Patterns of chromosome 18 loss of heterozygosity in multifocal ileal neuroendocrine tumors

Zhouwei Zhang1,2 | Netta Mäkinen1,2 | Yosuke Kasai3 | Grace E. Kim4 | Begoña Diosdado5 | Eric Nakakura3 | Matthew Meyerson1,2,6

1Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, Massachusetts
2Cancer Program, Broad Institute of Harvard and MIT, Cambridge, Massachusetts
3Department of Surgery, University of California San Francisco, San Francisco, California
4Department of Pathology, University of California San Francisco, San Francisco, California
5Division of Molecular Carcinogenesis, OncoDe Institute, The Netherlands Cancer Institute, Amsterdam, The Netherlands
6Departments of Genetics and Medicine, Harvard Medical School, Boston, Massachusetts

Correspondence
Matthew Meyerson, Dana-Farber Cancer Institute, 450 Brookline Ave, Boston, MA 02215.
Email: matthew_meyerson@dfci.harvard.edu

Funding information
Neuroendocrine Tumor Research Foundation

Abstract
Ileal neuroendocrine tumors (NETs) represent the most common neoplasm of the small intestine. Although up to 50% of patients with ileal NETs are diagnosed with multifocal disease, the mechanisms by which multifocal ileal NETs arise are not yet understood. In this study, we analyzed genome-wide sequencing data to examine patterns of copy number variation in 40 synchronous primary ileal NETs derived from three patients. Chromosome (chr) 18 loss of heterozygosity (LOH) was the most frequent copy number alteration identified; however, not all primary tumors from the same patient had evidence of this LOH. Our data revealed three distinct patterns of chr18 allelic loss, indicating that primary tumors from the same patient can present different LOH patterns including retention of either parental allele. In conclusion, our results are consistent with the model that multifocal ileal NETs originate independently. In addition, they suggest that there is no specific germline allele on chr18 that is the target of somatic LOH.

KEYWORDS
chromosome 18, copy number variation, high-throughput sequencing, ileal neuroendocrine tumor, loss of heterozygosity

1 INTRODUCTION

Small intestinal neuroendocrine tumors (SI-NETs) represent the most common neoplasm of the small bowel, accounting for 40% of all small intestinal malignancies. The estimated annual age-adjusted incidence is 1.05/100 000 persons in the United States and has increased approximately 4-fold since 1973. SI-NETs are generally slow-growing, well-differentiated tumors that are mostly found in the terminal ileum (ileal NETs), but show often high metastatic potential. About 50% of the patients have distant metastases at diagnosis, and the 5-year survival rate for those patients is around 50%. For patients with metastatic disease, effective treatment modalities are limited.

Previous high-throughput sequencing studies have revealed that ileal NETs have a low somatic mutation rate. To date, the most frequent genomic event identified in these tumors is loss of heterozygosity (LOH) at chromosome (chr) 18, occurring in approximately 60% of cases, and the only recurrent mutations identified to date are loss-of-function mutations in cyclin-dependent kinase inhibitor 1B in 8% to 10% of tumors. Indeed, in a recent whole genome sequencing study of 2520 metastatic cancer/normal pairs, SI-NETs were found to be the one tumor type in which a candidate driver gene was rarely found; 18 of 37 SI-NETs had no identified candidate driver compared to 16 of 2483 other cancers.

The majority of previous studies have focused on single primary tumors from each patient. However, up to 50% of patients with ileal...
NETs are diagnosed with multiple synchronous primary tumors. The molecular genetic mechanisms by which these multifocal lesions develop remain mostly unknown. One hypothesis is that the multifocal tumors may arise as a result of an unrecognized germline mutation. By analyzing LOH patterns at six candidate genetic loci using PCR-based method, Katona et al. have suggested multifocal ileal NETs may originate independently. We report here the first high-throughput sequencing-based copy number profiling of multifocal primary ileal NETs. We not only confirm that chr18 LOH is the most common somatic copy number change in multifocal ileal NETs, but also reveal distinct patterns of chr18 allelic loss in individual tumors from the same patient. Our results corroborate the hypothesis that multiple primary ileal NETs may develop independently and provide novel insights into the molecular mechanism of chr18 LOH.

