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Ｈ．知的財産権の出願・登録状況

１．特許取得
該当せず

２．実用新案登録
該当せず

３．その他
該当せず

様式第１９

学　会　等　発　表　実　績

委託業務題目「希少がんである神経内分泌腫瘍の個別化医療開発に向けたがん抑制遺伝子 PHLDA3 の機能解析」
機関名　国立がん研究センター研究所

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発表した成果（発表題目、口頭・ポスター発表の別）	発表者氏名	発表した場所（学会等名）	発表した時期	国内・外の別
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（注１） 発表者氏名は、連名による発表の場合には、筆頭者を先頭にして全員を記載すること。

（注２） 本様式はexcel形式にて作成し、甲が求める場合は別途電子データを納入すること。

Ⅰ．がんゲノム解析の治療への応用

4. 新規がん抑制遺伝子*PHLDA3*によるAkt経路の制御機構と治療への展開
―膵神経内分泌腫瘍の個別化医療開発をめざして

山口陽子，斉藤　梢，陳　妤，大木理恵子

多くのがんで機能喪失が認められるp53経路の研究は，がん制御機構を解明するうえで重要である．われわれはp53の機能解析を通して，がん遺伝子*Akt*の抑制因子をコードする新規がん抑制遺伝子*PHLDA3*を同定した．*PHLDA3*遺伝子は，膵臓の神経内分泌腫瘍（neuroendocrine tumor：NET）において高頻度にヘテロ接合性の喪失LOHが認められ，その頻度は膵NETのがん抑制遺伝子として有名な*MEN1*遺伝子と同等であった[1]．本稿では，膵NETの新規がん抑制因子PHLDA3によるAkt抑制を介した膵NET抑制機能について解説した後，PHLDA3研究を応用した新規がん診断・治療法の開発について考察する．

はじめに

1）がん抑制遺伝子p53の機能

*p53*遺伝子はがんの約半数において変異や欠失が認められる．また，*p53*遺伝子が野生型である場合，p53の活性を制御する因子や，p53の標的遺伝子が不活性化していることが報告されている[2]．このように*p53*は重要ながん抑制遺伝子であり，がんを理解するうえでp53研究は欠くことができない．

p53は転写因子であり，DNAダメージや低酸素状態，がん遺伝子の活性化などのさまざまなストレスに応答し特定の塩基配列に結合して，標的遺伝子を転写誘導する．p53は標的遺伝子を介して細胞周期停止や細胞死を誘導し，細胞のがん化を防いでいる．p53タンパク質は，転写活性化ドメインを含むN末端ドメイン，DNA結合ドメイン，C末端ドメインから構成される（**図1A**）．がんにおいて*p53*遺伝子にみられる変異の多くがDNA結合ドメインに生じることが，p53の転

[キーワード&略語]
p53，PHLDA3，Akt，膵神経内分泌腫瘍，エベロリムス

Gsk3β：glycogen synthase kinase 3-beta
Mdm2：mouse double minute 2
mTOR：mammalian target of rapamycin
PHLDA3：pleckstrin homology-like domain, family A, member 3
PTEN：phosphatase and tensin homolog
S6：ribosomal protein S6
S6k：ribosomal protein S6 kinase

Repression of the Akt pathway by a novel tumor suppressor gene *PHLDA3*
―towards development of tailor-made therapies for pancreatic neuroendocrine tumors

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PHLDA3 is a novel tumor suppressor of pancreatic neuroendocrine tumors

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The molecular mechanisms underlying the development of pancreatic neuroendocrine tumors (PanNETs) have not been well defined. We report here that the genomic region of the *PHLDA3* gene undergoes loss of heterozygosity (LOH) at a remarkably high frequency in human PanNETs, and this genetic change is correlated with disease progression and poor prognosis. We also show that the *PHLDA3* locus undergoes methylation in addition to LOH, suggesting that a two-hit inactivation of the *PHLDA3* gene is required for PanNET development. We demonstrate that PHLDA3 represses Akt activity and Akt-regulated biological processes in pancreatic endocrine tissues, and that *PHLDA3*-deficient mice develop islet hyperplasia. In addition, we show that the tumor-suppressing pathway mediated by MEN1, a well-known tumor suppressor of PanNETs, is dependent on the pathway mediated by PHLDA3, and inactivation of PHLDA3 and MEN1 cooperatively contribute to PanNET development. Collectively, these results indicate the existence of a novel PHLDA3-mediated pathway of tumor suppression that is important in the development of PanNETs.

