Endocrine Pathology (2019) 30:56–63
https://doi.org/10.1007/s12022-018-9564-1

In Situ Hybridization Analysis of Long Non-coding RNAs MALAT1 and HOTAIR in Gastroenteropancreatic Neuroendocrine Neoplasms

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Published online: 2 January 2019

Abstract

Recent studies suggest onco-regulatory roles for two long non-coding RNAs (lncRNAs), MALAT1 and HOTAIR, in various malignancies; however, these lncRNAs have not been previously examined in neuroendocrine neoplasms (NENs) of gastroenteropancreatic origins (GEP-NENs). In this study, we evaluated the expressions and prognostic significance of MALAT1 and HOTAIR in 83 cases of GEP-NENs (60 grade 1, 17 grade 2, and 6 grade 3 tumors) diagnosed during the years 2005–2017. Expression levels of MALAT1 and HOTAIR were digitally quantitated in assembled tissue microarray slides labeled by chromogenic in situ hybridization (ISH) using InForm 1.4.0 software. We found diffuse nuclear expression of both HOTAIR and MALAT1 in all primary tumors of GEP-NENs with variable intensities. By multivariate model which adjusted for age and histologic grade, high expression of HOTAIR was associated with lower presenting T and M stages and subsequent development of metastases ($P < 0.05$). MALAT1 expression was associated with presenting T stage and development of metastases ($P < 0.05$). In summary, MALAT1 and HOTAIR are commonly expressed in GEP-NENs. High expression of either lncRNA showed grade-independent associations with clinically less aggressive disease.

Keywords HOTAIR · MALAT1 · Gastroenteropancreatic neuroendocrine neoplasms · siRNA · Proliferation · Invasion

Introduction

Neoplasms with neuroendocrine differentiation in the digestive system include well-differentiated neuroendocrine tumors (NETs, grades 1–3), neuroendocrine carcinomas (NECs), and mixed neuroendocrine-non-neuroendocrine neoplasms [1]. NEC typically presents at an advanced stage with adverse clinical outcome. In contrast, grade 1 to 2 NETs are mostly indolent although the prognosis varies significantly with location of origin, size, depth of invasion, proliferative activity, and syndromic associations. The group of tumors with well-differentiated morphology but a Ki-67 index over 20% has been recently recognized as grade 3 NETs in the pancreas by the 2017 World Health Organization (WHO) classification of endocrine tumors [1]. Although such tumors were categorized as NECs in the gastrointestinal tract by the 2010 WHO classification of tumors of the digestive system, experts have recently suggested distinguishing gastrointestinal grade 3 well-differentiated NETs from poorly differentiated NECs based on their differential prognosis [2]. As recently reviewed [3–5], decades of investigation into the genetics of these tumors have led to the discovery of shared genetic defects and promising prognostic markers. Nevertheless, the underlying molecular events leading to the development of these neoplasms remain poorly understood and patient outcome largely unpredictable.

Non-coding RNAs (ncRNAs) constitute a rapidly expanding area of molecular biology. Instead of being translated into protein products, ncRNAs are now known to participate in a myriad of pre- and post-transcriptional gene regulatory mechanisms. Research thus far conducted on ncRNAs in gastroenteropancreatic neuroendocrine neoplasms (GEP-NENs) have largely focused on microRNAs (miRNAs), a subclass of ncRNAs that act as gene silencers by forming inhibitory complexes with messenger RNAs. In a recent report of microarray miRNA profiling of pancreatic neuroendocrine neoplasms, divergent expression patterns were observed

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between tumors, nonneoplastic islets, and exocrine pancreatic tissue [6]. This study also noted prognostic associations with multiple miRNAs such as miR-642 and miR-210 [6]. Other miRNAs have shown associations with tumoral hormone synthesis, metastatic status, and treatment history with somatostatin analogs in GEP-NENs as recently reviewed [7]. Based on these findings, ncRNAs have elicited considerable investigative interest as promising predictors of tumor behavior.

Long noncoding RNAs (lncRNAs) which are greater than 200 bases in length constitute another subclass of ncRNAs. LncRNAs possess remarkable versatility in gene regulation by interactions with DNA-binding proteins, histone-modifying enzymes, pre-mRNA splicing machineries as well as intracytoplasmic mRNA translation and degradation mechanisms [8, 9]. Surprisingly, although recent studies have found multiple aberrantly expressed lncRNAs in adenocarcinomas of the digestive system [10], there are very few reports about the expression of lncRNAs in gastrointestinal and pancreatic neuroendocrine neoplasms. Recent studies have examined the role of epigenetic gene regulation by a lncRNA, maternally expressed gene 3 (MEG3), in menin deficiency-associated pancreatic neuroendocrine tumors [11, 12]. MEG3 has been shown to downregulate c-MET after being activated by menin in cell line models of pancreatic neuroendocrine tumors [11] and was therefore considered to be a key tumorigenic mediator in patients affected by the MEN1 syndrome. Of late, Wei et al. performed transcriptome analysis on three cases of GEP-NENs and noted downregulation of a lncRNA LncNEN885 in tumors compared to adjacent normal tissue [13]. They further demonstrated the inhibitory effect of LncNEN885 on epithelial-mesenchymal transition and tumor invasiveness [13]. These findings point to the potential utility of lncRNAs in understanding the biology of GEP-NENs.

