Somatic alterations of *CDKN1B* are associated with small bowel neuroendocrine tumors

Jessica E. Maxwell^a, Scott K. Sherman^a, Guiying Li^a, Allen B. Choi^a, Andrew M. Bellizzi^b, Thomas M. O'Dorisio^c, and James R. Howe^{a,*}

^aDepartment of Surgery, Carver College of Medicine, University of Iowa, Iowa City, Iowa

^bDepartment of Pathology, Carver College of Medicine, University of Iowa, Iowa City, Iowa

^cDepartment of Internal Medicine, Carver College of Medicine, University of Iowa, Iowa City, Iowa

Abstract

CDKN1B, a cyclin-dependent kinase inhibitor associated with G1 arrest, was recently proposed as an important tumor suppressor gene in small bowel neuroendocrine tumors (SBNETs). The rate of frameshift mutations in SBNET primaries are reportedly 7.4%, and hemizygous deletions are 6.7%. We set out to confirm the role of *CDKN1B* mutations and copy number variants (CNVs) in primary SBNETs, and whether these are also found in pancreatic neuroendocrine tumors (PNETs). Genomic DNA was isolated from 90 primary SBNETs and 67 PNETs. Coding exons of *CDKN1B* were amplified by PCR and sequenced. CNV analysis was performed by quantitative PCR, p27 expression was evaluated using immunohistochemistry. In SBNETs, three frameshifts, one missense mutation, and three CNVs were observed. The total rate of *CDKN1B* alterations was 7.0% (6 of 86; 95% confidence interval (CI) 3.2–4.4%). The frameshift rate was 3.5% (95% CI 1.1–9.8%). One SBNET patient had a hemizygous deletion of *CDKN1B*, and two patients had duplications (3.4%; 95% CI –0.41–7.2%). One PNET patient had a duplication, and two patients had hemizygous deletions (4.8%; 95% CI –0.44–10%). Alterations of cell-cycle control due to alterations in *CDKN1B* may be one mechanism by which SBNETs develop, which could have implications for new treatment modalities.

Keywords

Neuroendocrine tumor; small bowel neuroendocrine tumor; pancreatic neuroendocrine tumor; p27; CDKN1B; frameshift mutation

Over the past 40 years, the incidence of neuroendocrine tumors (NETs) in the United States has nearly tripled from 1.09 to 3 cases per 100,000 each year (1). This timespan has also seen the introduction of NET-specific treatments, such as octreotide, that improve the quality of life for many patients. Unfortunately, this drug is not cytotoxic to neuroendocrine cells and does not significantly affect a patient's overall survival (2). Drugs targeting the specific

*Corresponding author. james-howe@uiowa.edu.

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molecular pathways disrupted in tumors are used for medullary thyroid carcinomas (3) and pancreatic neuroendocrine tumors (4,5), but drug development specific for small bowel NETs (SBNETs) has been frustrated by the lack of knowledge of the molecular mechanisms that are important in the initiation and progression of these neoplasms.

Three groups have reported on gene mutations and copy number variations in SBNETs (6–8). In two of these studies, the *CDKN1B* gene was altered in approximately 8.5–10% of SBNETs, suggesting a role in SBNET carcinogenesis (7,8). *CDKN1B* encodes for the protein p27, which inhibits the human cyclin E-Cdk2 and cyclin D-Cdk4 complexes, ultimately preventing the cell from moving into the synthesis phase of the cell cycle (9,10). In mice, homozygous knockouts of p27 have alterations of endocrine signaling in the hypothalamic-pituitary-ovarian axis, leading to pituitary hyperplasia and infertility (11).