p53 | PH domain | everolimus | p53 target gene | mTOR

Neuroendocrine tumors (NETs) arise from cells of the endocrine and nervous systems, and are found in tissues such as lung, pancreas and pituitary (1–3). NETs often produce, store and release biogenic amines and polypeptide hormones, and secretary granules containing these products provide a diagnostic marker for NETs. The mechanisms underlying the development of NETs remain unclear to date, due to the low incidence of these tumors and due to the lack of suitable experimental model systems, including genetically engineered mouse models. Pancreatic NET (PanNET), which is probably the best-studied NET, is the second-most common pancreatic tumor, having an incidence of ~1 per 100,000 individuals. Patients having late-stage PanNET often harbor tumors that are unresectable or metastatic and face limited treatment options. Accordingly, the prognosis of patients having metastatic PanNET is the worst among the NET subtypes, with a 5-y survival rate of 27–43% (1). Recently, the drug Everolimus has shown promise in the treatment of PanNETs (4), providing a significant improvement in progression-free survival. Everolimus is an inhibitor of mammalian target of rapamycin (mTOR), a downstream mediator of the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) pathway. The striking efficacy of Everolimus demonstrates the importance of the PI3K/Akt pathway in the pathology of PanNETs.

In agreement with these clinical results, studies on pancreatic endocrine cell lines have identified the PI3K/Akt signaling pathway as a major proliferation and survival pathway in these cells (5). Activated Akt phosphorylates substrates such as mTOR and controls various biological processes, including protein synthesis, proliferation, cell growth, and survival. Regulation of pancreatic islet β-cell proliferation, cell size, and apoptosis by Akt has been demonstrated using various mouse models. For example, transgenic mice overexpressing constitutively active Akt in β-cells exhibit increased β-cell proliferation and cell size and decreased induction of apoptosis (6).

Recently, the results of whole exomic sequencing of 10 PanNET specimens were published, revealing several key genetic alterations (7). In particular, genes in the PI3K/Akt pathway, i.e., TSC2, PTEN, and PIK3CA, were mutated in 15% of PanNETs. However, this represents only a subset of PanNETs, and may not fully explain the remarkable clinical results achieved by Everolimus in the majority of PanNET patients.

Previously, we have shown that Pleckstrin homology-like domain family A, member 3 (PHLDA3) is a novel p53-regulated repressor of Akt. The PHLDA3 contains a PH domain that, we showed, competes with the PH domain of Akt for binding to membrane lipids, thereby inhibiting Akt translocation to the cellular membrane and its activation. We also showed that

Significance

Pancreatic neuroendocrine tumors (PanNETs) are a rare pathology, and molecular mechanisms underlying their development have not been well defined. This article shows that a two-hit inactivation of the *PHLDA3* gene is required for PanNET development: methylation of the locus and loss of heterozygosity. PHLDA3 functions as a suppressor of PanNETs via repression of Akt activity and downstream Akt-regulated biological processes. In addition, the tumor-suppressing pathway mediated by MEN1, a well known suppressor of PanNETs, is dependent on the pathway mediated by PHLDA3, and inactivation of PHLDA3 and MEN1 cooperatively contribute to PanNET development. A novel PHLDA3-mediated pathway of tumor suppression that is important in the development of PanNETs is demonstrated, and the findings may contribute to personalized medicine of PanNET patients.

Author contributions: R.O. designed research; R.O., K. Saito, Y.C., R.S., M.M., Y.A., and H.S. performed research; T. Kawase, N.H., G.Y., S.Y., N.S., R.D., T. Kosuge, K. Shimada, B.T., T.T., Y.K., and S.S. contributed new reagents/analytic tools; R.O. and K. Saito analyzed data; R.O., H. Namiki, Y.T., T.S. and H. Nakagama supervised the research; and R.O. wrote the paper.