In this study, we examined two lncRNAs, MALAT1 and HOTAIRxx, because both lncRNAs have previously shown regulatory effect in various cancers [14–19]. Previous studies have noted heterogenous function of MALAT1, which acts as a tumor suppressor in brain, breast, and colon cancers [20–23] but appears to be pro-oncogenic in lung, gastric, pancreatic, and urological cancers [24]. Reports on HOTAIR have largely supported an oncogenic function in breast, gastrointestinal, urological, and gynecologic cancers [25]. The pro-oncogenic property of MALAT1 and HOTAIR have been attributed to the activation of Wnt/β-catenin pathway [25, 26] and the induction of epithelial-mesenchymal transition (EMT) through epigenetic regulation of EMT-related genes including E-cadherin (downregulated by MALAT1 [26]) and matrix metalloproteinases (induced by HOTAIR [25]). On the other hand, several anti-survival and anti-migratory pathways have been noted mediated by MALAT1, including: (1) the inhibition of the viability of a human glioma cell line by suppressing the prooncogenic miRNA miR-155 while upregulating the tumor-suppressing protein FBXW7 [22] and (2) the inhibition of metastasis formation by suppressing transcription factor TEAD [23]. MALAT1 and HOTAIR have not been previously investigated in GEP-NENs. This study aims to explore the expression and outcome-predicting significance of both lncRNAs in a well-characterized clinical cohort of gastroenteropancreatic neuroendocrine neoplasms.

Material and Methods

Tissue Microarrays

Tissue microarrays (TMAs) were constructed and included normal tissues ($N = 78$), primary ($N = 83$), and metastatic ($N = 17$) neuroendocrine neoplasms of gastrointestinal (esophagus through rectum) and pancreatic origin from a total of 83 patients diagnosed between 2005 and 2017. Cases were identified by searching the institutional electronic medical record system and confirmed by immunohistochemical analysis (details provided below). Each case was represented by duplicate 0.6-mm cores which were prepared using a manual tissue arrayer (Beecher Instruments, Sun Prairie, WI). The study was approved by the Institutional Review Board at the University of Wisconsin-Madison.

Immunohistochemistry

Identified cases of neuroendocrine neoplasms were confirmed and graded by immunohistochemical analyses performed on Ventana BenchMark Ultra system (Ventana Medical Systems, Inc., Tucson, AZ) according to the manufacturer's protocols. Primary antibodies used included Ki-67 (1:50 dilution with Van Gogh Yellow; Biocare, Pacheco, CA), chromogranin A (clone LK2H10, prediluted; Ventana Medical Systems, Inc., Tucson, AZ), synaptophysin (polyclonal, prediluted; Cell Marque, Rocklin, CA), and insulinoma-associated protein 1 (INSM1; clone SC-271408 from Santa Cruz Biotechnology, Dallas, TX; 1:1000 dilution with Van Gogh Green from Biocare, Pacheco, CA). In addition, manual immunohistochemistry staining was performed using anti-somatostatin receptor 2 (clone UMB1; 1:500 dilution with phosphate buffered saline with Tween 20; Abcam, Cambridge, United Kingdom). The manual protocol included antigen retrieval in citric acid pH 6.0 in Biocare Decloaker for 3 min, followed by overnight incubation with the UMB1 antibody at 4 °C. All immunolabeled markers were visualized with DAB. Positive and negative controls were prepared with each analysis. Staining was scored based on percentage of positive cells: negative (0%), focal (0–25%), and diffuse (> 25%).

In Situ Hybridization (ISH)

TMAs were probed for MALAT1 and HOTAIR expression using the RNAscope 2.5 HD-Brown Manual Assay

(Advanced Cell Diagnostics, Newark, CA) as per manufacturer's recommendations with the following modifications: antigen retrieval was performed in a Biocare Decloaker for 3 min, protease digestion for 30 min, and probe incubation overnight at 40 °C. The probes used are hs-MALAT1 (400811), hs-HOTAIR (312341), hs-PPIB (positive control, 313901), and dapB (negative control, 310043) (Advanced Cell Diagnostics). Expression levels of all probes were visualized with DAB.

Automated Multispectral Image Quantitation

The hybridized TMA slides were visualized with the Vectra slide scanner (PerkinElmer, Waltham, MA). InForm 1.4.0 software (PerkinElmer, Waltham, MA) was used to segment each tissue core into architectural compartments (tumor cells versus stroma) and subcellular compartments (nucleus versus cytoplasm). Expression levels of MALAT1 and HOTAIR were quantitated for each tissue core as average nuclear optical density (OD) as previously described [27]. Results from duplicated cores were averaged for each case to obtain a representative score.

Statistical Analysis

Digitally quantified expression levels of HOTAIR and MALAT1 in a total of 83 primary tumors were included for statistical analysis. Cases were stratified into high versus low-expressing groups using the median expression level in the cohort as the cutoff value for each biomarker. Biomarker associations with tumor histologic grade, genetic syndrome (specifically multiple endocrine neoplasia type 1), and clinical outcomes (lymphovascular invasion, pathologic T and M stages at diagnosis and development of distant metastases during follow-up) were first analyzed using univariate logistic regression models. Multivariate models were then performed by including patient age and histologic grade as covariates. Proliferation and invasion assay readouts were analyzed using a one-sample t test. All reported P values are two-sided and $P < 0.05$ was used to define statistical significance. Statistical analyses were conducted using SAS software (SAS Institute Inc., Cary NC), version 9.4.