In humans, germline mutations of *CDKN1B* leading to dysfunctional p27 cause multiple endocrine neoplasia 4 (MEN4). The phenotype for this disease most closely resembles that of MEN1, with pituitary and parathyroid adenomas predominating, although the limited number of patients reported has made delineation of the full disease spectrum difficult (12,13). A study that investigated gastroenteropancreatic neuroendocrine tumors found that patients with loss of nuclear p27 expression had worse survival rates compared with those who had normal expression (57 months vs. 140 months, $P = 0.037$) (14). Many studies in mice and humans support the fact that haploinsufficiency of *CDKN1B* leads to loss of its tumor suppressor function. In patients with a known somatic mutation and concomitant cancer, variable levels of p27 expression are seen, but are usually depressed (15).

The evidence supporting the role of *CDKN1B* in endocrine tumorigenesis is compelling, although examination of additional series of SBNETs would help to confirm its importance. We therefore set out to determine the frequency of *CDKN1B* mutations in a cohort of primary and metastatic SBNETs to corroborate the mutation rates proposed in the previous report by Francis et al. (7) and more recently by Crona et al. (8). We also wanted to examine whether *CDKN1B* alterations are seen in PNETs, due to the clinical overlap of the MEN1 and MEN4 syndromes. PNETs develop in MEN1 patients, but these have not been described in the rare reports of MEN4, which are caused by germline *CDKN1B* mutations. Finally, we inspected the level and location of expression of p27 in patients with mutated and wild type *CDKN1B* in primary SBNETs and PNETs.

Materials and methods

Patient samples

This is a retrospective, single-institution study. All patients were enrolled under an Institutional Review Board–approved protocol from 2004 to 2013. Primary tumors, metastases, and associated normal tissue were collected at the time of surgery from 90 patients with SBNETs and 67 patients with PNETs. Tissues were stored at -20°C in RNAlater solution (Life Technologies, Carlsbad, CA). Tumor cellularity was estimated by examining whole sections of paraffin-embedded tissues. Genomic DNA (gDNA) was isolated from 90 SBNET primary tumors, 52 liver metastases, 80 lymph node metastases, 4 peritoneal nodules, and 85 normal small bowel samples using the Qiagen DNeasy Blood &

Tissue Kit (Venlo, Netherlands). In PNET patients, genomic DNA was isolated from 67 primary tumors, 2 liver metastases, 3 lymph node metastases, and 5 normal pancreatic tissues using the same methods.

Detection of somatic mutations in NET tumors within the coding exons of *CDKN1B*

To detect somatic exonic or splice-site mutations in *CDKN1B*, gDNA from 86 SBNET and 67 PNET primary tumors was amplified using the PCR primers and conditions outlined in Table 1. The PCR primers amplify the two coding exons (exons 1 and 2) and their intron/exon boundaries. Exon 1 was split into two parts and amplified using two primer pairs. Amplicons were purified over a silica-membrane column (QIAquick PCR Purification Kit, Qiagen) and then bidirectionally sequenced with the same primers. Somatic mutations were analyzed with Lasergene 11 software (DNASTAR Inc., Madison, WI).

The genomic DNA of metastases derived from mutated primary tumors was amplified and sequenced to determine the mutational concordance in these primary-metastasis sets. Four pairs were from patients with primary SBNETs, and one pair was from a PNET patient. To investigate the possibility of these mutations being in the germline, this procedure was also performed in the normal small bowel or pancreas tissues. The frequency of de novo *CDKN1B* mutations arising in the metastases from patients with wild type primaries was investigated by sequencing the metastases and normal tissue from 61 patients with SBNETs who did not have somatic mutations in their primary tumors. This was also performed in four PNET patients.

Accession codes for *CDKN1B* somatic mutations

The three frameshift mutations discovered in SBNETs were submitted to the NCBI ClinVar database (<http://www.ncbi.nlm.nih.gov/clinvar/>). The accession numbers for these mutations are SCV000189147, SCV000189148, and SCV000189149.

