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LOSS of *DPC4/SMAD4* EXPRESSION IN PRIMARY GASTROINTESTINAL NEUROENDOCRINE TUMORS IS ASSOCIATED WITH CANCER-RELATED DEATH AFTER RESECTION

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Abstract

Background—Gastrointestinal neuroendocrine tumors (GINET) have frequent loss of *DPC4/SMAD4* expression, a known tumor suppressor. The impact of *SMAD4* loss on GINET aggressiveness or cancer-related patient outcomes is not defined. We examined the expression of *SMAD4* in resected GINET and its impact on oncologic outcomes.

Methods—Patients who underwent complete surgical resection for GINET were retrospectively identified (n=38). Immunohistochemical staining for *SMAD4* expression was scored by a blinded pathologist and correlated with clinicopathologic features and oncologic outcomes.

Results—Twenty-nine percent of the tumors were *SMAD4*-neg and 71% *SMAD4*-pos. Median overall survival was 155 (95% CI, 102 – 208) months. Loss of *SMAD4* was associated with reduced median disease-free survival (28 [95% CI, 16 – 40] months compared to 223 [95% CI, 3 – 443] months for *SMAD4*-pos patients (p=0.03) and median disease-specific survival (*SMAD4*: 137 [95% CI, 81 – 194] months vs. *SMAD4*-pos: 204 [95% CI, 143 – 264] months; p=0.04). This translated into a reduction in median overall survival (*SMAD4*-neg: 125 (95% CI, 51 – 214) months vs. *SMAD4*-pos: 185 (95% CI, 138 – 232) months; p=0.02).

Conclusion—Consistent with the known biology of the *DPC4/SMAD4* gene, an absence of protein expression in the primary GINET tumor was negatively associated with outcomes after surgical resection.

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Keywords

Neuroendocrine; Gastrointestinal Neuroendocrine; *DPC4/SMAD4*

Introduction

The term neuroendocrine tumor (NET) describes various malignant tumors arising from neuroendocrine cells throughout the body, first described as “carcinoid tumors” by Oberndorfer in 1907 (1). These tumors are uncommon, with an age-adjusted annual incidence of 2.5–4.5 per 100,000 (2, 3). They are considered generally more indolent than other epithelial malignancies; however ongoing studies continue to define subsets with discrete differences in cancer-related outcomes. A comprehensive review of NETs within the Surveillance, Epidemiology, and End Results (SEER) database SEER data found the primary tumor site to be a powerful predictor of survival duration, suggesting distinct biologic differences by anatomic location. (4)

Similarly, advances in genetic analysis of NETs offer the opportunity to further characterize and stratify these tumors into useful groupings. Prior investigations have identified a correlation between anatomic site and patterns of genetic aberration. The most consistent finding is the frequent loss of genetic material on chromosome 18 in NETs arising in the small intestine. This has been observed in 43–100% of small bowel NETs (5–14) which strongly suggests loss of crucial tumor suppressor gene(s) in this location. However, mutational analyses of several putative tumor suppressor genes at 18q21–18q22 failed to identify mutations (8, 10, 15). Indeed, massive parallel exome sequencing of 48 small bowel NETs (SBNET) identified a low average mutation rate and, outside of 10% of patients having frameshift mutations in p27, no recurrently mutated genes. Interestingly, 46% of NETs did demonstrate some degree of copy number loss of *SMAD4*, suggesting a potential role of *SMAD4* in GINET carcinogenesis (16). Together, these data suggest that SBNET is a genetically unique subset of NET and that loss of genetic material at chromosome 18 drives clinical outcome.