Results

Clinical Characteristics

As summarized in Table 1, a total of 83 patients were represented on the tissue microarrays, including 60 (72%) grade 1, 17 (20%) grade 2, and 6 (8%) grade 3 neuroendocrine neoplasms based on morphology and Ki-67 indices. The six grade 3 cases consisted of ampullary ($n = 2$) and pancreatic ($n = 3$) well-differentiated G3 NETs and one esophageal large cell neuroendocrine carcinoma. The majority of patients were middle-age adults with nearly equal gender distribution. At diagnosis, 24 (29%) patients had regional lymph node involvement and nine patients (four grade 1, three grade 2 and two grade 3 cases; 11%) had distant metastases to liver (nine patients) and bone (one patient). Patients were followed for a median (range) period of 22.5 (0.3 to 114.9) months. The majority (78%) had disease-free survival at end point; however, three patients with grade 3 tumors died of metastatic disease to liver and bone, and in a case with esophageal tumor, mediastinum, pleural space, and peritoneum.

Syndromic association was noted in seven (8%) patients, including six (7%) cases with multiple neuroendocrine neoplasia type 1 (MEN1) and one patient with von Hippel-Lindau syndrome (VHL). MEN1-associated cases tended to present at younger ages (median [range] = 29.1 [17.8 to 49.5] years) as grade 1 to 2, stage T1 to T2 tumors with favorable clinical course. Over a median (range) follow-up of 28.6 (1.3 to 44.0) months, all MEN1 cases had disease-free survival except for one patient who developed a second pancreatic

Table 1 Clinical characteristics of cohort ($N = 83$)

Characteristics	N (%)
Age at diagnosis, median (range) years	55 (18–80)
Gender, M:F	39 (47%): 44 (53%)
Familial cases	7 (8%)[a]
Tumor size, median (range) centimeters	2 (0–13)
Histologic grade 1:2:3	60 (72%):17 (20%):6 (7%)[b]
Location	
Esophagus and stomach	3 (4%)
Small intestine	26 (31%)
Appendix	8 (10%)
Colorectal	3 (4%)
Pancreas	43 (52%)
Lymphovascular invasion	26 (31%)
Lymph node involvement at diagnosis	24 (29%)
Distant metastasis	
At initial diagnosis	9 (11%)
By follow-up endpoint	13 (16%)
Follow-up and outcome	
Median (range) months	28 (0–115)
Alive without disease	65 (78%)
Alive with disease	9 (11%)
Died of disease	3 (4%)
Died of unrelated causes	6 (7%)

[a] Included six patients with multiple endocrine neoplasia type 1 and one patient with von Hippel-Lindau syndrome

[b] Six cases of grade 3 tumors included esophageal large cell neuroendocrine carcinoma ($n = 1$), ampullary ($n = 2$), and pancreatic ($n = 3$) G3 well-differentiated neuroendocrine tumors

neuroendocrine tumor, but no metastatic disease. In contrast, the only VHL-associated case in the cohort was a 54-year-old woman who had grade 3 stage T3 pancreatic neuroendocrine tumor with clear cell features (Fig. 2D). Although there was no tissue-proven metastatic neuroendocrine tumor, she subsequently succumbed to concurrent metastatic colonic adenocarcinoma at 59.2 months after resection of the neuroendocrine tumor.

Morphology and Immunoprofile

All cases showed classic nested and trabecular histologic architecture (Fig. 2) with diffuse synaptophysin immunoreactivity. Chromogranin A was diffusely expressed by 76 (92%) cases, focally by 4 (5%) cases and was negative in 3 (4%) cases. Among the entire cohort of GEP-NENs, insulinoma-associated protein 1 (INSM1) was diffusely positive in 21 (25%) (Fig. 1A), focally in 46 (55%), and was negative in 16 (19%) patients. In the pancreas, the sensitivity was 88% with 38 out of 43 tumors showing at least focally positive staining. In the gastrointestinal tract, sensitivity of INSM1 was 73% with 29 out of 40 tumors showing positive staining. It is noteworthy that INSM1 staining was often focal and tended to be retained in most grade 2 (positive in 88%) and grade 3 (positive in 67%) tumors. Somatostatin receptor 2 (SSTR2) expression was overall diffuse in 69 (83%) (Fig. 1B), focal in 9 (11%), and negative in 5 (6%) patients with GEP-NENs. Among the five tumors which were negative for SSTR2 (four grade 1 and one grade 3 tumors), there was regional lymph node involvement in one patient but no distant metastases. All five patients remained disease-free during a median (range) follow-up of 22 (1–66) months. Among the three patients who died of progressive grade 3 tumors, one patient received somatostatin analog therapy (octreotide) but failed to halt disease progression despite strong tumor expression of SSTR2. None of the immunomarkers showed significant association with tumor location or histologic grade. Non-neoplastic islet cells in normal pancreatic tissues represented in the TMAs consistently showed immunoreactivity for synaptophysin, chromogranin A, INSM1, and SSTR2.

In Situ Hybridization

Predominantly nuclear labeling was observed for both HOTAIR and MALAT1 (Fig. 2) in all tumors with variable intensity. Both lncRNAs were also detected in gastrointestinal mucosal epithelia, exocrine pancreas, and pancreatic islets with moderate to strong intensity. All tissue cores in the TMAs showed adequate positive control labeling for PPIB. Of note, the seven syndromic cases (six MEN1 and one VHL) consistently displayed diffuse strong expression for both HOTAIR and MALAT1 (Fig. 2D–I).

Table 2 summarized logistic regression analyses for associations between clinical characteristics and tumor expression of HOTAIR and MALAT1 as determined by in situ hybridization. Correlations between clinical characteristics and tumor histologic grade were also examined. Not surprisingly, histologic grade was a significant predictor of lymphovascular invasion, advanced presenting stage, and development of distant metastases during study period ($P < 0.05$) and was therefore corrected for in multivariate analyses. In both univariate and multivariate models, higher HOTAIR expression was associated with lower presenting T and M stages as well as less frequent development of distant metastases by the end of follow-up ($P < 0.05$). Higher MALAT1 expression was associated with lower presenting T stage and decreased risk for developing distant metastatic disease during the study period ($P < 0.05$). Neither lncRNAs showed correlation with histologic grade, MEN1 syndrome, or lymphovascular invasion.