Determination of *CDKN1B* copy number variation

Copy number variation in *CDKN1B* was determined for all primary SBNETs (n = 90) and PNETs (n = 64) using the Taqman Copy Number Assay (Life Technologies). The *CDKN1B*-specific probes were positioned at the most 5' end of exon 1 (Hs02136152_cn) and 3' end of exon 3 of the gene (Hs00515405_cn), and were run concurrently. Reactions were performed in quadruplicate. Each patient sample was run once with the internal control ribonuclease P RNA component H1 (*RNase P*), which was positioned at chromosome 14q11.2. All assays were repeated with a second internal control, telomerase reverse transcriptase (*TERT*), which derives from 5p15.33. This was done to ensure that potential duplication of chromosome 14 had not affected the copy number variant (CNV) results, which Kulke et al. found in a subset of SBNETs (16). Results from each control probe were used to calculate the mean delta-delta threshold cycle (ddCt) relative to the two *CDKN1B* probes, and the CopyCaller software (Version 2.0, Life Technologies) was used to determine confidence levels for each. Loss of the gene was called when the ddCt was >0.5; a gain was called when the ddCt was <-0.5. When confidence levels were low using one control (< 80%), they were repeated. If confidence remained low, the results with the other control were used to determine loss or gain. If results with both probes had high confidence values,

but the gain/loss interpretations were different, the sample was considered discrepant. When confidence levels of both controls were 80% despite repeats, they were not considered in the analysis.

Examination of p27 expression using immunohistochemistry in mutated tumors

Immunohistochemistry was performed using a monoclonal antibody against p27 (clone SX53G8; Dako, Carpinteria, CA). Staining was performed on 4- μ m tissue sections cut from formalin-fixed, paraffin-embedded tissues. Each section was deparaffinized, rehydrated, and subjected to heat-induced epitope retrieval in citrate buffer (pH 6). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide. The sections were incubated with the primary antibody at a dilution of 1:500 for 1 hour at room temperature, and detected with the Dako Envision Kit. Sections from normal pancreas served as the positive control. Citrate buffer was substituted for the primary antibody in the negative control.

Each sample was examined by a gastrointestinal pathologist and assigned an H-score to quantify the level of expression of p27. The H-score is calculated as the product of the extent (% of cells) and intensity (0, none; 1+ faint; 2+ moderate; 3+ strong) of staining. If multiple sections of the same tumor were stained, the average H-score was reported. The nuclear and cytoplasmic expression of p27 was determined for every sample.

Statistical analysis

All statistical analyses were performed in the R software (version 3.1.0, The R Foundation, Vienna, Austria). *P*-values were obtained using the Fisher exact test. Confidence intervals (CIs) were determined using Wilson's CI calculation; 95% CIs were constructed for purposes of comparison of each investigator's mutation rate (e.g., Francis et al. (7), Banck et al. (6), Crona et al. (8)), because they were not provided in the manuscripts. The mutation rates were considered as not significantly different if the 95% CIs were overlapping, and if the *P*-value for the calculated z score was >0.05 (17).

Results

Tumor cellularity assessment of SBNETs and PNETs

Tumor cellularity was estimated for 75 primary SBNETs (87% of total tumors; Supplementary Table S1), 59 SBNET metastases (56%), 50 primary PNETs (75%), and 2 PNET metastases (40%). The median cellularity of SBNET primary tumors was 72% (range: 22–90%); 90% of tumors were >50% cellularity, 64% were 70%, and only 5% of tumors were <40% cellularity. In SBNET metastases, the median cellularity was 86% (range: 16–94%); 98% of metastases were 50% cellularity, 91% were >70%, and only 2% were <40% cellularity. In PNET primaries, the median cellularity of the primary tumors was 80% (34–96%); 94% were above 50% cellularity, 74% were at 70%, and only 4% were <40% cellularity. The two PNET metastases measured had 80% and 88% cellularity, respectively.

***CDKN1B* sequencing alterations in SBNETs**

Of the 86 primary SBNETs sequenced, 3 had frameshift mutations that resulted in a premature stop codon (3.5%; 95% CI 1.1–9.8%); Table 2; Supplemental Figure S1). Mutational concordance was determined for all of the metastases and normal small bowel samples of these patients. The frame-shift mutations found in the primary tumors were observed in all of the patients' associated metastases, but were not present in their normal tissue. This resulted in a primary-metastasis frameshift mutation concordance rate of 100%.

