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Deletion of Chromosome 1, But Not Mutation of *MEN-1*, Predicts Prognosis in Sporadic Pancreatic Endocrine Tumors

Sydney S. Guo, M.D., Alan Y. Wu, M.D., Mark P. Sawicki, M.D.

Department of Surgery, West Los Angeles VA Medical Center and the UCLA School of Medicine, Los Angeles, CA 90073, USA

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Abstract. Pancreatic endocrine tumors (PETs) may be sporadic or inherited in the multiple endocrine neoplasia type 1 (*MEN-1*) syndrome. The inherited form is caused by mutations of the *MEN-1* gene, which functions as a tumor suppressor gene and maps to chromosome 11q13. These tumors tend to have a better prognosis than their sporadic counterparts, which often have mutations of the *MEN-1* gene. Previous molecular analyses of sporadic PETs suggest a high frequency of loss of heterozygosity (LOH) at chromosome 1 as well as mutation of *MEN-1*. In this study we correlate abnormalities of *MEN-1* and chromosome 1 LOH with the biological behavior of sporadic PETs. Loss of heterozygosity for markers at chromosome 11q13 and mutation of *MEN-1* were equally frequent in tumors with or without liver metastases. Mutation of *MEN-1* is more frequent in gastrinomas than in non-gastrinomas. Loss of heterozygosity for markers on chromosome 1 is more frequent in PETs with liver metastases. These results suggest a molecular tumor model in which there is a dichotomy in the development of benign and malignant PETs.

Pancreatic endocrine tumors (PETs) may be sporadic or occur as part of the inherited multiple endocrine neoplasia type 1 syndrome (*MEN-1*). Approximately 75% of PETs occur as sporadic tumors, with the remaining 25% found to be associated with *MEN-1*. They are rare tumors, with an overall frequency of approximately less than 1 in 100,000 [1]. These tumors are hypothesized to arise from precursor cells that are part of the neuroendocrine system.

As a group, PETs have strikingly different behaviors. Their clinical manifestations vary greatly depending on the function of the secreted neuroendocrine hormone. The most frequent of these are insulinomas, followed closely by gastrinomas, occurring at a frequency of approximately 4 to 5 per million population and 3 to 4 per million population, respectively [1]. Other pancreatic endocrine tumors include non-functional tumors, VIPoma, glucagonoma, somatostatinoma, PPoma, GRFoma, ACTHoma, and PTH-RBoma.

Regardless of the specific tumor type, it is difficult to distinguish between benign and malignant PETs because they have similar histological appearances. Although primary hepatic gastrinomas have been described, there is general agreement that malignancy is shown by hepatic involvement. Malignant potential is variable among PETs. Whereas glucagonomas are almost always malignant, only about one third of gastrinomas, and less than 5% of insulinomas are malignant [2–4]. Great controversy surrounds the question of whether gastrinomas with metastases to the lymph nodes are malignant or benign. Some patients are thought to have primary nodal tumors, and resection has resulted in cure. As these tumors are histologically similar, benign and malignant gastrinomas are best distinguished by their hepatic involvement [4, 5]. Other types of PETs are thought not to arise de novo within lymph nodes, and such tumors are always considered metastatic. Knowledge of the molecular and genomic differences between malignant and benign tumors will have obvious clinical impact, as well as contribute toward our understanding of tumorigenesis and cellular differentiation.

Several genetic loci have been implicated in the pathogenesis of PETs. The best-characterized genetic alteration is mutation of the *MEN-1* tumor suppressor gene. This gene is not only responsible for the inherited *MEN-1* syndrome but is also frequently mutated in sporadic PETs. It is currently unknown whether mutation of *MEN-1* is associated with prognosis for these tumors. We recently reported a tumor-suppressor gene–containing region on chromosome 1 that is frequently deleted in malignant PETs.

