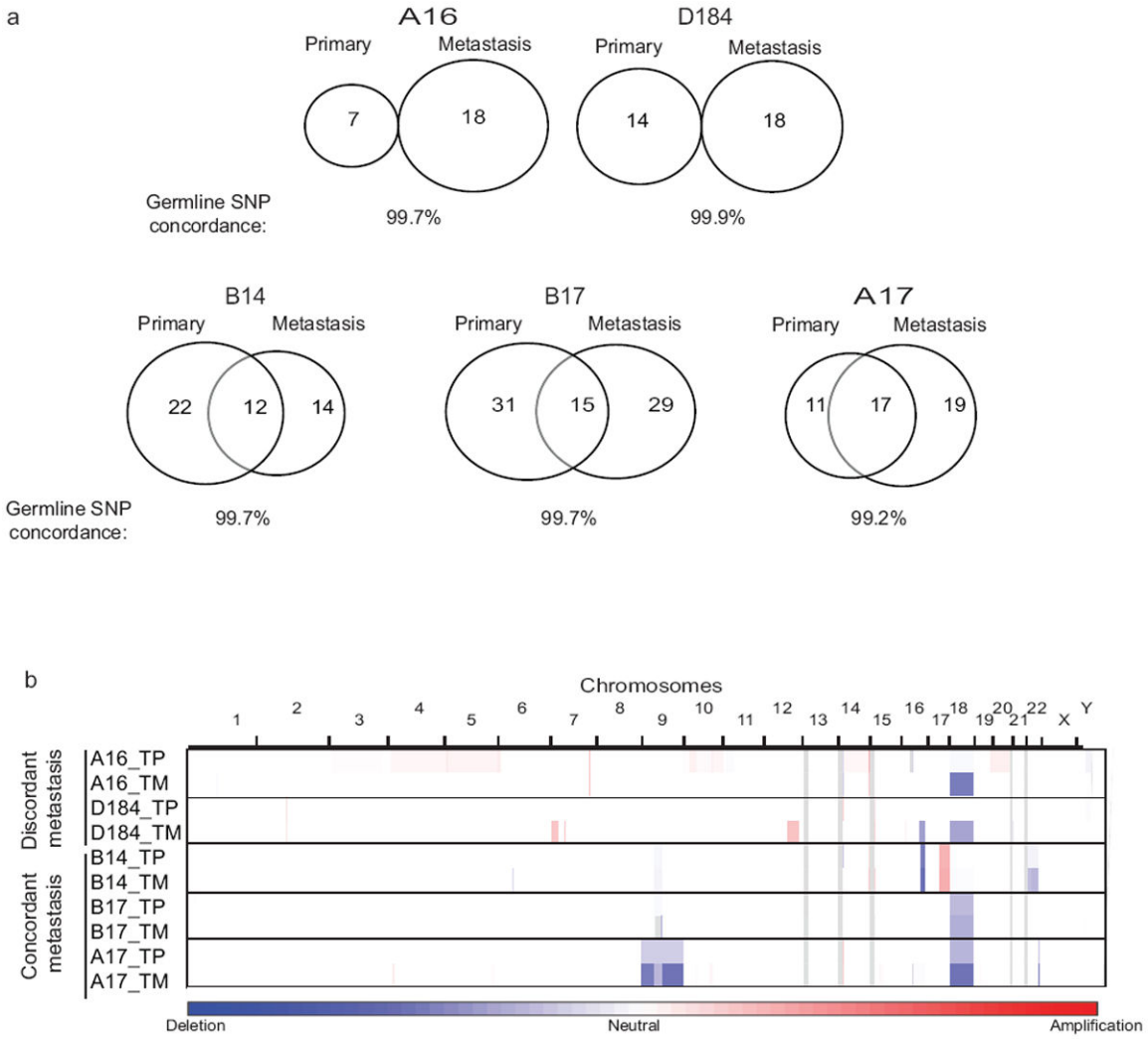

Figure 2.

Somatic mutation and copy number discordance between primary and metastatic cases.

a) Venn diagram depicting the concordance and discordance in somatic mutation calls in the primary or metastatic lesion analyzed in 5 cases. b) Copy number profiles for concordant and discordant primary and metastatic tumors.