Abstract. Background/Aim: Low-grade pancreatic neuroendocrine tumors (LG-PNETs) behave unpredictably. The aim of the study was to identify biomarkers that predict PNET metastasis to improve treatment selection. Patients and Methods: Five patients with primary non-metastatic LG-PNETs, six with primary LG-PNETs with synchronous or metachronous metastases (M-PNETs), and six metastatic to liver LG-PNETs (ML-PNETs) from the group of six M-PNET patients were selected. RNA data were normalized using iterative rank-order normalization. Student's t-test identified differentially-expressed genes in LG-PNETs versus M-PNETs. A 2-fold difference in expression was considered to be significant. Results were validated with an independent dataset of LG-PNETs and metastatic LG-PNETs. Results: Overall, 195 genes had a >2-fold change (in either direction). A total of 29 genes were differentially overexpressed in M-PNETs. Erythrocyte membrane protein band 4.1-like 5 (EPB41L5) had a 2.07-fold change increase in M-PNETs and the smallest p-value. EPB41L5 was not statistically different between M-PNETs and ML-PNETs. EPB41L5 differential expression between primary and metastatic LG-PNETs was confirmed by immunohistochemistry. Conclusion: These results support further investigation into whether EPB41L5 is a biomarker of PNETs with high risk for metastases.

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Pancreatic neuroendocrine tumors/carcinomas (PNETs/PECAs) represent about 1-2% of all pancreatic tumors. Recently, however, it has been shown that PNETs have higher prevalence and malignant potential and result in considerable morbidity and mortality (1-4). This may be the result of increased physician awareness, increased use of advancements in imaging modalities, and the protracted clinical course of the disease (1). The oncogenic drivers responsible for the PNET metastatic phenotype have yet to be uncovered. As a result, these tumors are difficult to manage clinically because of their tendency to behave unpredictably (2). Indeed, even when considering low-grade PNETs (LG-PNETs), the presence of metastasis significantly affects patient survival and renders the tumors resistant to currently available therapies (5). Thus, the identification of a biomarker capable of identifying LG-PNETs at higher risk of metastasis may guide the clinical management of these tumors (3, 4).

Published reports have shown that molecular alterations in PNETs include genomic alterations (GAs) in DNA damage repair genes MUTYH, CHEK2, and BRCA2 and inactivation of tumor suppressor genes and gene rearrangements that occur in MTAP, ARID2, SMARCA4, MLL3, CDKN2A, and SETD2 (6-12). Molecular analyses of PNETs have uncovered alterations in the pathways responsible for chromatin remodeling, DNA damage repair, activation of mammalian target of rapamycin (mTOR) signaling, and telomere maintenance. Moreover, gene expression profiling (GEP) of PNETs identified a subgroup of these tumors that are associated with hypoxia-inducible factor signaling (12). PNETs have also been reported to exhibit epithelial mesenchymal transition (EMT) by signaling that involves SLUG-mediated EMT through increased expression of the cancer stem-cell markers DCLK1 and cathepsin Z (9, 13, 14). Therefore, it is possible that the
molecular comparison of LG-PNETs at high and low risk of metastasis may uncover prospective genes that are capable of reliably predicting PNET metastases in low-grade tumors.

In this study, GEP was used to compare primary LG-PNETs without synchronous or metachronous metastases, primary LG-PNETs with synchronous or metachronous metastases (M-PNETs), and metastatic to liver LG-PNETs (ML-PNETs) that developed in the M-PNET patients. The results were validated using immunohistochemistry and tissue microarray (TMA) technology.

Materials and Methods

Frozen tissue samples from 5 primary LG-PNETs, 6 primary M-PNETs, and 6 ML-PNETs (from the same group of M-PNET patients) were selected from the Pathology Tissue Core files of the Moffitt Cancer Center. The slides and pathology reports from each case were reviewed by 2 pathologists (DC and NA) to confirm diagnoses.

RNA was extracted from the frozen samples and analyzed by using an Affymetrix U133 2.0 GeneChip (Thermo Fisher Scientific, Waltham, MA, USA). Data were normalized using iterative rank-order normalization (15), and Student t test was performed to identify differentially expressed genes between LG-PNETs, M-PNETs, and ML-PNETs. Differential expression was defined by a \( p \)-value<0.05 and a minimum 2-fold difference in expression.