SMAD4 is a well-known tumor suppressor gene originally recognized as “deleted in pancreatic carcinoma locus 4” (*DPC4*) located on human chromosome 18q21.1 (17, 18). Loss of expression of the *SMAD4* protein is the most commonly observed consequence of genetic mutation. This loss of function is critical as *SMAD4* is a lynchpin within the TGF- β canonical signaling pathway (19). Importantly, loss of *SMAD4* occurs in numerous solid organ neoplasms and is associated with tumor progression and metastasis (20, 21). Identification of molecular markers with primary NETs associated with cancer progression is clinically important. Once distant metastasis occurs, the median survival decreases from 111 to 56 months. Furthermore, there is currently no effective systemic therapy for distant metastatic SBNET. Therefore, it is critical to increase our understanding of the biology of distant metastasis development in GINET.

Based on these preclinical data, we hypothesized that loss of *SMAD4* would be associated with reduced oncologic outcomes. The goal of our study was to evaluate cancer-specific end-points in patients with primary, resected GINET in relation to *SMAD4* status.

Methods

1. Patient Data

With the approval of the Institutional Review Board (PA12-0021) of The University of Texas MD Anderson Cancer Center, the Pathology Archive was searched for primary gastrointestinal NET (GINET) specimens with complete clinically and pathologic data from 1990–2009. All GINET diagnoses were confirmed by an MDACC pathologist. A clinically-annotated tissue microarray (TMA) was constructed using fifty- two individual tumor samples from patients diagnosed with GINET using formalin-fixed, paraffin-embedded archival tissue blocks and a tissue microarrayer (Beecher Instruments, Sun Prairie, WI), as previously described (22). Patients with synchronous metastatic adenocarcinoma of another tissue origin were excluded from analysis (n=8), leaving 44 individual tumor samples for SMAD4 immunohistochemistry in the TMA. Nineteen patients did not undergo curative-intent surgery for treatment of GINET and were excluded from analysis. A prospectively maintained institutional tumor bank was further queried to identify additional cases of primary resected GINET. Twenty additional tumors with complete clinical and pathologic data were identified and included. Therefore a total of 38 patients were identified to have surgically resected GINETs with complete clinical and pathological data and comprised the patient cohort.

2. Immunohistochemistry

SMAD4 immunohistochemical analysis was done at the clinical core laboratory at MD Anderson using a Lab-Vision 480-2D immunostainer (ThermoFisher, Fremont, CA, USA). All reactions were visualized with diaminobenzidine (DAB) as a chromogen. Positive and negative controls were included in each run for the antibody used. Isotype controls for antibody were used as negative controls as previously described (23). SMAD4 antibody was purchased from Leica Biosystems Inc., (#NCL-DPC4, Buffalo Grove, IL, USA) and was diluted in 1:40. All images were captured via an Olympus DP72 camera attached to an Olympus BX51 microscope and processed via cellSens software (Olympus Scientific, Waltham, MA, USA). SMAD4 staining, cytoplasmic, nuclear or both, was scored using the following criteria: 0, no staining of tumor cells; 1+ faint staining of the tumor cells as compared to surrounding normal pancreatic parenchyma; 2+, moderate to strong tumor cell staining, of similar or greater intensity compared to surrounding normal pancreatic parenchyma. The percentage of tumor cells staining were noted as well. For statistical analysis, SMAD4 score = 0 was considered SMAD4 negative, whereas scores of 1+ or 2+ in greater than or equal to 10% of tumor cells were classified as “SMAD4-positive.” Representative micrographs are shown in (Figure 1).

3. Statistical Analysis

Patients were divided into dichotomous groups based on SMAD4 score and the clinical oncologic outcomes data were analyzed. Categorical variables were compared using χ^2 or Fisher's exact test. Wilcoxon's rank sum test (t-test) and the Kruskal-Wallis test (ANOVA) were used to compare the distributions of continuous variable among different groups. Overall survival (OS) and disease-specific survival (DSS) were measured from date of surgery to date of death owing to any cause or date of GINET-specific death, respectively.