The functions of the MEN-1 protein, *menin* and molecular mechanisms leading to sporadic pancreatic endocrine tumor formation have not been completely elucidated. Here we present a synthesis of our previously reported data suggesting prognostic roles of chromosome 1, chromosome 11, and *MEN-1*. We propose that in the multi-step molecular model for pancreatic endocrine tumorigenesis, there is a dichotomy in the development of benign and malignant PETs. That is, we hypothesize that, genetically, the population of PETs with hepatic involvement is distinct from those without liver metastases.

Materials and Methods

Tumor DNA Extraction and Molecular Analysis

Sporadic pancreatic endocrine tumors were obtained after operation and immediately snap-frozen in liquid nitrogen as previously

Correspondence to: M.P. Sawicki, M.D., Department of Surgery, West Los Angeles VA Medical Center and the UCLA School of Medicine, 11301 Wilshire Boulevard, Los Angeles, CA 90073, USA, e-mail: msawicki@ucla.edu

Table 1. Molecular analysis of 26 sporadic pancreatic endocrine tumors studied for *MEN-1* mutation and loss of heterozygosity on chromosome 11 and chromosome 1.

Tumor no.	Phenotype	Primary	Tumor in lymph nodes	Hepatic metastasis	Chromosome 11 deletion	MENIN mutation	Chromosome 1 deletion
1T	Gastrinoma	Peripancreatic	Y	N	N	Y	N
2T	Gastrinoma	Peripancreatic	Y	N	N	Y	N
7T	Gastrinoma	Peripancreatic	Y	N	N	N	N
8T	Gastrinoma	Duodenum	Y	N	Y	Y	N
20T	Nonfunctional	Pancreatic head	N	N	[a]	N	N
22T	Insulinoma	Pancreatic tail	N	N	N	N	Y
25T	Gastrinoma	Periduodenal	Y	N	Y	Y	N
26T	Gastrinoma	Pancreatic head	N	N	N	N	Y
39T	VIP	Pancreas body	N	N	Y	N	N
65T	Nonfunctional	Pancreatic tail	N	N	Y	N	Y
66T	Gastrinoma	Duodenum	Y	N	N	Y	N
67T	Gastrinoma	Liver	N	N	Y	Y	N
72T	Gastrinoma	Duodenum	N	N	N	N	N
89T	Gastrinoma	Peripancreatic	N	N	Y	N	N
111T	Nonfunctional	Pancreatic head	Y	N	[a]	N	N
118T	Gastrinoma	Liver	N	N	[a]	N	N
124T	Insulinoma	Pancreatic tail	N	N	N	N	N
150T	Gastrinoma	Peripancreatic	Y	N	[a]	N	N
4T	Gastrinoma	Pancreatic head	N	Y	N	N	Y
12T	Nonfunctional	Pancreatic tail	N	Y	N	N	Y
14T	Gastrinoma	Pancreatic tail	N	Y	Y	Y	Y
28T	Nonfunctional	Pancreatic tail	N	Y	N	N	N
36T	Nonfunctional	Pancreatic head	N	Y	Y	N	Y
43T	VIP	Pancreas	N	Y	Y	Y	Y
83T	Nonfunctional	Pandreatic body	N	Y	Y	Y	Y
120T	Gastrinoma	Pancreatic head	N	Y	[a]	N	Y

VIP: vasoactive intestinal peptide; N: no; Y: yes.
[a]The loss of heterozygosity study was inconclusive.

described [6]. Frozen-section analysis confirmed that the tumors contained minimal adjacent normal tissue. Constitutional DNAs were obtained from peripheral blood leukocytes of each patient. DNAs from tumor and normal tissues were isolated by phenol: chloroform extraction as previously described and stored at 4°C until molecular analysis [6]. Chromosome 1 loss of heterozygosity (LOH) analysis was performed by microsatellite analysis as previously described [7]. Chromosome 11 LOH was performed by Southern blot and microsatellite analysis as previously described [6–8]. *MEN-1* mutation analysis was performed by single-strand conformation polymorphism (SSCP) as previously reported [9].