The authors declare no conflict of interest.

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PHLDA3 may have a tumor suppressive function (8). However, there has hitherto been no reported role for PHLDA3 in human tumors, and its in vivo function has remained elusive. In this report, we demonstrate that the *PHLDA3* gene is a novel tumor suppressor, inactivation of which can lead to the development of PanNETs. We show that the *PHLDA3* genomic locus undergoes LOH and that the *PHLDA3* promoter is methylated at a high frequency in PanNETs. Furthermore, analysis of *PHLDA3*-deficient mice showed that these mice frequently develop islet hyperplasia as a result of enhanced islet cell proliferation and an increase in islet cell size. Collectively, these results indicate that PHLDA3 functions as a tumor suppressor in PanNETs.

Results

Frequent LOH at the *PHLDA3* Gene Locus in PanNETs. The *PHLDA3* gene is located at 1q31, a locus that has been reported to have a high frequency of LOH in two NETs derived from pancreas: insulinomas and gastrinomas (9, 10). We therefore speculated that the *PHLDA3* locus may undergo LOH in PanNETs, and analyzed the *PHLDA3* locus for LOH using microsatellite markers surrounding the gene in 54 PanNET samples (Fig. 1 *A–D*; clinical diagnosis for each sample is shown in *SI Appendix*, Fig. S1*A*). As shown in Fig. 1*B*, out of 54 PanNETs, 50 samples were informative and 36 samples showed LOH at the *PHLDA3* locus. The incidence of LOH at the *PHLDA3* locus (72%) is remarkably high, and was comparable to the reported LOH incidence of the *Multiple endocrine neoplasia type 1 (MEN1)* gene, which has the highest reported incidence of genomic changes in PanNETs (11). Within the region analyzed, the LOH frequency peaks near the *PHLDA3* locus, suggesting that LOH of the *PHLDA3* gene is critical for PanNET development (Fig. 1*D*). This tendency becomes clearer when samples that exhibit partial LOH within this region (PanNET 1–18) were analyzed (Fig. 1*D*, blue line). A strikingly high incidence of LOH at the *PHLDA3* locus indicates the importance of this PHLDA3-regulated tumor suppression pathway in PanNETs. Most of the PanNETs analyzed in this study are nonfunctional, and we found no associations between *PHLDA3* LOH and specific PanNET type or insulin/glucagon positivity, to the extent that we examined this (*SI Appendix*, Fig. S1).

PHLDA3 and MEN1 Cooperatively Suppress PanNET. The most outstanding genomic aberration previously reported in PanNETs was the mutation and LOH of the *MEN1* gene, a tumor suppressor gene associated with multiple endocrine neoplasia type 1