2 | MATERIALS AND METHODS

2.1 | Patients and tissue samples

Our sample set consisted of 40 de-identified synchronous primary tumors and matched adjacent normal ileal mucosa specimens from three ileal NET patients (Figure 1; Table S1). A piece of each tissue sample was obtained, freshly frozen in liquid nitrogen, and stored at -80°C. All tumor specimens were stained with hematoxylin and eosin (H&E) and the presence and density of tumor cells were verified before sequencing. Each patient provided informed consent in accordance with the protocols approved by the Institutional Review Boards of the Dana-Farber Cancer Institute and the University of California San Francisco.

2.2 | Whole-exome and linked-read whole-genome sequencing

Genomic DNA (gDNA) was extracted from fresh frozen tumor and adjacent normal ileum samples. Tissue specimens from patient 1 were sequenced using the 10x Genomics linked-read WGS approach (10x Genomics, Pleasanton, California), as detailed before. Briefly, gDNA libraries were sequenced on an Illumina HiSeq platform to generate 151-bp paired-end reads with a mean depth of 60x coverage for tumor specimens, and 30x coverage for the tumor adjacent normal tissue. Whole-exome sequencing (WES) was performed for patients 2 and 3, as previously described. In brief, exonic sequences were enriched using the Agilent V2 capture probe set (Agilent, Santa Clara, California) and sequenced by 76-bp paired-end reads using the Illumina Genome Analyzer IIx system (Illumina, San Diego, California) with a mean coverage of 80x for each base. Sequencing reads were aligned to the human reference genome GRCh37 (hg19).

2.3 | Copy number variation and chr18 LOH mapping

For patient 1, somatic copy number changes and LOH in tumor samples were detected using a modified TITAN pipeline (https://github.com/gavinha/TitanCNA_10X_snakemake), and for patients 2 and 3, using CapSeg, allelic CapSeg, and ABSOLUTE algorithms in the Clonal Evolution Exome Suite. Detailed LOH mapping was performed for tumors showing chr18 LOH by completing the following steps: (a) identifying germline heterozygous SNPs in the normal samples using the following filters: read depth >10 and variant allele frequency between 0.4 and 0.6; (b) retrieving the allelic depth of the SNPs in the corresponding tumor.

FIGURE 1  Representative images of multifocal ileal NETs from patient 1. A, Resected segment of ileum, showing multifocal NETs indicated by white arrows. B, Hematoxylin and eosin, and C, Chromogranin A staining of representative primary tumor tissue to confirm its neuroendocrine origin.
samples if the total read depth of a given SNP is >10 in the tumor samples; (c) applying a binomial test to the read counts of the reference and alternative alleles of each SNP with the null hypothesis of 0.5, meaning that both alleles are expected to occur in half of the reads; (d) preserving SNPs with FDR-adjusted $P < 0.1$ as LOH-informative SNPs; (e) retaining tumor samples that have at least 1000 LOH-informative SNPs (WGS, patient 1) or at least 100 LOH-informative SNPs (WES, patient 2 and 3) for further analysis; (f) assigning the retained allele of each LOH-informative SNP by comparing read counts of the reference and alternative alleles (Table S2).

3 | RESULTS

3.1 | Chr18 LOH is common in multifocal ileal NETs

Copy number analysis identified hemizygous loss of chr18 (log2 [copy number/2] < -0.1) to be the most common genomic alteration in multifocal ileal NETs (Figure S1), consistent with previous reports.\(^6,8\) The frequency of chr18 LOH was 55% (6 of 11 primary tumors) in tumors from patient 1, 39% (7 of 18 primary tumors) in tumors from patient 2, and 27% (3 of 11 primary tumors) in tumors from patient 3. Other chromosomal copy number changes included gains of chromosomes 4, 5, 7, 14, and 20; none of these changes were present in tumors from more than one patient.

3.2 | Multifocal ileal NETs show different patterns of allelic loss at chr18

By comparing retained SNP alleles between tumor samples with chr18 LOH, we observed three different chr18 LOH patterns in multifocal ileal NETs. Individual tumors with chr18 allelic loss from the same patient can lose either (a) the same parental allele, (b) different parental alleles with consistent allelic loss across chromosomal arms, or (c) different parental alleles in the short (p) and long (q) arms of chr18 (Figure 2). Copy number analysis of patient 1 identified six primary ileal NETs displaying chr18 LOH. Based on 359 common LOH-informative SNPs, two of the tumors (T5 and T8) had lost the same parental allele, while three of the tumors (T6, T7, and T9) had lost the other parental allele (Figure 2; Tables S3 and S4). Both groups had consistent allelic loss patterns across both chromosomal arms. Interestingly, one tumor (T2) from patient 1 had lost different parental alleles in the p and q arms of chr18. In other words, the lost allele of T2 in 18p was the same as that of T5 and T8, while the allele of T2 lost in 18q was the same as that of T6, T7, and T9 (Figure 2; Tables S3 and S4). For patient 2, five out of seven tumors with chr18 LOH were included in LOH mapping. By comparing retained alleles of 116 common LOH-informative SNPs, four of the five tumors had lost the same parental allele of the whole chr18 (T1, T6, T12 and T14), while one tumor (T9) had lost the other parental allele of the whole chr18 (Figure 2; Tables S3 and S5). Lastly, two out of three primary tumors displaying chr18 LOH from patient 3 (T1 and T2) were analyzed and found to have lost the same parental allele (Figure 2; Tables S3 and S6).