Statistical Analysis

We compared the proportion of malignant tumors with and without chromosome 1 LOH via an exact permutation χ^2 test (Statxact, Cytel Corp.). Malignant tumors were defined as those pancreatic endocrine tumors with multiple hepatic metastases. Tumors within lymph nodes were not considered malignant.

Results

Twenty-six PETs were studied for chromosome 11 LOH, *MEN-1* mutation, and chromosome 1 LOH. All patients underwent exploratory laparotomy, with staging and resection of the tumors and/or biopsy of the metastases and lymph nodes. In some cases, only the hepatic metastasis or the lymph node tumor was available for molecular study. Phenotypically, these 26 tumors included 15 gastrinomas, 7 nonfunctional tumors, 2 insulinomas, and 2 VIPomas. Hepatic involvement was found in 8 tumors, and lymph node involvement was found in 8 tumors as well. Interestingly, these two sets of tumors did not overlap (Table 1).

Of the 26 sporadic pancreatic endocrine tumors in our study sample, 9 had *MEN-1* mutations. Mutation analysis of each of the 9 coding exons and their flanking splice donor sites of *MEN-1* was performed using SSCP analysis. Of the 9 tumors with *MEN-1* mutation 7 were gastrinomas, one was a VIPoma, and the remaining was a nonfunctional tumor. Only 3 tumors with *MEN-1* mutations had hepatic metastases, and these included a gastrinoma, a VIPoma, and a nonfunctional tumor. Five other tumors with *MEN-1* mutations had lymph node involvement, and the remaining tumor had no evidence of either hepatic or lymph node disease. All the tumors with *MEN-1* mutation and no hepatic involvement were gastrinomas. Four tumors had LOH at chromosome 11 without mutation of *MEN-1*. Most of these were non-gastrinomas.

The 9 tumors with *MEN-1* mutations were graphed according to the amino acid position of the mutation onto a schema of *MEN-1* cDNA (Fig. 1) [9]. Included in this figure are all of the reported mutations in sporadic PETs [10–16]. The relationship between these mutation and known functional domains is shown. There is no correlation between tumor phenotype and the type of mutation, although the frequency of *MEN-1* mutation is highest in gastrinomas. In our series, *MEN-1* mutation was equally frequent in tumors with or without metastases.

Twelve microsatellites spanning both arms of chromosome 1 were used in the study of LOH at chromosome 1. Using primers for each of these tri- or tetra-nucleotide polymorphic markers and the polymerase chain reaction (PCR), LOH analysis was performed on the PCR fragments. A total of 10 tumors were found to