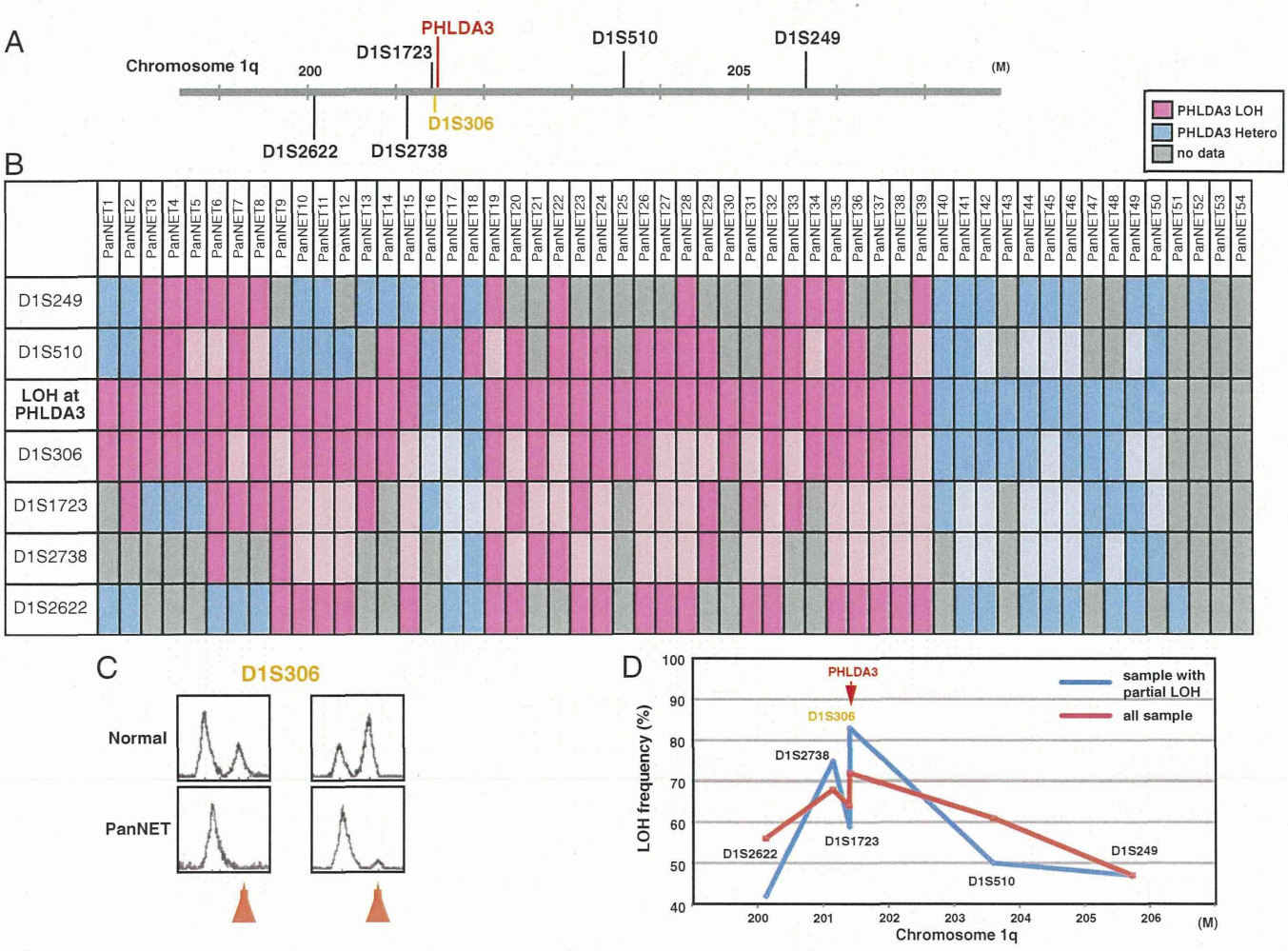
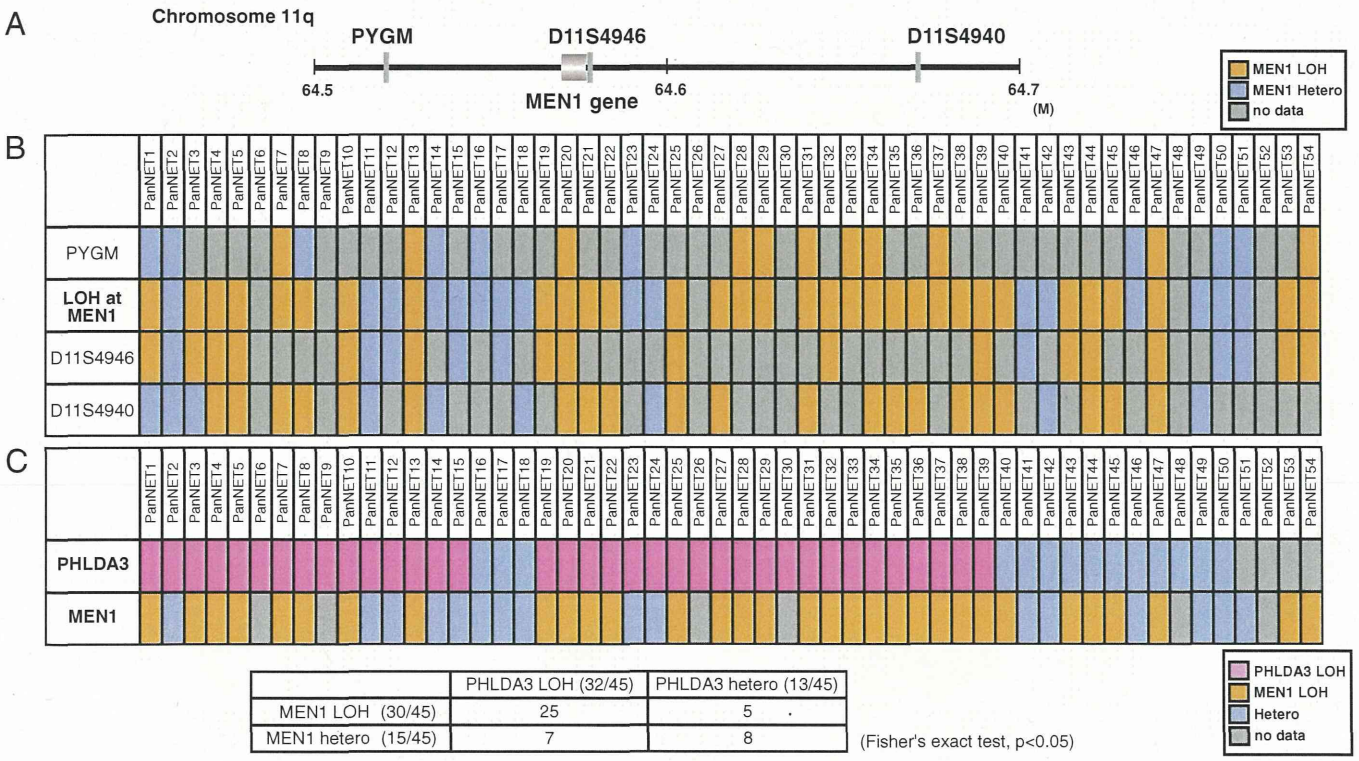