Disease-free survival (DFS) was measured from the date of surgical resection to time of pathological or radiographic evidence of recurrent or metastatic disease or date of last follow-up, if recurrence-free. Univariate Kaplan and Meier methods were used to estimate OS, DSS and DFS. All tests were two sided. P-values less than 0.05 were considered statistically significant. The Cox proportional hazards regression model, in which a backward elimination process was used for variable selection with an entry and removal limit of $P < 0.1$ and $P < 0.05$, respectively, was applied to identify independent factors associated with DSS. All analyses were performed using SPSS version 23.0 (IBM corp., Chicago, IL) and Graphpad software (La Jolla, CA USA).

Results

1. Clinicopathologic Patient Results

A total of 45 tumors were evaluated for *SMAD4* expression by IHC. Seven specimens (16%) had insufficient tissue for evaluation and were excluded, leaving a total of 38 tumors with complete clinical and pathologic information available for evaluation (Table 1). Thirty (78.9%) were of small bowel origin and eight (21.1%) arose in the large bowel. Median age at diagnosis was 59 years. Sixteen patients were female. At the time of diagnosis, all patients underwent curative-intent surgical resection of their primary disease and radiographically-evident metastases (when applicable). The majority of tumors were T3 and lymph node metastases were common, with 31 patients (81.5%) having lymph node positive disease.

2. SMAD4 IHC

Twenty-nine percent ($n=11$) of the tumors had an IHC score of 0 and were classified as *SMAD4*-negative. Nineteen tumors had an IHC score of 1+ and 8 had a score of 2+, for a total of 27 (71%) of tumors classified as *SMAD4*-positive. There was no difference in the stage distribution or lymphovascular invasion based on *SMAD4* status (Table 1).

3. Cancer-related outcomes

Median follow-up time was 76 months (77 months for survivors) with a median overall survival of 155 (95% CI, 102 – 208) months. Median DFS for the entire cohort was 49 months (95% CI, 10 – 87 months), with a DSS of 204 (95% CI, 123 – 283) months. Of the 38 patients who underwent curative-intent surgery, 22 developed recurrent disease. First recurrence events included: 9 liver metastases, 5 peritoneal metastases, 2 local recurrences and 3 simultaneous liver and local recurrences (Table 1). 82% of patients ($n=9$) with tumors that were *SMAD4*-neg recurred compared to 48% of patients ($n=13$) with *SMAD4*-pos tumors ($p=0.04$).

A total of 20 patients died during the follow-up period; of these, 14 died of metastatic NET and 6 died of other causes. Univariate analysis of factors associated with DSS (Table 2) demonstrated that loss of *SMAD4* was associated with reduced median DSS ($p=0.04$). There were no other clinical or pathologic factors identified that were associated with DSS.

Loss of *SMAD4* (*SMAD4*-neg) was associated with a shorter DFS (43 [95% CI, 16–40] months) compared to patients who expressed *SMAD4* (*SMAD4*-pos; 223 [95% CI, 3–443]

months, $p=0.01$; Figure 2A). Patients with no expression of SMAD4 within the primary tumor were significantly more likely to die a cancer-related death than those whose primary tumor retained SMAD4 expression (137 [95% CI, 81 – 194] vs. 204 [95% CI, 143 – 264] months respectively; $p=0.04$; Fig. 2B). This translated into a reduced overall survival, (SMAD4-negative: 125 [95% CI, 51 – 214] months vs. SMAD4-positive: 185 [95% CI, 138 – 232] months; $p=0.02$; Figure 2C). Finally, when SMAD4 status was combined with LN status in assessing overall patient survival, patients with no lymph node metastases and retained SMAD4 expression in the primary tumor experienced significantly improved survival (Fig. 2D). Although SMAD4 status was significant for DFS and DSS on univariate analysis, we were unable to identify any clinical factors associated with survival by multivariate analysis.