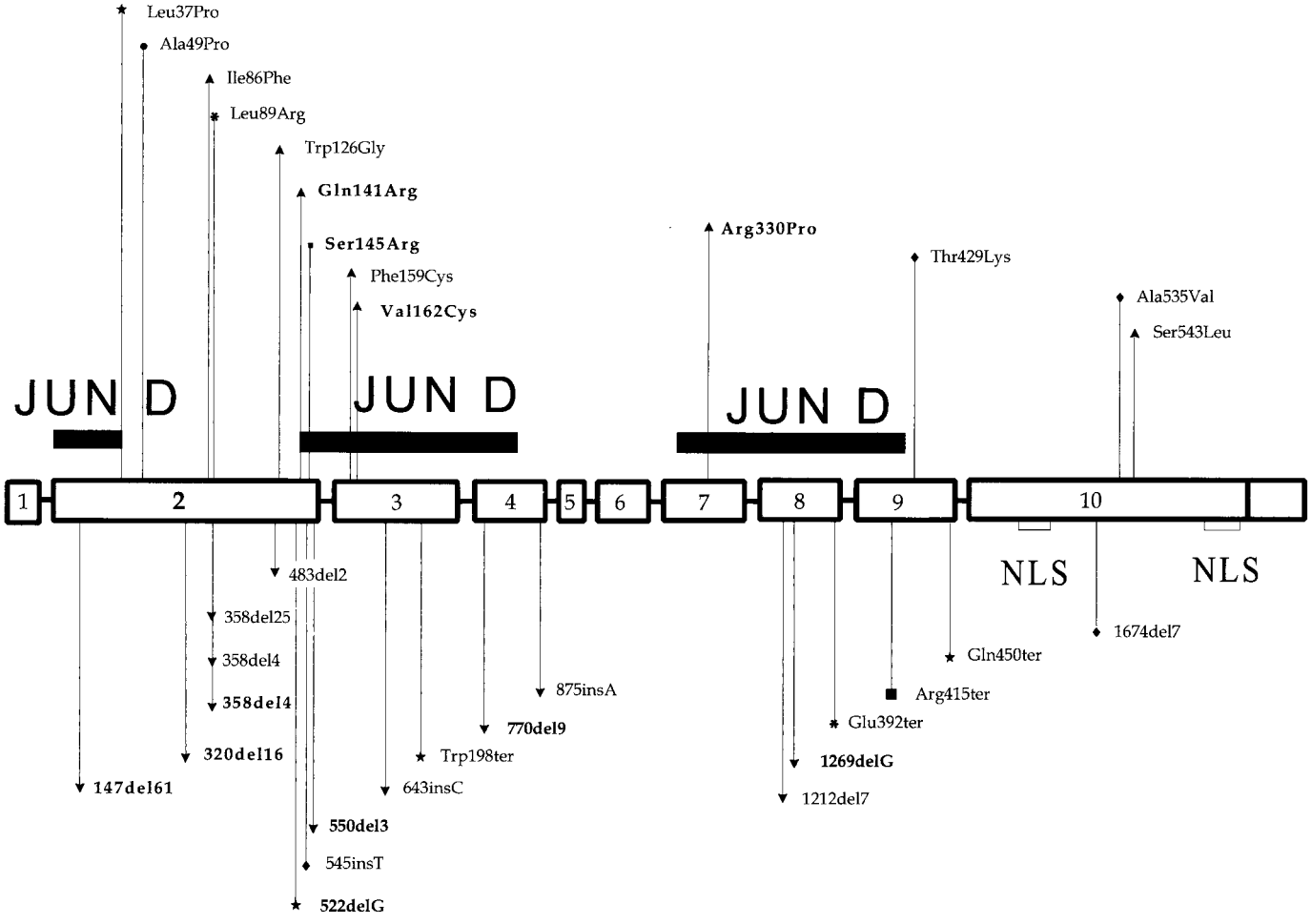

Fig. 1. In our series, 26 sporadic pancreatic endocrine tumors (PETs) were studied and multiple endocrine neoplasia type 1 (*MEN-1*) mutation was found in 9 tumors. These are mapped to a *MEN-1* cDNA schema and labeled in boldface. Other *MEN-1* mutations in sporadic PETs reported in the literature are also shown. **Top**: missense mutations. **Bottom**: nonsense mutations, deletions, and insertions. For missense and nonsense mutations, the numbers indicated represent the amino acid where the mutation occurs. For deletion and insertion mutations, the numbers refer to the nucleotide position of mutation. The amino acid (aa) positions of the three JunD binding sites are aa1–40, aa139–242, and aa323–428, respectively. The amino acid positions of the two nuclear localization signals are: aa479–497 and aa588–608. ▲: gastrinoma; ■: nonfunctional; ◆: insulinoma; ★: VIPoma; ●: somatostatinoma; ∗: glucagonoma. There does not seem to be any correlation between genotype and phenotype.

have LOH at chromosome 1. Phenotypically, these included 5 gastrinomas, 2 VIPomas, and 3 nonfunctional tumors (Table 1). Significantly, of the 8 tumors with hepatic metastases, 7 had LOH at chromosome 1. On the other hand, only 3 of 18 tumors without hepatic involvement had LOH at chromosome 1 ($p < 0.05$). If one were to believe that lymph node involvement is consistent with benign behavior in pancreatic endocrine tumors, then it is also significant that none of the tumors with lymph node involvement ($n = 8$) had LOH at chromosome 1. Three of 26 tumors had both *MEN-1* mutations and LOH at chromosome 1, and all three were malignant with hepatic disease and no lymph node involvement. One was a gastrinoma and 2 were nonfunctional tumors. The remaining tumor with hepatic disease had neither chromosome 1 deletion nor *MEN-1* mutation. In our tumor group no tumor with *MEN-1* mutation alone was associated with hepatic disease.