4 | DISCUSSION

Ileal NETs represent the most common neoplasm of the small intestine with a high incidence of multiple synchronous primary tumors at diagnosis. Our understanding of the tumor multifocality, however, remains limited with respect to the molecular mechanisms underlying their tumorigenesis and effects on clinical management.\(^11,12,20\) Here, we utilized high-throughput sequencing to characterize copy number alterations present in multifocal ileal NETs to better understand the molecular genetic background of these lesions.

Most large-scale sequencing studies to date have concentrated on sequencing single primary tumors from ileal NET patients.\(^6,7\) This is, to our knowledge, the first comprehensive copy number profiling study of multifocal primary ileal NETs. The observed copy number changes...
The origin of multifocal ileal NETs is not well understood. Previous studies have proposed that multifocal ileal NETs may arise due to germline predisposition or as independent clones.13,14 Meanwhile, our analysis addresses two limitations from previous results, we show that chr18 loss occurs in many, but not all, primary ileal NETs.6,7,22,23 Tissue karyotyping and fluorescence in situ hybridization analysis may be required for further confirmation. Second, we could not map the LOH pattern for all tumors displaying chr18 LOH because of a small number of analyzable LOH-informative SNPs found in some tumors. This may be explained by a lower amount of tumor cells in those tissue samples. Last, our current data were not able to pinpoint the exact mechanism of how a tumor (T2 from patient 1) lost different parental alleles in p and q arms of chr18. One possibility is that homologous recombination occurs at chr18, and one of the recombined copies is lost during tumorigenesis. Alternatively, this tumor might undergo loss of the two arms of chr18 separately, indicating that the chr18 LOH of ileal NETs may represent two independent genomic events (Figure 2). Harnessing long-read sequencing technologies to generate ultra-long reads that cover both chromosomal arms may be required to reveal the exact mechanism.24,25

In conclusion, our data demonstrate that chr18 LOH is commonly found in multifocal ileal NETs. The distinct LOH patterns in individual tumors from the same patients suggest that there is not a particular germline allele on chr18 that predisposes somatic LOH.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Joshua Francis, Dr. Aruna Ramachandran, Dr. Rameen Beroukhim, and Dr. William Sellers for their encouragement, guidance, and advice with this project.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article. However, Dr. Meyerson declares the following general conflicts of interest: research support from Bayer, Ono, Novo, and Janssen; patent licensing royalties from LabCorp; and serving as scientific advisory board chair and consultant for OriGene.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are currently being submitted to the European Genome-phenome Archive.

ORCID

Zhouwei Zhang https://orcid.org/0000-0001-6362-9031

REFERENCES

1. Bilimoria KY, Bentrem DJ, Wayne JD, Ko CY, Bennett CL, Talamonti MS. Small bowel cancer in the United States: changes in epidemiology, treatment, and survival over the last 20 years. Ann Surg. 2009;249(1):63-71.
2. Modlin IM, Lye KD, Kidd M. A 5-decade analysis of 13,715 carcinoid tumors. Cancer. 2003;97(4):934-959. https://doi.org/10.1002/cncr.11105.
3. Dasari A, Shen C, Halperin D, et al. Trends in the incidence, prevalence, and survival outcomes in patients with neuroendocrine tumors in the United States. JAMA Oncol. 2017;3(10):1335-1342.
4. Yao JC, Hassan M, Phan A, et al. One hundred years after “carcinoid”: epidemiology of and prognostic factors for neuroendocrine tumors in

included loss of chr18 and gains of chr4, chr5, chr7, chr14, and chr20. These findings are in agreement with what others and we have previously reported when studying single primary ileal NETs from different individuals.6,7,21 More importantly, our data corroborate that chr18 loss is the most prominent copy number alteration in ileal NETs, regardless of whether tumors are unifocal or multifocal. Of note, not all primary tumors from the same patient displayed chr18 loss.