Results were validated by using an independent cohort of formalin-fixed paraffin-embedded tissues from 24 primary and 7 metastatic LG-PNETs and TMA technology. Formalin-fixed paraffin-embedded TMA tissue was cut into 4 \( \mu \)m sections. The sections were stained using a Ventana Discovery XT automated system (Ventana Medical Systems, Tucson, AZ, USA) as per manufacturer’s protocol, with proprietary reagents. Briefly, slides were deparaffinized on the automated system with EZ Prep solution (Ventana). Heat-induced antigen retrieval method was used in Cell Conditioning 1 (Ventana). The slides were incubated for 2 h with rabbit primary antibody that reacts to EPB41L5 (#NBP2-30920, Novus Biological, Littleton, CO, USA) was used at a 1:50 dilution in Dako antibody diluent (Carpenteria, CA, USA). Then the slides were incubated for 16 min with Ventana Omnimap Anti-Rabbit Secondary Antibody. The detection system used was the Ventana ChromoMap kit, and slides were counterstained with Hematoxylin. Slides were then dehydrated and coverslipped per standard laboratory protocol.

The EPB41L5 immunostaining reaction was measured semiquantitatively by using the Allred scoring system. Briefly, 1 represented positive EPB41L5 immunoreactivity in 1% of the tumor, 2 represented 1% to 10%, 3 represented 10% to 33%, 4 represented 33% to 67%, and 5 represented greater than 67%. The intensity of the stain was scored as weak (1), moderate (2), or strong (3). The sum of the percent and intensity scores was used to determine an overall total immunohistochemistry score (0 to 8).

Statistical analysis. Iterative rank-order normalization was used to normalize the data. Student t test was used to identify differentially expressed genes in LG-PNETs versus M-PNETs, which were defined by a \( p \)-value<0.05 (unadjusted). A minimum of a 2-fold difference in expression was considered to be significant. Student t test was also used for the immunohistochemical analysis of an independent dataset of LG-PNETs and M-PNETs, and a \( p \)-value <0.05 was required to determine statistical significance.

Table I. EPB41L5 immunohistochemical protein expression in an independent set of primary and metastatic LG-PNETs.

| Characteristics       | Tissue microarray sample type | Primary PNET (n=24) | Metastatic PNET (n=7) | \( p \)-Value |
|-----------------------|-------------------------------|---------------------|----------------------|--------------|
| Age, y               | Median (Range)                | 57 (27-76)          | 63 (24-78)           |              |
| Gender               |                               |                     |                      |              |
| Female, n (%)        | 12 (50)                       | 4 (57)              |                      |              |
| Male, n (%)          | 12 (50)                       | 3 (43)              |                      |              |
| EPB4115 IHC Score, median (range) | 4.0 (0.0-5.0) | 5.0 (3.2-5.0)    | 0.05                 |

EPB41L5: Erythrocyte membrane protein band 4.1-like 5; IHC: immunohistochemistry; LG-PNETs: low-grade pancreatic neuroendocrine tumors; M-PNET: pancreatic neuroendocrine tumor.

Results

Patient demographics. The demographical and immunohistochemical data of the patients studied are summarized in Table I and presented in Figure 1. The 5 patients with primary LG-PNETs included 3 men and 2 women with a mean age of 66 years; the 6 patients with primary M-PNETs and ML-PNETs included 3 men and 3 women with a mean age of 59 years. All tumors were well differentiated grade 1 PNETs (16).

Gene Expression Profiling (GEP). In order to compare gene expression between LG-PNET, M-PNET, and ML-PNET cases, 1483 probe sets were used and 1029 differentially expressed genes were identified (\( p<0.05 \)). A total of 195 genes had a greater than 2-fold change, 29 of which were differentially overexpressed in M-PNET cases. Among those genes that were shown to have statistically significant differential expression, EPB41L5 (variant 225855_at) \( p=0.005 \) had a fold change of 2.07 between cases of LG-PNETs and M-PNETs. Interestingly, the expression of EPB41L5 was not statistically different when comparing M-PNETs and ML-PNETs \( p=0.3 \) (Figure 1). Using TMA containing an independent cohort of 24 primary LG-PNETs and 7 M-PNETs, a near significant difference in EPB41L5 immunohistochemical expression was observed between primary and metastatic PNETs \( p=0.05 \) (Figure 2).