Discussion

It is very difficult to predict the prognosis of a pancreatic endocrine tumor without evidence of hepatic metastasis at the time of staging work-up. In addition, for gastrinomas there is much controversy regarding the nature of lymph node–containing tumors. In this study we have attempted to establish the molecular basis of these biological observations with studies on LOH at chromosome 1, chromosome 11, and mutation of the *MEN-1* gene.

The data presented suggest that malignant progression of PETs is associated with deletion of chromosome 1 but not with mutation of *MEN-1. MEN-1* is mutated equally in tumors with and without liver metastasis. However, LOH at chromosome 1 indicating deletion of a tumor suppressor gene occurs predominantly in tumors with hepatic metastasis. Of the 8 tumors with malignant behavior

and liver disease, 7 harbored LOH at chromosome 1. In contrast, of the 18 benign PETs, only 3 had LOH at chromosome 1. Therefore, LOH at chromosome 1 is predictive of hepatic metastasis ($p < 0.05$), and is equally predictive of the lack of lymph node involvement (0 of 8 tumors) for gastrinomas.

For gastrinomas there has been suspicion among some investigators that nodal involvement actually represents multifocality and does not signify metastasis. Here we present preliminary evidence that nodal involvement may indeed be a sign of the benign nature of the tumor. We have found that no tumor had both hepatic involvement and nodal disease. Furthermore, all three gastrinomas that metastasized to the liver had chromosome 1 LOH as well as *MEN-1* mutations, whereas in 11 of 12 gastrinomas without liver metastasis no LOH at chromosome 1 was found. This indicates that these may be two distinct populations of gastrinomas at the molecular level.

From our compilation of *MEN-1* mutations in PETs, we have found that there is no correlation between tumor phenotype and the type of mutation. *MEN-1* mutations encompass the entire length of the *MEN-1* coding transcript. There are mutations reported outside of the currently known functional regions (JunD binding domains and nuclear localization signals). These may affect the tertiary structure of *MEN-1* or they may represent binding regions for yet undiscovered binding partners of the menin protein. Our series of *MEN-1* mutations studied shows that without LOH at chromosome 1, *MEN-1* mutation leads predominantly to gastrinomas with nodal, but not hepatic involvement.

From the present study, we construct a tentative proposal for defining the molecular pathways leading to the development of pancreatic endocrine tumors. Starting with the putative neuroendocrine stem cell, there are four possible pathways. The first pathway begins with mutation in the *MEN-1* gene followed by deletion in chromosome 1. All three tumors with both mutations behaved in a malignant manner, metastasizing to the liver. A second pathway consists of *MEN-1* mutation only, and these tumors tend to become gastrinomas and behave in a benign fashion. The third pathway consists of an upstream mutation of an unknown gene, followed by deletions in chromosome 1. This leads to various functional and nonfunctional PETs with predominantly hepatic involvement and no lymph node involvement. The last pathway consists of mutations in an as yet unknown tumor suppressor(s) or oncogene(s) that is not *MEN-1* and that does not reside on chromosome 1.