The significance of our study is three-fold. First, consistent with previous results, we show that chr18 loss occurs in many, but not all, primary ileal NETs.6,7 Second, we demonstrate that primary ileal NETs display mixed allelic loss patterns in chr18 among individual tumors from the same patient. These results suggest that multifocal ileal NETs are likely to be polyclonal, which is largely in line with the findings of Katona et al.14 Meanwhile, our analysis addresses two limitations from the earlier study,14 which we were able to overcome thanks to advances in genome analysis technology during the intervening years. Katona et al. only assessed LOH patterns for two microsatellite loci on the 18q arm, which might not reflect LOH patterns across the entire chr18, while we mapped LOH patterns across the whole chromosome. Furthermore, Katona et al. used a less sensitive PCR-based genotyping method, whereas we refined the definition by conducting high-throughput sequencing. Third, given the frequent chr18 LOH in ileal NETs, it would be reasonable to infer that the reason for chr18 LOH is to delete a wild type copy of a tumor suppressor gene in the setting of a germline loss-of-function mutation. However, the loss of either parental allele, in tumors from the same patient, rules out this possibility. Thus, our data suggest that it is improbable that there exists a germline loss-of-function mutation in a tumor suppressor gene on chr18. Rather, we conclude that it is more likely that the oncogenic effect of chr18 allelic loss may be due to a dosage impact on the expression of one, several or many genes within the chromosome.

There are some limitations in our current study. First, our interpretation of LOH pattern assumed that the hemizygous loss of chr18 in ileal NET is more likely to occur in a single parental allele as previously reported.6,7,22,23 Tissue karyotyping and fluorescence in situ hybridization analysis may be required for further confirmation. Second, we could not map the LOH pattern for all tumors displaying chr18 LOH because of a small number of analyzable LOH-informative SNPs found in some tumors. This may be explained by a lower amount of tumor cells in those tissue samples. Last, our current data were not able to pinpoint the exact mechanism of how a tumor (T2 from patient 1) lost different parental alleles in p and q arms of chr18. One possibility is that homologous recombination occurs at chr18, and one of the recombined copies is lost during tumorigenesis. Alternatively, this tumor might undergo loss of the two arms of chr18 separately, indicating that the chr18 LOH of ileal NETs may represent two independent genomic events (Figure 2). Harnessing long-read sequencing technologies to generate ultra-long reads that cover both chromosomal arms may be required to reveal the exact mechanism.24,25

In conclusion, our data demonstrate that chr18 LOH is commonly found in multifocal ileal NETs. The distinct LOH patterns in individual tumors from the same patients suggest that there is not a particular germline allele on chr18 that predisposes somatic LOH.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Joshua Francis, Dr. Aruna Ramachandran, Dr. Rameen Beroukhim, and Dr. William Sellers for their encouragement, guidance, and advice with this project.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article. However, Dr. Meyerson declares the following general conflicts of interest: research support from Bayer, Ono, Novo, and Janssen; patent licensing royalties from LabCorp; and serving as scientific advisory board chair and consultant for OriGene.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are currently being submitted to the European Genome-phenome Archive.

ORCID

Zhouwei Zhang https://orcid.org/0000-0001-6362-9031

REFERENCES

1. Bilimoria KY, Bentrem DJ, Wayne JD, Ko CY, Bennett CL, Talamonti MS. Small bowel cancer in the United States: changes in epidemiology, treatment, and survival over the last 20 years. Ann Surg. 2009;249(1):63-71.
2. Modlin IM, Lye KD, Kidd M. A 5-decade analysis of 13,715 carcinoid tumors. Cancer. 2003;97(4):934-959. https://doi.org/10.1002/cncr.11105.
3. Dasari A, Shen C, Halperin D, et al. Trends in the incidence, prevalence, and survival outcomes in patients with neuroendocrine tumors in the United States. JAMA Oncol. 2017;3(10):1335-1342.
4. Yao JC, Hassan M, Phan A, et al. One hundred years after "carcinoid": epidemiology of and prognostic factors for neuroendocrine tumors in
35,825 cases in the United States. J Clin Oncol. 2008;26(18):3063-3072.

5. Norlén O, Stålberg P, Öberg K, et al. Long-term results of surgery for small intestinal neuroendocrine tumors at a tertiary referral center. World J Surg. 2012;36(6):1419-1431.

