Cancer Genetics 208 (2015) 564–570

Cancer Genetics

ORIGINAL ARTICLE

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], James R. Howe [a,*]

[a] *Department of Surgery, Carver College of Medicine, University of Iowa, Iowa City, Iowa;* [b] *Department of Pathology, Carver College of Medicine, University of Iowa, Iowa City, Iowa;* [c] *Department of Internal Medicine, Carver College of Medicine, University of Iowa, Iowa City, Iowa*

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 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

Received April 24, 2015; received in revised form August 27, 2015; accepted August 28, 2015.

* Corresponding author.
E-mail address: james-howe@uiowa.edu

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http://dx.doi.org/10.1016/j.cancergen.2015.08.003

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 Lasergene11 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 was replaced 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

Table 1 PCR 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.