In our study a total of 10 tumors were found to have neither *MEN-1* mutations nor LOH at chromosome 1. These tumors do not generally involve the liver (9 of 10), but may produce many hormonal phenotypes. Other studies have implied additional genetic loci in the tumorigenesis of these PETs. These include p16 [17], HER-2-neu [18], p53 [18], chromosome 3, chromosome 8, and possibly others [19]. We have also reported previously a possible second tumor suppressor on chromosome 11 [8]. Recently it was reported that in approximately 50% of nonfunctional PETs the transcriptional factor Smad4 is mutated [20]. This is in contrast with our finding that *MEN-1* mutations tend to lead to functional PETs. Because menin interacts with the transcriptional factor JunD, we hypothesize that JunD and Smad4 have important and divergent functions in PETs. These findings support the hypothesis that a dichotomy exists in the molecular pathogenesis of these PETs. Further characterization of this tumor population will lead to candidate gene(s) and contribute to a step-wise molecular model of tumorigenesis.

We have tried to explain some of these biological observations of PET behavior at the molecular level. Further studies will lead to the positional cloning of other tumor-suppressor genes involved in PETs, and join *MEN-1* as tumor suppressors frequently mutated in PETs. Additional study will also identify gene(s) that may be useful in the prediction of benign or malignant behavior of a given PET. The long-term goal of these studies is to generate a step-wise molecular model of tumorigenesis.

Résumé. Les tumeurs pancréatiques endocrines (TPE) peuvent être sporadiques ou héréditaires, dans le cadre du syndrome MEN (multiple endocrine neoplasia) de type 1 (*MEN-1*). La forme héréditaire est en rapport avec des mutations du gène *MEN-1*, qui fonctionne comme un gène de suppression tumorale et se trouve au niveau du chromosome 11q13. Ces tumeurs tendent à avoir un meilleur pronostic que leur contre parti sporadique, qui souvent présente des mutations du gène *MEN-1*. Des analyses moléculaires des TEP sporadiques suggèrent une prévalence élevée de perte d'hétérozygoticité au niveau du chromosome 1 tout comme une mutation de *MEN-1*. Dans cette étude, nous avons corrélé les anomalies perte d'hétérozygoticité de *MEN-1* et du chromosome 1 avec le comportement biologique des TPE sporadiques. Les pertes d'hétérozygoticité pour les marqueurs au niveau de chromosome 11q13 et la mutation de *MEN-1* ont été retrouvées avec la même fréquence dans les tumeurs avec ou sans métastases hépatiques. La mutation de *MEN-1* est plus fréquente dans les gastrinomes que dans les tumeurs non gastrinomes. La perte d'hétérozygoticité comme marqueur du chromosome 1 est plus fréquent en cas de TPE avec métastases hépatiques. Ces résultats suggèrent un modèle tumoral moléculaire où existe une dichotomie dans le développement des TEP bénignes et malignes.

Resumen. Los tumores endocrinos del páncreas (pancreatic endocrine tumors, PETs) del síndrome de neoplasia endocrina múltiple tipo 1 (multiple endocrine neoplasia type 1, *MEN-1*) pueden ser esporádicos o hereditarios. La forma hereditaria es causada por mutación del gen *MEN-1*, que funciona como un gen supresor tumoral y se ubica en el cromosoma 11q13. Estos tumores tienden a un mejor pronóstico que los de tipo esporádico, los cuales frecuentemente exhiben mutaciones del gen *MEN-1*. Previos análisis moleculares de los PETs esporádicos sugieren una alta frecuencia de LOH en el cromosoma 1, así como mutación de *MEN-1*. En el presente estudio hemos correlacionado las anormalidades de *MEN-1* y del cromosoma 1 LOH con el comportamiento biológico de los PETs esporádicos. LOH para marcadores en el cromosoma 11q13 y la mutación de *MEN-1* aparecieron con igual frecuencia en tumores con y sin metástasis hepáticas. La mutación de *MEN-1* es más frecuente en los gastrinomas que en los tumores no-gastrinomas. LOH para marcadores en el cromosoma 1 es más frecuente en los PETs con metástasis hepáticas. Estos resultados sugieren un modelo tumoral molecular donde existe una dicotomía en cuanto al desarrollo de PETs benignos y malignos.

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