A total of 17 patients had both primary and metastatic tumors represented in the tissue microarray. By paired *t* test, HOTAIR expression levels were consistently lower in metastatic tumors compared to the patient-matched primary tumors with a median (range) difference of − 0.10 (− 0.44 to 0.21) ($P = 0.006$). There was no significant difference in the expression of MALAT1.

Discussion

In this study, we examined the expression and prognostic relevance of HOTAIR and MALAT1 in gastroenteropancreatic neuroendocrine neoplasms. In a cohort of 83 cases, both

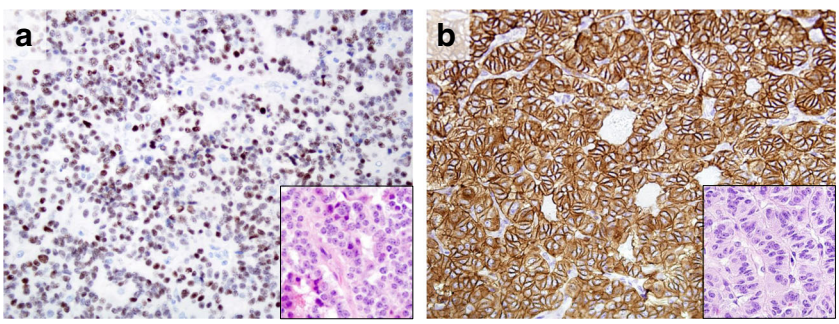

Fig. 1 Focal to diffuse INSM1 (**A**) and SSTR2 (**B**) expression was seen in 80% and 94% of gastroenteropancreatic neuroendocrine neoplasms, respectively. Insets: hematoxylin and eosin stains

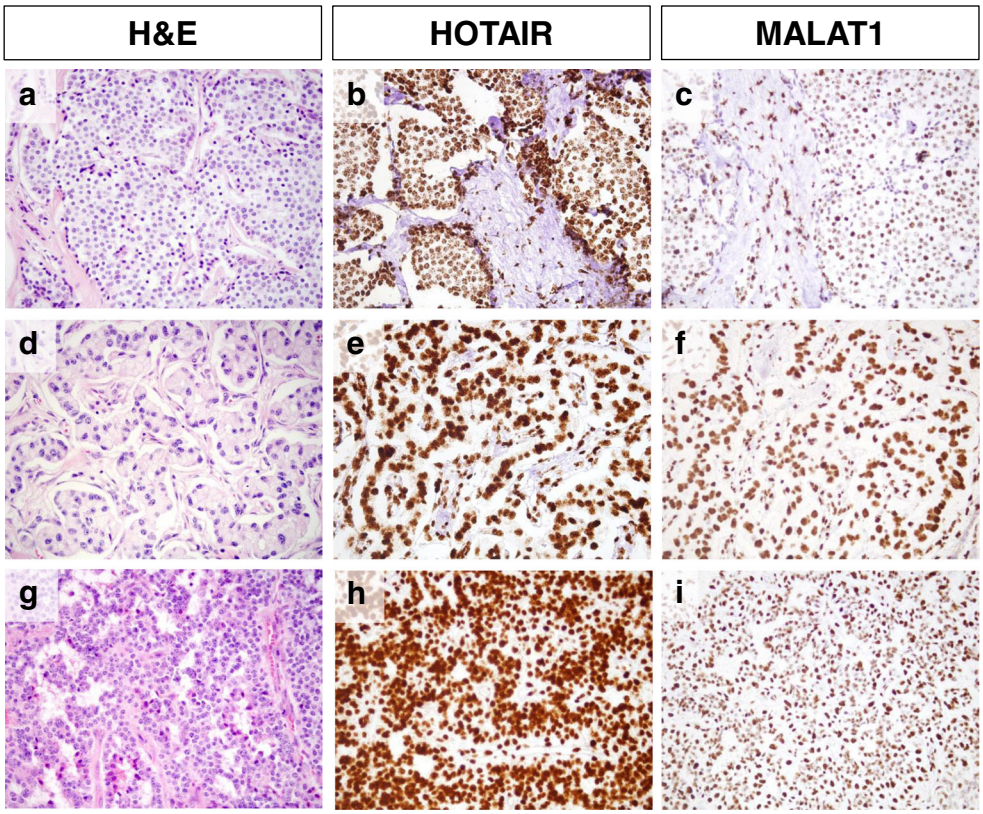

Fig. 2 **a**–**c** A patient with grade 1 jejunal neuroendocrine tumor which showed diffuse expression of HOTAIR and MALAT1. **d**–**f** Grade 3 pancreatic neuroendocrine tumor with clear cell features in a patient with von Hippel-Lindau syndrome. **g**–**i** Grade 2 pancreatic neuroendocrine tumor in a patient with multiple endocrine neoplasia type 1

lncRNAs were universally expressed but labeling intensity was notably variable, suggesting potential diagnostic and prognostic utility. We further demonstrated that HOTAIR and MALAT1 expression independently correlated with less aggressive tumor behavior with reduced risk for metastatic disease and favorable survival. The correlations remained significant after adjusting for the effect of histologic grade, suggesting that both lncRNAs may provide new prognostic information not reflected by the current grading system.