Fig. 1. Frequency of LOH at the *PHLDA3* gene locus in PanNETs. (*A*) Chromosomal locations of *PHLDA3* gene and microsatellite markers used in this study. D1S306 is located just next to the *PHLDA3* gene (32 kb upstream). (*B*) Microsatellite analysis of the *PHLDA3* gene locus region. PanNET samples were analyzed for LOH around the *PHLDA3* gene locus. Because D1S306 is located next to the *PHLDA3* gene, the LOH status of the *PHLDA3* gene was determined from the LOH status of the D1S306 locus. For some loci with no data (not informative or data unavailable), the LOH status of the locus was determined from the surrounding LOH status (shown in faint pink and faint blue). (*C*) Representative microsatellite analysis results. In normal tissues, two peaks derived from maternal and paternal alleles were detected, whereas in tumors, one allele was lost (shown by orange arrows), indicating LOH at the locus. (*D*) LOH frequency for each microsatellite marker. Frequencies from all samples (shown by red line) and frequencies from samples showing LOH partially within the analyzed region (PanNET1-18, shown by blue line) are shown.

(7, 11). It has been reported that ~50% of PanNET cases exhibit LOH of the *MEN1* gene. Therefore, we analyzed whether *MEN1* LOH is observed in our PanNET samples. As shown in Fig. 2 *A* and *B*, 32 samples out of 48 informative samples showed LOH at the *MEN1* locus. Frequent LOH was observed at the *MEN1* locus in our samples (67%), confirming previous studies. We next combined the LOH data for the *PHLDA3* and *MEN1* loci. As shown in Fig. 2*C*, LOH at the *PHLDA3* and *MEN1* loci did not show a mutually exclusive pattern, which would be expected if PHLDA3 and MEN1 were on the same tumor suppressing pathway. Interestingly, we observed a significant frequency of double LOH, i.e., occurring at both the *PHLDA3* and *MEN1* loci (25 of 45 samples, $P < 0.05$ by Fisher's exact test). These data suggest that development of PanNET involves the functional loss of both pathways.