Discussion

In this study, GEP was used to analyze tumor tissues from primary LG-PNETs, primary M-PNETs, and ML-PNETs. EPB41L5 was identified as a potential biomarker for LG-PNETs that have a high propensity for metastasis. As there are behavioral differences between primary and metastatic PNETs (17), this finding may have clinical significance.
Historically, clinical attempts to use serum tumor markers have been diagnostically and prognostically limited to cases of non-functional pancreatic neuroendocrine neoplasms (PNENs). The sensitivity of serum tumor markers is typically suboptimal (<50%) among patients with PNENs that are small and nonmetastatic. When patients with metastatic PNENs are considered, serum tumor marker sensitivity may be as low as 60%. Furthermore, a PNET tissue biomarker that is able to distinguish LG-PNETs at risk of metastasis from those that are not would enormously benefit appropriate management of this disease. Research has focused on distinguishing GAs that drive the biological behavior of PNETs from those that drive PECAs (18-23). GAs in genes involved in chromatin remodeling, such as MEN1, DAXX, and ATRX, as well as those in phosphatase and tensin homolog (PTEN) loss, have been found to be enriched in G1, G2, and G3 PNETs, whereas PECAs have been determined to be enriched in GAs that lead to the inactivation of TP53 and/or RB1 and to EMT (5, 19, 20, 24-29). Here, we propose EPB41L5 as a potential new biomarker for LG-PNET with metastatic potential.

EPB41L5 is a member of the band 4.1 superfamily and is also a FERM (protein 4.1, Ezrin, Radixin, Moesin) domain protein (30). EPB41L5 is essential for maintaining the kidney filtration barrier by sustaining podocyte adhesion in vivo. It is also essential for maintaining actomyosin activity upregulation and focal adhesion stabilization (31). Moreover, EPB41L5 is a mesenchymal-specific protein that is induced during EMT of mammary epithelial cells (30). EMT is a reversible process that occurs during normal cellular processes associated with wound repair (32).

In carcinogenesis, EMT precedes invasion and involves a dysregulation of adhesion molecules, loss of apico-basal polarity, disintegration of tight junctions, and acquisition of a variable cell shape that facilitates cell movement, invasion, and metastasis (32, 33). Interestingly, ZEB1 induces EPB41L5, which binds to AMAP1 in the Arf6-AMAP1-EPB41L5 pathway during EMT (34, 35). EPB41L5 subsequently binds

Figure 1. EPB41L5 (variant 225855_at) has a fold change of 2.07 between cases of LG-PNETs and M-PNETs (p=0.005). The expression of EPB41L5 was not statistically different when comparing M-PNETs and ML-PNETs (p=0.3).
Figure 2. The weak EPB41L5 immunostaining of a primary LG-PNET without metastasis (A) compared to the strong and diffuse EPB41L5 immunostaining of a metastatic LG-PNET (B). The metastatic LG-PNET (B) shows a stronger and more diffuse EPB41L5 immunoreactivity than the weak and focal EPB41L5 immunoreactivity seen in the primary LG-PNET (A).
to p120 catenin to sequester p120 from E-cadherin complexes, thereby promoting E-cadherin internalization. EPB41L5 also binds to paxillin, which mediates the disruption of E-cadherin-based cell-cell adhesion, ultimately promoting cell motility. Therefore, studies indicating that EPB41L5 plays an essential role during the metastatic process in renal cancer and breast cancer were not surprising (36-39).

Poor outcomes have been correlated with overexpression of EPB41L5 among patients with breast cancer, squamous cell carcinoma of the tongue, and upper urinary tract urothelial carcinoma (35-40). Given that these prior studies have characterized the reliable prognostic value of EPB41L5 in other cancer types, it is plausible that EPB41L5 may represent a new biomarker for LG-PNETs with high propensity of metastasis. The statistically significant differential expression of EPB41L5 between LG-PNETs and M-PNETs is a finding that supports further investigation in a larger patient population, as EPB41L5 may have prognostic value as a biomarker for identifying LG-PNETs with a higher risk of progression and metastasis.

Finally, recent neuroendocrine tumor therapeutic efforts have included the investigation of inhibitors that target signaling in the Wnt/β-catenin, PI3K/AKT/mTOR, MET, and vascular endothelial factor pathways (8, 10, 11, 25, 41-59). PNETs and PECAs have been shown to differ in responsiveness to different therapies, depending on differences in types of drivers responsible for their biological behaviors (5, 8, 11, 19, 25, 27, 43, 46, 48, 53, 59-109). In light of these findings, it is possible that EPB41L5 is a novel target for LG-PNET therapies.

**Conclusion**

Within the limits of the investigated genes, our findings suggested that EPB41L5 is a differentially expressed gene that may have clinical value as a putative biomarker for risk stratification in cases of LG-PNETs with a propensity to metastasize.

**Conflicts of Interest**

The Authors declare that they have no competing interests. The funding agent played no role in the design, collection, analysis, interpretation of data, or writing of the study.

**Authors’ Contributions**

SS collected references and drafted the manuscript. JS and MS evaluated the slides, collected the data and drafted the manuscript. SAE performed the bioinformatics analysis. DB performed the statistical analysis on the TMA data. MC and JRS reviewed the final manuscript. NA selected the cases and supervised the gene expression profiling. DC evaluated the histologic and molecular data and finalized the manuscript.

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