One non-synonymous missense mutation was found in an SBNET primary, its lymph node metastasis, and its normal small bowel tissue (SBNET 162-1; Table 2). This resulted in a change at amino acid 173 from asparagine to serine. The mutation carries a minor allele frequency in the Exome Variant Server (18) of 0.0615%, but is not predicted to be deleterious. This is a heterozygous change and may be a germline single nucleotide polymorphism (SNP). The patient does not have any known family history of MEN1 or MEN4.

The concordance study was extended to the remainder of the SBNET cohort (n = 61) who did not have *CDKN1B* alterations in their primary tumors, but did have metastases available for analysis. No de novo *CDKN1B* mutations were detected in the 105 metastases tested.

Copy number variation of *CDKN1B* in SBNET patients

A total of 90 SBNET samples were tested with both control probes. Agreement between the assays using both controls was found in 87 cases (97%); 1 case was thrown out for low confidence with both probes; and in 2 samples (2.2%), the results with the 2 controls were discrepant. The overall rate of *CDKN1B* loss (after removing the case that was thrown out and discrepancies from the denominator) was found to be 1 of 87 (1.1%), and the gain was 2 of 87 (2.3%) (see Table 3).

***CDKN1B* sequencing alterations in PNETs**

A total of 67 PNET primary tumors were sequenced to identify somatic alterations in the coding exons of *CDKN1B*. No frameshift mutations were found. One non-synonymous missense mutation was discovered in this group, which resulted in amino acid 42 being changed from threonine to isoleucine (Table 2). The minor allele frequency has been reported as 0.0077%, and the change was predicted to be damaging (18). This may have been a germline SNP, because the change was identified in the primary tumor, lymph node metastasis, and normal pancreas tissue. The patient had no known familial syndrome (such as MEN4).

Four PNET patients had gDNA available from their associated metastases (two liver metastases and two lymph node metastases) and normal pancreas tissue for use in concordance experiments of exonic *CDKN1B* mutations. As with the wild type SBNETs, none of the metastases associated with wild type primary tumors displayed de novo mutations in the gene, nor did the normal tissues have *CDKN1B* mutations.

Copy number variation of *CDKN1B* in PNET patients

A total of 64 PNET samples were tested with both control probes. Agreement between the two probes was seen in 63 cases (98%). No samples were thrown out for low confidence with both probes. In one sample (1.6%), the results found with the two controls were discrepant. The overall rate of *CDKN1B* loss (after discrepancies were removed from the denominator) was 2 of 63 (3.2%), and the gain was 1 of 63 (1.6%) (see Table 3).

p27 expression in tumors with somatic mutations

Nuclear and cytoplasmic p27 expression was analyzed in 38 SBNET and 3 PNET patients, including the patients in whom somatic or missense mutations were found (Supplementary Table S1; Figure S1). The three SBNETs with frameshift mutations demonstrated reduced to absent nuclear p27 expression and variable cytoplasmic expression. In one case (SBNET 318-1), p27 expression was completely lost in the primary tumor and the associated lymph node and liver metastases. The other two cases (SBNET 243-1, SBNET 308-1) demonstrated weak nuclear p27 expression and a range of cytoplasmic expression in their primary tumors and metastases (Table 4; Figure 1). In all three samples, the normal small bowel tissue demonstrated exclusive nuclear p27 expression. The two cases with missense mutations (PNET 224-1, SBNET 162-1) demonstrated moderate to strong nuclear and cytoplasmic p27 expression (Table 4; Figure 2) in their primary tumors. Patients without frameshift or missense *CDKN1B* mutations had variable p27 nuclear (range: 0–275) and cytoplasmic (range: 0–225) expression in their primary tumors. The normal small bowel samples from the patients with wild type *CDKN1B* tended toward high nuclear p27 expression and low to no cytoplasmic expression, but this was not consistent across all samples (Supplementary Table S1).