LOH at the *PHLDA3* Locus Is Correlated to Poor Prognosis in PanNET Patients. To select the proper treatment for each PanNET patient, prediction of disease prognosis is important. We observed that LOH at the *PHLDA3* locus was associated with advanced stage PanNETs, whereas absence of LOH was associated with lower tumor grades. These associations were statistically significant ($P < 0.01$, by Fisher's exact test), suggesting that the LOH is associated with a malignant phenotype in PanNETs (Fig. 3*A*). We next analyzed the relationship between LOH at the *PHLDA3* locus and the prognosis of PanNET patients. As shown in Fig. 3*B*, patients exhibiting LOH at the *PHLDA3* locus seemed to have a poorer prognosis compared with the patients without LOH. However, the observed difference did not achieve statistical significance, probably due to the relatively small numbers of patients analyzed. On the other hand, LOH at the *MEN1* locus had no influence on tumor grade or patient survival, as has been reported (7) (Fig. 3 *C* and *D*). It will be necessary to extend these studies with larger numbers of patients before reaching a firm conclusion, however these results suggest that the PHLDA3-regulated pathway, but not the MEN1-regulated pathway, may be a critical determinant of the prognosis of PanNET patients.

The *PHLDA3* Gene Undergoes Aberrant Methylation in Addition to LOH in PanNETs. Because we found frequent LOH at the *PHLDA3* locus in PanNETs, we next examined how the remaining allele is inactivated. The *PHLDA3* gene is mutated in several cancers: 11 mutations in lung, urinary, and large intestine cancer are reported in the COSMIC database (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic), and mutation in lung cancer was reported by Yoo et al. (12). Therefore, we analyzed the *PHLDA3* ORF genomic sequence in our PanNET samples, but did not find any mutations within the coding regions (*SI Appendix*, Fig. S2). We next analyzed *PHLDA3* mRNA expression levels in these PanNETs. As shown in Fig. 4*A*, *PHLDA3* expression was significantly lower in samples showing LOH at the *PHLDA3* locus compared with samples without LOH. We noticed a CpG island overlapping the promoter region and the first exon of the *PHLDA3* gene (Fig. 4*B*). We therefore analyzed whether the methylation status of the *PHLDA3* gene is related to *PHLDA3* transcription levels. Specifically, we analyzed DNA methylation levels within the first exon of the *PHLDA3* gene, because it has been reported that methylation of the first exon is tightly linked to transcriptional silencing (13). Analysis of *PHLDA3* mRNA expression levels in four cancer cell lines revealed that *PHLDA3* is highly expressed in LNCaP and MDA-MB-M468 cells, whereas expression is very low in DLD1 and H1299 cells (Fig. 4*C*). Analysis of DNA methylation levels by methylation-specific PCR revealed detectable methylation only in DLD1 and H1299 cells, cell lines with low *PHLDA3* expression (Fig. 4*D*). It is of note that, in H1299 cells, a human lung NET cell line, *PHLDA3* mRNA expression was remarkably low and methylation was remarkably high. We further treated H1299 cells with 5-aza-C to demethylate the *PHLDA3* gene. As shown in Fig. 4*E*, 5-aza-C treatment resulted in decreased methylation at the *PHLDA3* gene and



	PHLDA3 LOH (32/45)	PHLDA3 hetero (13/45)
MEN1 LOH (30/45)	25	5
MEN1 hetero (15/45)	7	8

(Fisher's exact test, p<0.05)

Fig. 2. Relationship between PHLDA3 and MEN1 tumor-suppressing pathways. (*A*) Chromosomal locations of the *MEN1* gene and microsatellite markers used in this study. (*B*) Analysis of LOH at the *MEN1* locus. The LOH status of the *MEN1* gene was determined from the LOH status of either of the informative markers. (*C*) Relationship between LOH status of the *PHLDA3* and *MEN1* loci. In total, 45 samples informative for both *PHLDA3* and *MEN1* loci were analyzed.