Discussion

This study is the first to identify that loss of SMAD4 expression within the primary GINET tumor is associated with reduced cancer survival after surgical resection. We performed immunohistochemical staining for SMAD4 expression of formalin-fixed paraffin embedded primary NET tumor of 38 patients who underwent resection at our institution. Patients whose primary exhibited retention of SMAD4 experienced improved oncologic outcomes when compared to those with loss of SMAD4 expression. SMAD4 loss was associated with a significant decrease in disease-free and GINET-specific survival. Additionally, loss of SMAD4 expression was associated with the presence of lymph node metastases within the specimen, and patients with lymph node metastases and loss of SMAD4 within the primary tumor fared worse with an overall time to recurrence of only 28 months (Fig. 2D).

SMAD4 is a well-described tumor suppressor gene that was originally identified as “deleted in pancreatic carcinoma locus 4” (*DPC4*). Point mutations or deletions that result in the loss of expression of the SMAD4 protein are the most common structural mutations observed. This loss of function is critical, as SMAD4 is a critical for TGF- β canonical signaling (19) and thereby regulates cell proliferation, migration, bone development, differentiation, and apoptosis (24). Importantly, loss of SMAD4 protein expression is observed in numerous cancers, including pancreas, breast and prostate adenocarcinoma, and SMAD4 loss is often associated with tumor progression and metastasis (20, 21, 25). Chaudhry et al first demonstrated the intimate relationship between NET and TGF- β (26). Kidd et al used KRJ-I cells derived from GINET (27) to show that loss of SMAD4 produced upregulation of c-myc, and inhibition of the cell cycle checkpoint inhibitor, p21^{waf1} resulting in cell proliferation and tumorigenesis. Together, these data implicate the loss of SMAD4 as a major contributor to neuroendocrine tumor cell proliferation and progression.

For patients presenting with loco-regionally advanced GINET, surgical resection of the primary tumor and draining regional lymph nodes remains the only potentially curative therapy (28). Unfortunately, about half of these patients will recur and die of disease. Molecular markers to predict progression and/or metastasis are lacking and this has spurred increasing molecular characterization studies. A consistent finding of prior studies has been the frequent loss of large portions of genetic material on chromosome 18 in NETs arising in the small intestine, which has been observed in 43–100% of small bowel NETs (5–14, 29).

This consistent finding strongly suggests loss of crucial tumor suppressor gene(s) in this chromosomal location. Chromosome 18 harbors several candidate tumor suppressor genes, including deleted in colon cancer (*DCC*), *SMAD4*, and *SMAD2*. More detailed mutational analyses of several putative tumor suppressor genes at 18q21–18q22 has failed to identify candidate mutation loci (6, 8, 10) including massive parallel exome sequencing of 48 SINETs which identified a low general mutation rate and no recurrently mutated genes (16). Even though direct sequencing of the *SMAD4* gene from GINET tumors has not identified structural mutations, we identified loss of SMAD4 protein expression in 29% of primary tumors. This is consistent with previous exome sequencing data, which demonstrated *SMAD4* copy number loss in 22/48 (46%) of small bowel NET (16). In addition, epigenetic mechanisms including hypermethylation (30) or micro RNA targeting (31) could be responsible for SMAD4 protein loss. Additionally, SMAD proteins, including SMAD4, are degraded through ubiquitination which can modify the levels of SMAD4 within the cytoplasm of GINET cancer cells (32). Regardless of the mechanism, loss of SMAD4 expression has repeatedly been demonstrated as a prognostic factor associated with tumor progression and cancer-related death in numerous solid tumors (25, 33, 34).

These data should be evaluated in the context of their limitations. First, this was a non-randomized, retrospective cohort study of selected patients treated at a single institution. In addition, the small sample limited our ability to perform substantive multivariable analyses. The majority of patients had lymph node positive disease, making it difficult to assess the impact of SMAD4 in patients with lymph node negative disease. However, although the sample size of the current investigation was relatively small and therefore will require validation, these results suggest that expression of SMAD4 in the primary tumor could be used as a prognostic marker.