While MALAT1 has been recognized as oncogenic lncRNAs in the literature [28, 29], several recent studies have started to reveal its antitumoral properties [20–23]. Expression of MALAT1 has been shown to be significantly lower in glioma compared to normal brain tissue [21, 22]. Overexpression of MALAT1 appeared to be a favorable survival predictor for patients with glioma [22]. Also, MALAT1 suppresses the viability and invasiveness of glioma cell lines [21, 22]. Two independent studies have proposed two potentially overlapping oncoregulatory pathways affected by MALAT1 in glioma. Han et al. showed that MALAT1 suppressed the expression of phosphorylated ERK1/2 and invasion-related enzyme matrix metalloproteinase 2 [21]. Cao et al. were able to demonstrate that MALAT1 downregulates the expression of prooncogenic miRNA miR-155 while upregulating the tumor-suppressing protein FBXW7 [22]. FBXW7 has been showed to be a downstream target of ERK activation by increased ubiquitination and degradation [30]. On the other hand, in breast and colon cancers, Kwok et al. found that PTEN upregulates MALAT1 by sequestering several miRNAs that target both PTEN and MALAT1 [20]. Furthermore, knockdown of MALAT1 led to upregulation of pro-migratory genes *EpCAM* and *ITGB4* in a colon cancer cell line [20]. Kim et al. also observed the inhibition of ITGB4 by MALAT1 in breast cancer cell lines and further demonstrated that the inhibition was mediated by the sequestration of protumorigenic TEAD family of transcription factors in a YAP-independent fashion [23]. In the current study, we found that MALAT1 is associated with favorable clinical outcome in gastroenteropancreatic neuroendocrine neoplasms. Further functional studies are needed to reveal the underlying mechanisms.

In the current study, we found HOTAIR to be diffusely expressed by gastroenteropancreatic neuroendocrine neoplasms. Furthermore, high expression of HOTAIR was associated with lower tumor stage. HOTAIR was previously shown to play an essential role in neuroendocrine differentiation of prostate cancer [31] but has not been analyzed in primary neuroendocrine tumors. While HOTAIR has been mostly reported as a pro-oncogenic lncRNA which regulates several survival- and migration-related genes in the literature (recently reviewed [16]), it was found to be downregulated in several brain tumors including medulloblastoma and ependymoma compared to normal brain parenchyma [32]. In the current study, normal pancreatic islets represented in tissue microarray cores appeared to diffusely express HOTAIR in a visually similar intensity compared to tumors; however, quantitative comparison of

Table 2 Association of lncRNA expression with clinical characteristics

	Univariate		Multivariate[a]	
Characteristic/factor	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Histologic grade (G2/G3 vs. G1)				
HOTAIR	1.17 (0.44–3.09)	0.7542		
MALAT1	1.66 (0.63–4.55)	0.3085		
Inheritance (MEN1 vs. sporadic)				
Histologic grade	0.50 (0.03–3.34)	0.5375		
HOTAIR	–	0.9339		
MALAT1	5.13 (0.78–100.69)	0.1439		
LVI (present vs. absent)				
Histologic grade	5.11 (1.82–15.11)	**0.0023**		
HOTAIR	0.44 (0.16–1.14)	0.0971	0.39 (0.13–1.12)	0.0893
MALAT1	0.61 (0.23–1.56)	0.3055	0.46 (0.15–1.28)	0.1443
T stage at diagnosis (T2–4 vs. T1)				
Histologic grade	5.50 (1.96–16.51)	**0.0016**		
HOTAIR	0.29 (0.10–0.77)	**0.0149**	0.20 (0.05–0.60)	**0.0068**
MALAT1	0.38 (0.14–0.96)	**0.0440**	0.24 (0.07–0.71)	**0.0140**
M stage at diagnosis (M1 vs. M0)				
Histologic grade	3.89 (0.94–17.24)	**0.0605**		
HOTAIR	0.11 (0.01–0.62)	**0.0390**	0.08 (0.00–0.53)	**0.0271**
MALAT1	–	0.9462	–	0.9357
Distant metastasis at endpoint (present vs. absent)				
Histologic grade	5.87 (1.71–22.01)	**0.0057**		
HOTAIR	0.25 (0.05–0.91)	**0.0496**	0.18 (0.03–0.75)	**0.0293**
MALAT1	0.13 (0.02–0.53)	**0.0110**	0.06 (0.01–0.31)	**0.0031**

Abbreviations: *OR* ratio of the odds, *CI* confidence interval, *MEN1* multiple endocrine neoplasia type 1

Significance is defined by P value < 0.05

[a] Included patient age and tumor histologic grade as covariates

expression between GEP-NENs and their non-neoplastic counterparts (pancreatic islets and gastrointestinal intramucosal neuroendocrine cells) was not feasible due to software limitations. Given the functional versatility commonly seen with lncRNAs, we speculate a possible tumor-suppressive aspect of HOTAIR based on the observed associations with tumor stage and metastatic prospect although this hypothesis needs to be further tested by functional experiments.

Although this study examined only MALAT1 and HOTAIR in endocrine tumors, a growing number of lncRNAs have recently been described in endocrine tumors such as ROR and PVT1 in papillary thyroid carcinomas [33]. Regulatory functions of lncRNAs in endocrine tumors such as in thyroid tumors and in exosomes derived from thyroid tumors have also been recently reported [34].