Discussion

Until recent years, the genetic changes leading to SBNET tumorigenesis have been poorly understood. Francis et al. (7) were the first to suggest that somatic mutations in *CDKN1B* may be driver mutations in SBNET initiation. In that report, 3 groups of SBNET tumors were analyzed for somatic alterations of *CDKN1B*—their own 50 patients (containing both primary tumors and metastases), a set of 48 primary tumors from Banck et al. (6), and an extension set of 81 SBNET primaries and metastases. In their 50 patients, they found a 10% rate of frameshift mutations (5 of 50; 95% CI 4.3–21.4%). Two frameshifts were found in the Banck et al. SBNETs (4.2% mutation rate; 95% CI 1.2–12.7%), and seven were found in the extension set (9.9% mutation rate; 95% CI 4.2–16.8%). Overall, the frameshift rate in the entire Francis set was 7.8% (14 of 179; 95% CI 4.7–12.7%).

Crona et al. (8) evaluated SBNETs from 200 patients (150 primary SBNETs) for *CDKN1B* mutations and found 13 with insertions or deletions, 4 nonsense mutations, and 1 loss of a stop codon for an overall mutation rate of 8.5% (reported as 17 of 200; 95% CI 4.6–12.4%). They selected tumors with >40% cellularity, and in 11 of 13 cases where normal tissue was available, were able to confirm that the changes were somatic.

We found a lower rate of frameshift mutations in our 86 SBNET primary tumors at 3.5% (3 of 86; 95% CI 1.1–9.8%). Although this was lower than the overall rate seen in Francis et al. and Crona et al., the overlap of the 95% CIs and the Z test for proportions of independent groups confirms that no significant difference existed between the rates found (17).

Copy number variation of *CDKN1B* may be another mechanism for tumor development in small bowel neuroendocrine tissues. The CNV rate in our SBNETs was 3.4% (3 of 87; 95% CI –0.41–7.2%), whereas the rate found by Francis et al. (n = 50) was 14% (95% CI 4.4–23.6%). Although the 95% CIs did overlap, the z statistic was 2.31 ($P = 0.02$), which suggested that the proportion of CNV rates in these two groups was significantly different. In our SBNETs, there were no primary tumors with both a frameshift mutation and a CNV of *CDKN1B*. The overall rate of these two types of alterations was 7.0% in our SBNETs (6 of 86; 95% CI 3.2–14.4%).

As in other types of NETs, p27 is reportedly important in the progression of pancreatic islet cells into the S phase of the cell cycle (19). It follows that disruption of this pathway could lead to PNET tumorigenesis. We tested primary PNETs, their metastases, and normal tissues for somatic mutations and CNV of *CDKN1B*. Interestingly, in contrast with SBNETs, no frameshift mutations were found in PNET primaries or their associated metastases. *CDKN1B* CNVs were seen in 4.8% of primary PNETs, and the overall *CDKN1B* alteration rate was 4.8% (3 of 64; 95% CI –0.44–10%) as compared with 7.0% in SBNETs. The significance of this small rate of CNVs in PNETs is unclear, and evidence is insufficient from the current study to determine the importance of these changes to PNET tumorigenesis.

It is likely that the events driving SBNET initiation are different from those in PNETs, which would explain the lack of frameshift mutations of *CDKN1B* in PNETs. For instance, the PI3K/Akt/mTOR pathway is crucial to many aspects of normal cell growth and survival, and disruptions of this pathway are seen in a large number of cancer types, including both PNETs and SBNETs, thus making it an attractive treatment target in NETs (20). However, despite the widespread importance of this pathway, everolimus (an mTOR inhibitor) has been FDA-approved only for use in PNETs and has not yet been proven useful in SBNETs (21,22). *CDKN1B* and its protein product are important in most cell types, but are known to be disrupted in only a handful of cancer types. From our study, *CDKN1B* appears to be more commonly disrupted in SBNETs than PNETs. Currently, no highly effective SBNET-specific chemotherapeutics are available. Knowing that *CDKN1B* mutations are likely drivers of SBNET tumorigenesis in a subgroup of tumors highlights this pathway as a promising target for development of novel, SBNET-specific drugs.