6. Francis JM, Kiezun A, Ramos AH, et al. Somatic mutation of CDKN1B in small intestine neuroendocrine tumors. Nat Genet. 2013;45(12):1483-1486.

7. Banck MS, Kanwar R, Kulkarni AA, et al. The genomic landscape of small intestine neuroendocrine tumors. J Clin Invest. 2013;123(6):2502-2508.

8. Kulke MH, Freed E, Chiang DY, et al. High-resolution analysis of genetic alterations in small bowel carcinoid tumors reveals areas of recurrent amplification and loss. Genes Chromosomes Cancer. 2008;47(7):591-603.

9. Priestley P, Baber J, Lolkema MP, et al. Pan-cancer whole-genome analyses of metastatic solid tumours. Nature. 2019;575(7781):210-216.

10. Gangi A, Siegel E, Barmparas G, et al. Multifocality in small bowel neuroendocrine tumors. J Gastrointest Surg. 2018;22(2):303-309.

11. Choi AB, Maxwell JE, Keck KJ, et al. Is multifocality an indicator of aggressive behavior in small bowel neuroendocrine tumors? Pancreas. 2017;46(9):1115-1120.

12. Yantiss RK, Odze RD, Farraraye FA, Rosenberg AE. Solitary versus multiple carcinoid tumors of the ileum: a clinical and pathologic review of 68 cases. Am J Surg Pathol. 2003;27(6):811-817.

13. Sei Y, Zhao X, Forbes J, et al. A hereditary form of small intestinal carcinoid associated with a germline mutation in inositol polyphosphate multikinase. Gastroenterology. 2015;149(1):67-78.

14. Katona TM, Jones TD, Wang M, Abdul-Karim FW, Cummings OW, Cheng L. Molecular evidence for independent origin of multifocal neuroendocrine tumors of the enteropancreatic axis. Cancer Res. 2006;66(9):4936-4942.

15. Viswanathan SR, Ha G, Hoff AM, et al. Structural alterations driving castration-resistant prostate cancer revealed by linked-read genome sequencing. Cell. 2018;174(2):433-447.

16. Imieliński M, Berger AH, Hammerman PS, et al. Mapping the hallmarks of lung adenocarcinoma with massively parallel sequencing. Cell. 2012;150(6):1107-1120.

17. Stachler MD, Taylor-Weiner A, Peng S, et al. Paired exome analysis of Barrett’s esophagus and adenocarcinoma. Nat Genet. 2015;47(9):1047-1055.

18. Carter SL, Cibulskis K, Helman E, et al. Absolute quantification of somatic DNA alterations in human cancer. Nat Biotechnol. 2012;30(5):413-421.

19. Giannikou K, Malinowska IA, Pugh TJ, et al. Whole exome sequencing identifies TSC1/TSC2 Biallelic loss as the primary and sufficient driver event for renal angiomyolipoma development. PLoS Genet. 2016;12(8):e1006242.

20. Burke AP, Thomas RM, Elsayed AM, Sobin LH. Carcinoids of the jejunum and ileum: an immunohistochemical and clinicopathologic study of 167 cases. Cancer. 1997;79(6):1086-1093.

21. Walter D, Harter PN, Battke F, et al. Genetic heterogeneity of primary lesion and metastasis in small intestine neuroendocrine tumors. Sci Rep. 2018;8(1):3811.

22. Tönnies H, Toilat MR, Ramel C, et al. Analysis of sporadic neuroendocrine tumours of the enteropancreatic system by comparative genomic hybridisation. Gut. 2001;48(4):536-541.

23. Zhao J, de Krijger RR, Meier D, et al. Genomic alterations in well-differentiated gastrointestinal and bronchial neuroendocrine tumors (carcinoids); marked differences indicating diversity in molecular pathogenesis. Am J Pathol. 2000;157(5):1431-1438.

24. Jain M, Olsen HE, Turner DJ, et al. Linear assembly of a human centromere on the Y chromosome. Nat Biotechnol. 2018;36(4):321-323.

25. Tomaszkiewicz M, Rangavittal S, Cechova M, et al. A time- and cost-effective strategy to sequence mammalian Y chromosomes: an application to the de novo assembly of gorilla Y. Genome Res. 2016;26(4):530-540. https://doi.org/10.1101/gr.199448.115.

SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Zhang Z, Mäkinen N, Kasai Y, et al. Patterns of chromosome 18 loss of heterozygosity in multifocal ileal neuroendocrine tumors. Genes Chromosomes Cancer. 2020;59:535-539. https://doi.org/10.1002/gcc.22850