The molecular features of GEP-NENs have been characterized by several recent studies [35–37]. Somatic mutations have been identified in *SMARCB1*, *TP53*, *STK11*, *RET*, *BRAF*, *KRAS*, *PIK3CA*, *PTEN*, *CTNNB1*, *APC*, *FBXW7*, *ATM*, and *IDH1* [35–37]. Furthermore, prior studies have found the downregulation of ATRX (alpha thalassemia/mental retardation syndrome X-linked)/DAXX (death-domain associated protein) to be associated with aggressive clinical course in pancreatic neuroendocrine neoplasms [38]. Several of these genes, including *PTEN*, *FBXW7*, and genes involved in the Wnt/β-catenin pathway, have been shown to be regulated by MALAT1 and HOTAIR in breast and colon cancers [20, 22, 25, 26] but their molecular crosstalk has yet to be examined in GEP-NENs. No studies have reported on the relation between ATRX/DAXX and either MALAT1 or HOTAIR. There has been very scarce information in the literature regarding lncRNAs in GEP-NENs. Individual studies have found two lncRNAs, MEG3 and LncNEN885, to be dysregulated in GEP-NENs [11, 13]. Of note, MEG3 has been shown in cell line models to be regulated by menin and potentially involved in the pathogenesis of pancreatic neuroendocrine tumors arising in the MEN1 syndrome [11] and was therefore considered to be a key tumorigenic mediator in patients affected by the MEN1 syndrome. Whether there is any molecular interaction between MEG3 and LncNEN885 with MALAT1 and HOTAIR has yet to be studied. Prior studies have also suggested molecular differences between pancreatic

and gastrointestinal tumors [35–37]. In the current study, we attempted to analyze pancreatic and gastrointestinal tumors separately but this was limited by the total number of cases.

Immunohistochemical studies with insulinoma-associated protein 1 (INSM1) expression supported recent observations that INSM1 is a neuroendocrine marker in multiple organs including lung, gastrointestinal tract, pancreas, skin (in Merkel cell carcinomas), prostate, head, and neck [39–44]. Tanigawa et al. previously observed high level of INSM1 expression (100%) in a cohort of 25 pancreatic neuroendocrine neoplasms (20 grade 1, 4 grade 2, and 1 grade 3 tumors) and in the neuroendocrine component of a mixed neuroendocrine-non-neuroendocrine neoplasm case but no expression in pancreatic solid-pseudopapillary neoplasms, a major morphologic differential diagnosis [42]. In our cohort, we noted an overall sensitivity of 81% (67 of 83 tumors with positive staining) which was similar to published reports [41].

Somatostatin analogs (SSAs) have been clinically used for decades as an effective and well-tolerated treatment for metastatic well-differentiated gastroenteropancreatic neuroendocrine tumors [45]. Consistent with previous reports [46], 94% of tumors (78 of 83 cases) showed expression of somatostatin receptor 2 (SSTR2) in the current cohort. Of note, all five patients with tumors which were negative for SSTR2 remained disease-free at the end of study period, suggesting that lack of SSTR2 expression may not be an adverse outcome predictor. On a separate note, one patient in our cohort with tumor that strongly expressed SSTR2 failed to respond to somatostatin analog treatment, suggesting that SSTR2 expression may not always be a reliable biomarker for therapeutic response. A remarkable knowledge gap still exists in terms of various contributing oncogenic pathways in GEP-NENs and resistance mechanisms against SSAs. Previous trials have noticed survival benefit of everolimus (an mTOR inhibitor) and sunitinib in patients who progressed after SSA treatment [45].

In conclusion, neuroendocrine neoplasms of the digestive system represent a biologically diverse group of tumors with highly variable clinical behavior. Current prognostication has been largely based on histological grade, proliferative activity, and the TNM staging system. This study provided evidence that HOTAIR and MALAT1 may have prognostic significance in gastroenteropancreatic neuroendocrine tumors. The associations with tumor behavior were independent of histologic grade and may therefore provide novel prognostic utility.

Acknowledgements The authors thank the University of Wisconsin Translational Research Initiatives in Pathology Laboratory, in part supported by the University of Wisconsin Department of Pathology and Laboratory Medicine and University of Wisconsin Carbone Cancer Center grant P30 CA014520, for use of its facilities and services.

Dr. Chu and Dr. Lloyd received a Research and Development Award from the Department of Pathology and Laboratory Medicine, University of Wisconsin Hospital and Clinics to conduct the study.

Compliance with Ethical Standards

Conflict of Interest The authors declare that there are no conflicts of interest.