Normally, p27 is expressed in the nucleus, is phosphorylated, and then exported to the cytoplasm, where it is targeted for degradation via a ubiquitin-dependent proteolysis pathway (23). Cytoplasmic p27 expression has been correlated with more aggressive tumor behavior in gastric cancer (24), worse cancer-specific survival in clear cell renal cell carcinoma (25), and poorer 5-year survival in melanoma patients (23). The trend in our primary tumors with *CDKN1B* frameshift mutations toward lower levels of protein expression is not surprising, because this pattern is seen in a number of malignant and premalignant conditions, such as breast and prostate cancers (26). The cytoplasmic

expression of p27 was variable among the tumors and metastases, as well as within the cytoplasm of individual tumor cells. The intratumor variability is not surprising, given the findings of Crona et al. (8), though it is unknown how this scattered expression pattern affects neoplastic cells. p27 cytoplasmic expression was uniformly absent in the normal tissues. The two cases with missense mutations had uniformly high levels of p27 expression, which suggested that the mutations did not disrupt expression of the protein. Given the small number of patients with *CDKN1B* alterations in this study, clear clinical correlations cannot be drawn. In Crona et al., a specific phenotype could not be correlated with low p27 expression either, which, again was likely due to the relatively small number of mutated SBNETs in their cohort of patients. Thus, it is unclear whether levels of p27 expression determined by immunohistochemistry in NETs provide useful prognostic information.

A few reasons explain why the *CDKN1B* alteration rate in this study may be less than the rates found in Francis et al. (7) and Crona et al. (8). The first may be that the genomic DNA used in all of the assays was isolated from tumors that contained a mix of stroma and tumor cells. It is possible that the stromal component decreased the sensitivity of the assays, which led to a lower alteration rate. However, our SBNET and PNET sets had median cellularities of >70%, with only six primary tumors and one metastasis with <40% cellularity, which was the inclusion cutoff used by Crona et al. In Francis et al. and Banck et al. (6), the purity of their samples was not addressed, so we cannot be sure how this factor may have affected their mutation rates. Second, our study employed *CDKN1B*-specific qPCR assays to determine the gene copy number, whereas Francis et al. used exome sequencing. The qPCR assay used has been well validated, but it may be less sensitive than next generation sequencing methods (27). Deletion of a single allele causes a 0.5 cycle (Ct) difference by qPCR, which might be difficult to detect in samples with significant amounts of stroma. Again, our assessment of tumor cellularity showed that the majority were >70%, and we used a wild type calibrator sample in our CNV analysis. One control probe used, *RNase P*, is derived from chromosome 14q11.2, an area where Kulke et al. (16) found gains in four of seven SBNET primaries. This high rate of gain was not borne out in this study, where there was 97% agreement between both *RNase P* and *TERT1* from chromosome 5p15.33.

Many studies in both animals and humans have suggested the importance of *CDKN1B* as a haploinsufficient tumor suppressor gene (15), and recent studies have implicated it as a contributor to SBNET tumorigenesis. Our study supports the conclusion that *CDKN1B* may be involved in SBNET tumor initiation in a small subset of patients, thus opening the door for investigation of this gene as a target for future therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. p27 expression in tumor samples with frameshift mutations (200× magnification). Positive staining is indicated by the brown color. Images a–c demonstrates p27 expression in a series of samples from the same patient (SBNET 318-1), with a frameshift mutation in CDKN1B. Expression of the protein is seen in non-neoplastic cells at the bottom of the image (a), but is virtually knocked out in the primary tumor (a), and completely abolished in the liver metastasis in (b) and lymph node metastasis in (c). Normally, p27 is expressed in the nucleus of the cell. In (d), the primary tumor SBNET 243-1 demonstrates weak cytoplasmic staining. Cytoplasmic staining has been correlated with aggressive tumor behavior. In (e) and (f), two different cores from the same primary tumor for patient SBNET 308-1 are seen. The sample in (e) shows strong cytoplasmic expression of the protein in a small fraction of cells, whereas the sample in (f) demonstrates weak cytoplasmic expression of p27 in the neoplastic cells on the right side of the image.