In summary, consistent with the known biology of the *DPC4/SMAD4* gene, the absence of SMAD4 protein expression in the primary gastrointestinal neuroendocrine tumor is negatively associated with cancer-related outcomes after surgical resection. Future prospective studies are warranted to assess the validity of testing for SMAD4 and its prospective use in the care of patients with GINET.

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Figure 1. Immunohistochemical expression of *SMAD4* in primary, resected GINET. (A): *SMAD4* expression at 10x; (B): *SMAD4* loss at 10x; (C): *SMAD4* Expression at 40x; (D): *SMAD4* loss at 40x.

Figure 2.

Kaplan-Meier estimate by *SMAD4* status (A–C) of (A): Disease-free survival; (B): Disease specific survival; (C): Overall survival (D): Kaplan-Meier estimate of disease-free survival by *SMAD4* and lymph node (LN) status. *SMAD4*-Pos: *SMAD4* positive; *SMADneg*: *SMAD4* negative.

Table 1

Clinical data of patient study population

	<i>SMAD4</i> -positive	<i>SMAD4</i> -negative	<i>p</i> -Value
Number	27	11	
Age, median (range)	56 (36–77)	66 (41–88)	0.25
Gender, n (%)			0.7
Male	15 (55)	7 (64)	
Female	12 (45)	4 (36)	
Surgical Treatment			
Localized Disease n (%)			0.76
Small bowel resection	16 (59)	5 (46)	
Right Colectomy	9 (33)	4 (36)	
Local Excision-Duodenum	1 (4)	1 (9)	
Local Excision-Rectum	1 (4)	1 (9)	
Metastatic Disease n (%)	7	3	0.35
Hepatectomy	5 (19)	2 (18)	
Oophorectomy	2 (7)	0	
Peritoneum	0	1 (9)	
T-stage, n (%)			0.1
T1	2 (7)	0	
T2	3 (11)	3(27)	
T3	12 (44)	7 (67)	
T4	7 (26)	1(9)	
Unavailable	3(11)	0	
Tumor Size, mean (range)	2.1 (0–5.0)	2.2 (0–4.0)	
N-Stage, n (%)			0.1
N0	1(4)	0	
N1	21 (78)	10(91)	
Unavailable	5 (18)	1(9)	
M-stage			0.3
M0	20 (74)	8 (73)	
M1	7 (26)	3 (27)	
Lymphovascular Invasion, n (%)			0.14
Positive	14 (52)	6 (55)	
Unavailable/Negative	13 (48)	5 (45)	
Time to Recurrence (months)	223	28	0.01
Recurrence Location, n (%)	13 (48)	9 (82)	0.04
Liver	4 (31)	5 (56)	
Peritoneum	3 (23)	2 (22)	
Local Only	2 (15)	2 (22)	

	<i>SMAD4</i> -positive	<i>SMAD4</i> -negative	<i>p</i> -Value
Local + Liver	3 (23)	0	
Other	1 (8)	0	

Table 2

Univariate analysis of factors associated with DSS

	N	Median DSS (months)	p-Value
Age, n (%)			0.68
60	19	Not reached	
> 60	19	204	
Gender			0.27
Male	22	204	
Female	16	155	
T-stage, n (%)			0.11
T1	3	Not reached	
T2	5	132	
T3	19	137	
T4	8	155	
Unavailable	3	Not reached	
Tumor Size, mean (range)			0.12
2.1	16	225	
> 2.1	13	101	
N-Stage, n (%)			0.25
N0	2	Not reached	
N1	30	204	
Unavailable	6	156	
M-stage			0.65
M0	28	204	
M1	10	156	
Lymphovascular Invasion, n (%)			0.11
Negative	3	Not reached	
Positive	20	149	
Unavailable	15	225	
SMAD4, n (%)			0.04
Negative	11	135	
Positive	27	204	