References

1. Ohike N, Adsay NV, La Rosa S, Volante M, Zamboni G. 2017. WHO Classification of Tumours of Endocrine Organs, 10 ed. WHO Press, World Health Organization, Geneva.
2. Coriat R, Walter T, Terris B, Couvelard A, Ruszniewski P. 2016. Gastroenteropancreatic Well-Differentiated Grade 3 Neuroendocrine Tumors: Review and Position Statement. Oncologist 21:1191–1199.
3. Oberg K. 2009. Genetics and molecular pathology of neuroendocrine gastrointestinal and pancreatic tumors (gastroenteropancreatic neuroendocrine tumors). Curr Opin Endocrinol Diabetes Obes 16: 72–78.
4. Lewis MA, Yao JC. 2014. Molecular pathology and genetics of gastrointestinal neuroendocrine tumours. Current Opinion in Endocrinology, Diabetes and Obesity 21:22–27.
5. Amair-Pinedo F, Matos I, Sauri T, Hernando J, Capdevila J. 2017. The Treatment Landscape and New Opportunities of Molecular Targeted Therapies in Gastroenteropancreatic Neuroendocrine Tumors. Target Oncol 12:757–774.
6. Thorns C, Schurmann C, Gebauer N, et al. 2014. Global microRNA profiling of pancreatic neuroendocrine neoplasias. Anticancer Res 34:2249–2254.
7. Malczewska A, Kidd M, Matar S, Kos-Kudla B, Modlin IM. 2018. A Comprehensive Assessment of the Role of miRNAs as Biomarkers in Gastroenteropancreatic Neuroendocrine Tumors. Neuroendocrinology 107:73–90.
8. Yoon JH, Abdelmohsen K, Gorospe M. 2013. Posttranscriptional gene regulation by long noncoding RNA. J Mol Biol 425:3723–3730.
9. Rinn JL, Chang HY. 2012. Genome regulation by long noncoding RNAs. Annu Rev Biochem 81:145–166.
10. Yang Y, Junjie P, Sanjun C, Ma Y. 2017. Long non-coding RNAs in Colorectal Cancer: Progression and Future Directions. J Cancer 8: 3212–3225.
11. Modali SD, Parekh VI, Kebebew E, Agarwal SK. 2015. Epigenetic regulation of the lncRNA MEG3 and its target c-MET in pancreatic neuroendocrine tumors. Mol Endocrinol 29:224–237.
12. Parekh VI, Modali SD, Desai SS, Agarwal SK. 2015. Consequence of Menin Deficiency in Mouse Adipocytes Derived by In Vitro Differentiation. Int J Endocrinol 2015:149826.
13. Wei YL, Hua J, Liu XY, Hua XM, Sun C, Bai JA, Tang QY 2018. LncNEN885 inhibits epithelial-mesenchymal transition by partially regulation of Wnt/beta-catenin signalling in gastroenteropancreatic neuroendocrine neoplasms. Cancer Sci 109:3139–3148.
14. Pang EJ, Yang R, Fu XB, Liu YF. 2015. Overexpression of long non-coding RNA MALAT1 is correlated with clinical progression and unfavorable prognosis in pancreatic cancer. Tumour Biol 36: 2403–2407.
15. Zheng HT, Shi DB, Wang YW, Li XX, Xu Y, Tripathi P, Gu WL, Cai GX, Cai SJ 2014. High expression of lncRNA MALAT1 suggests a biomarker of poor prognosis in colorectal cancer. Int J Clin Exp Pathol 7:3174–3181.
16. Wu ZH, Wang XL, Tang HM, et al. 2014. Long non-coding RNA HOTAIR is a powerful predictor of metastasis and poor prognosis and is associated with epithelial-mesenchymal transition in colon cancer. Oncol Rep 32:395–402.