Figure 2.

p27 expression in primary tumor samples with missense mutations. (a) SBNET 162-1 demonstrates moderate to strong expression of p27 in the cytoplasm of the cell. The missense mutation in this sample was not predicted to be damaging per the Exome Variant Server (18). (b) PNET 224-1 also demonstrates moderate to strong expression of p27. In this case, protein expression was concentrated in the nucleus. In contrast with the missense mutation in SBNET 162-1, this mutation was predicted to be damaging.

Table 1PCR primer sequences and annealing temperatures used to detect somatic mutations in *CDKN1B*

Primer target	Forward primer sequence (5' → 3')	Reverse primer sequence (5' → 3')	Tm	Product size
Exon 1 (part 1)	GTA GGG GCG CTT TGT TTT G	ACC TTG CAG GCA CCT TTG	57	378 bp
Exon 1 (part 2)	ACC CCT AGA GGG CAA GTA CG	ATA CGC CGA AAA GCA AGC TA	57	360 bp
Exon 2	CTG ACT ATG GGG CCA ACT TC	TTT GCC AGC AAC CAG TAA GA	57	296 bp

Abbreviation: Tm, melting temperature.

Table 2

Mutations identified in *CDKN1B* in a set of 86 SBNET primary tumors and 67 PNET primary tumors

Tumor	Mutation	Location	Protein change
SBNET 243-1	Frameshift	c.127delCinsTAA	p.R43fs
SBNET 308-1	Frameshift	c.279_280insT	p.P94fs
SBNET 318-1	Frameshift	c.334delA	p.S112fs
SBNET 162-1	Missense	c.518A>G	p.N173S
PNET 224-1	Missense	c.125C>T	p.T42I

Table 3Copy number variation of *CDKN1B* for primary SBNET and PNETs

Tumor	Mutation	Location	Copy number variation
SBNET 175-1	<i>CDKN1B</i> deletion	c.?56_1590?del	Loss of one copy
SBNET 103-1	<i>CDKN1B</i> duplication	c.?56_1590?dup	Gain of one copy
SBNET 148-1	<i>CDKN1B</i> duplication	c.?56_1590?dup	Gain of one copy
PNET 251-1	<i>CDKN1B</i> deletion	c.?56_1590?del	Loss of one copy
PNET 358-1	<i>CDKN1B</i> deletion	c.?56_1590?del	Loss of one copy
PNET 324-1	<i>CDKN1B</i> duplication	c.?56_1590?dup	Gain of one copy

Table 4p27 expression in SBNETs and PNETs with somatic mutations^a

Mutation	Tissue	H-score	Location of expression
Frameshift (SBNET 243-1)	SBNET primary	11	Nucleus
	SBNET primary	66	Cytoplasm
	LN metastasis	10	Nucleus
	LN metastasis	225	Cytoplasm
Frameshift (SBNET 308-1)	SBNET primary	12	Nucleus
	SBNET primary	67	Cytoplasm
Frameshift (SBNET 318-1)	SBNET primary	0	Nucleus
	SBNET primary	0	Cytoplasm
	LN metastasis	0	Nucleus
	LN metastasis	0	Cytoplasm
	Liver metastasis	0	Nucleus
	Liver metastasis	0	Cytoplasm
Missense (SBNET 162-1)	SBNET primary	30	Nucleus
	SBNET primary	220	Cytoplasm
Missense (PNET 224-1)	PNET primary	225	Nucleus
	PNET primary	27	Cytoplasm
	LN metastasis	210	Nucleus
	LN metastasis	100	Cytoplasm

^aEach sample was assessed for the presence of p27 expression, which is quantified by the H-score. Cellular location of expression was also noted.