17. Kim K, Jutooru I, Chadalapaka G, Johnson G, Frank J, Burghardt R, Kim S, Safe S 2013. HOTAIR is a negative prognostic factor and exhibits pro-oncogenic activity in pancreatic cancer. Oncogene 32: 1616–1625.
18. Emadi-Andani E, Nikpour P, Emadi-Baygi M, Bidmeshkipour A. 2014. Association of HOTAIR expression in gastric carcinoma with invasion and distant metastasis. Adv Biomed Res 3:135.
19. Ge XS, Ma HJ, Zheng XH, Ruan HL, Liao XY, Xue WQ, Chen YB, Zhang Y, Jia WH 2013. HOTAIR, a prognostic factor in esophageal squamous cell carcinoma, inhibits WIF-1 expression and activates Wnt pathway. Cancer Sci 104:1675–1682.
20. Kwok ZH, Roche V, Chew XH, Fadieieva A, Tay Y. 2018. A non-canonical tumor suppressive role for the long non-coding RNA MALAT1 in colon and breast cancers. Int J Cancer https://doi.org/10.1002/ijc.31386, 143, 668, 678.
21. Han Y, Wu Z, Wu T, Huang Y., Cheng Z., Li X., Sun T., Xie X., Zhou Y., du Z. 2016. Tumor-suppressive function of long noncoding RNA MALAT1 in glioma cells by downregulation of MMP2 and inactivation of ERK/MAPK signaling. Cell Death Dis 7:e2123.
22. Cao S, Wang Y, Li J, Lv M, Niu H, Tian Y. 2016. Tumor-suppressive function of long noncoding RNA MALAT1 in glioma cells by suppressing miR-155 expression and activating FBXW7 function. Am J Cancer Res 6:2561–2574.
23. Kim J, Piao HL, Kim BJ, Yao F, Han Z, Wang Y, Xiao Z, Siverly AN, Lawhon SE, Ton BN, Lee H, Zhou Z, Gan B, Nakagawa S, Ellis MJ, Liang H, Hung MC, You MJ, Sun Y, Ma L 2018. Long noncoding RNA MALAT1 suppresses breast cancer metastasis. Nat Genet 50:1705–1715.
24. Zhao M, Wang S, Li Q, Ji Q, Guo P, Liu X. 2018. MALAT1: A long non-coding RNA highly associated with human cancers. Oncol Lett 16:19–26.
25. Hajjari M, Salavaty A. 2015. HOTAIR: an oncogenic long non-coding RNA in different cancers. Cancer Biol Med 12:1–9.
26. Hirata H, Hinoda Y, Shahryari V, Deng G., Nakajima K., Tabatabai Z.L., Ishii N., Dahiya R. 2015. Long Noncoding RNA MALAT1 Promotes Aggressive Renal Cell Carcinoma through Ezh2 and Interacts with miR-205. Cancer Res 75:1322–1331.
27. Zhang R, Hardin H, Huang W, Chen J, Asioli S, Righi A, Maletta F, Sapino A, Lloyd RV 2017. MALAT1 Long Non-coding RNA Expression in Thyroid Tissues: Analysis by In Situ Hybridization and Real-Time PCR. Endocr Pathol 28:7–12.
28. Amodio N, Raimondi L, Juli G, Stamato MA, Caracciolo D, Tagliaferri P, Tassone P 2018. MALAT1: a druggable long non-coding RNA for targeted anti-cancer approaches. J Hematol Oncol 11:63.
29. Tang Q, Hann SS. 2018. HOTAIR: An Oncogenic Long Non-Coding RNA in Human Cancer. Cell Physiol Biochem 47:893–913.
30. Ji S, Qin Y, Shi S, Liu X., Hu H., Zhou H., Gao J., Zhang B., Xu W., Liu J., Liang D., Liu L., Liu C., Long J., Zhou H., Chiao P.J., Xu J., Ni Q., Gao D., Yu X. 2015. ERK kinase phosphorylates and destabilizes the tumor suppressor FBW7 in pancreatic cancer. Cell Res 25:561–573.
31. Chang YT, Lin TP, Tang JT, Campbell M, Luo YL, Lu SY, Yang CP, Cheng TY, Chang CH, Liu TT, Lin CH, Kung HJ, Pan CC, Chang PC 2018. HOTAIR is a REST-regulated lncRNA that promotes neuroendocrine differentiation in castration resistant prostate cancer. Cancer Lett 433:43–52.
32. Chakravadhanula M, Ozols VV, Hampton CN, Zhou L, Catchpoole D, Bhardwaj RD. 2014. Expression of the HOX genes and HOTAIR in atypical teratoid rhabdoid tumors and other pediatric brain tumors. Cancer Genet 207:425–428.
33. Zhang R, Hardin H, Huang W, Buehler D, Lloyd RV. 2018. Long Non-coding RNA Linc-ROR Is Upregulated in Papillary Thyroid Carcinoma. Endocr Pathol 29:1–8.
34. Hardin H, Helein H, Meyer K, Robertson S, Zhang R, Zhong W, Lloyd RV 2018. Thyroid cancer stem-like cell exosomes: regulation of EMT via transfer of lncRNAs. Lab Invest 98:1133–1142.
35. Vijayvergia N, Boland PM, Handorf E, Gustafson KS, Gong Y, Cooper HS, Sheriff F, Astsaturov I, Cohen SJ, Engstrom PF 2016. Molecular profiling of neuroendocrine malignancies to identify prognostic and therapeutic markers: a Fox Chase Cancer Center Pilot Study. Br J Cancer 115:564–570.
36. Kim ST, Lee SJ, Park SH, Park JO, Lim HY, Kang WK, Lee J, Park YS 2016. Genomic Profiling of Metastatic Gastroenteropancreatic Neuroendocrine Tumor (GEP-NET) Patients in the Personalized-Medicine Era. J Cancer 7:1044–1048.
37. Kyriakopoulos G, Mavroeidi V, Chatzellis E, Kaltsas GA, Alexandraki KI. 2018. Histopathological, immunohistochemical, genetic and molecular markers of neuroendocrine neoplasms. Ann Transl Med 6:252.
38. Yadav R, Kakkar A, Sharma A, Malik PS, Sharma MC. 2016. Study of clinicopathological features, hormone immunoexpression, and loss of ATRX and DAXX expression in pancreatic neuroendocrine tumors. Scand J Gastroenterol 51:994–999.
39. Mukhopadhyay S, Dermawan JK, Lanigan CP, Farver CF. 2018. Insulinoma-associated protein 1 (INSM1) is a sensitive and highly specific marker of neuroendocrine differentiation in primary lung neoplasms: an immunohistochemical study of 345 cases, including 292 whole-tissue sections. Mod Pathol https://doi.org/10.1038/s41379-018-0122-7.
40. Lilo MT, Chen Y, LeBlanc RE. 2018. INSM1 is More Sensitive and Interpretable than Conventional Immunohistochemical Stains Used to Diagnose Merkel Cell Carcinoma. Am J Surg Pathol https://doi.org/10.1097/pas.0000000000001136, 42, 1541, 1548.
41. Rosenbaum JN, Guo Z, Baus RM, Werner H, Rehrauer WM, Lloyd RV. 2015. INSM1: A Novel Immunohistochemical and Molecular Marker for Neuroendocrine and Neuroepithelial Neoplasms. Am J Clin Pathol 144:579–591.
42. Tanigawa M, Nakayama M, Taira T, Hattori S, Mihara Y, Kondo R, Kusano H, Nakamura K, Abe Y, Ishida Y, Okabe Y, Hisaka T, Okuda K, Fujino K, Ito T, Kawahara A, Naito Y, Yamaguchi R, Akiba J, Akagi Y, Yano H 2018. Insulinoma-associated protein 1 (INSM1) is a useful marker for pancreatic neuroendocrine tumor. Med Mol Morphol 51:32–40.
43. Rooper LM, Bishop JA, Westra WH. 2018. INSM1 is a Sensitive and Specific Marker of Neuroendocrine Differentiation in Head and Neck Tumors. Am J Surg Pathol 42:665–671.
44. Xin Z, Zhang Y, Jiang Z, Zhao L, Fan L, Wang Y, Xie S, Shangguan X, Zhu Y, Pan J, Liu Q, Huang Y, Dong B, Xue W 2018. Insulinoma-associated protein 1 is a novel sensitive and specific marker for small cell carcinoma of the prostate. Hum Pathol 79: 151–159.
45. Uri I, Grozinsky-Glasberg S. 2018. Current treatment strategies for patients with advanced gastroenteropancreatic neuroendocrine tumors (GEP-NETs). Clin Diabetes Endocrinol 4:16.
46. Oberg KE, Reubi JC, Kwekkeboom DJ, Krenning EP. 2010. Role of somatostatins in gastroenteropancreatic neuroendocrine tumor development and therapy. Gastroenterology 139:742–753, 753.e741