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学位論文

Immunohistochemically Detected Expression of ATRX, TSC2, and PTEN Predicts Clinical Outcomes in Patients With Grade 1 and 2 Pancreatic Neuroendocrine Tumors

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ORIGINAL ARTICLE

Immunohistochemically Detected Expression of ATRX, TSC2, and PTEN Predicts Clinical Outcomes in Patients with Grade 1 and 2 Pancreatic Neuroendocrine Tumors

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ABSTRACT

Objective: The goal of this retrospective study was to clarify the clinical implications of immunohistochemically detected protein expression for genes that are frequently mutated in pancreatic neuroendocrine tumors (PNETs).

Background: The clinical management of PNETs is hindered by their heterogenous biological behavior. Whole-exome sequencing recently showed that five genes (*DAXX/ATRX, MEN1, TSC2,* and *PTEN*) are frequently mutated in PNETs. However, the clinical implications of the associated alterations in protein expression remain unclear.

Methods: We collected Grade 1 and 2 (World Health Organization 2017 Classification) primary PNETs samples from 100 patients who underwent surgical resection. ATRX, DAXX, MEN1, TSC2, and PTEN expression were determined immunohistochemically to clarify their relationships with prognosis and clinicopathological findings.

Results: Kaplan-Meier analysis indicated that loss of TSC2 (n=58) or PTEN (n=37) was associated with significantly shorter overall survival, and that loss of TSC2 or ATRX (n=41) was associated with significantly shorter recurrence-free survival. Additionally, loss of ATRX or TSC2 was significantly associated with nodal metastasis. In a

multivariate analysis, combined loss of TSC2 and ATRX (n=31) was an independent prognostic factor for shorter recurrence-free survival (hazard ratio 10.1, 95% confidence interval 2.1-66.9, p=0.003) in G2 PNETs.

Conclusions: Loss of ATRX, TSC2, and PTEN expression might be useful as a method of clarifying the behavior and clinical outcomes of Grade 1 and 2 PNETs in routine clinical practice. Combined loss of TSC2 and ATRX had an especially strong, independent association with shorter recurrence-free survival in patients with G2 PNETs. Loss of pairs in ATRX, TSC2, or PTEN would be useful for selecting the candidate for postoperative adjuvant therapy.

INTRODUCTION

Pancreatic neuroendocrine tumors (PNETs) are uncommon tumors that account for 1-2% of all pancreatic neoplasms[1, 2]. The main, well-known prognostic factor for PNETs is World Health Organization (WHO) histological grade, which classifies PNETs as G1, G2, G3, or neuroendocrine carcinoma (PNEC) based on mitotic counts and Ki-67 labeling indices[3]. PNEC is an extremely rare tumor that is associated with an exceptionally poor prognosis[4-6]. On the other hand, the biological behavior of well-differentiated PNETs (G1/G2) differs considerably between cases[7-12]. Some tumors grow slowly and do not recur, even after marginal excision by enucleation, but other tumors expand aggressively and metastasize rapidly. Although the Ki-67 proliferative index is a valuable prognostic factor that is currently employed in routine clinical practice, it remains difficult to predict which G1 and G2 tumors will recur and behave aggressively[12].

Whole-exome sequencing of PNETs has revealed several key genetic alterations[13]. Genes in the PI3K/Akt pathway, including *TSC2*, *PTEN*, and *PIK3CA*, were mutated in 15% of PNETs[13]. Mutually exclusive somatic inactivating mutations in either the *DAXX* or *ATRX* genes have been reported to be present in as much as 33% to

43% of PNETs in case series[13, 14]. Loss of function in these proteins leads to telomere dysfunction and results in impaired non-homologous end joining, alternate lengthening of telomeres, and general genomic instability[15, 16].

If it is possible to establish consistent associations between the above mentioned genetic alterations and clinical outcomes, then immunohistochemical analyses of the associated proteins could be deployed rapidly in routine clinical practice as a supplement to the WHO grading system. We therefore sought to investigate the associations of immunohistochemically determined protein expression with clinical outcomes and pathological characteristics in cases of G1 and G2 primary PNETs. Protein expression was evaluated for the genes *ATRX, DAXX, MEN1, TSC2,* and *PTEN.*

MATERIALS AND METHODS

This study was approved by institutional review boards of Kagawa University and each study center (No. H23-076).

Patients and Tissue Samples

We reviewed findings for 100 patients with PNETs who received resection of the

pancreas at 4 university hospitals affiliated with the Shikoku Consortium of Surgical Research (SCSR[17]), Kobe University Hospital and 3 related hospitals in Kagawa prefecture between September 1996 and November 2016. All tumor specimens were formalin-fixed and paraffin-embedded. The paraffin blocks from the 100 patients were prospectively prepared for pathologic and immunohistochemistry (IHC) studies at Kagawa University. To definitively confirm the diagnosis and grade according to the WHO 2017 classification[3], all cases were independently reviewed by a pathologist (KK) experienced in the diagnosis of PNETs. High grade pancreatic neuroendocrine carcinomas (in other words, G3 or PNEC cases under the WHO 2017 classification) were excluded. Mixed tumors with a pancreatic ductal adenocarcinoma, squamous, or acinar component (MiNEN under the WHO 2017 classification) were also excluded. For patients with multiple tumors, the largest tumor was assessed. Patients were followed up and overall survival was analyzed. Disease recurrences were generally identified using computed tomography imaging. Tumors were staged in accordance with the 7th edition of the TNM staging system for pancreas tumor issued by the American Joint Committee on Cancer[18].

Immunohistochemistry

Formalin-fixed paraffin-embedded samples were cut into sections (thickness, 4 μm) for immunolabeling. Immunohistochemical labeling was carried out using a Bond III and Bond Max automatic immunostainer (Leica Microsystems, Wetzlar, Germany) after incubation of the sample in a decloaking chamber for antigen activation. All samples were stained immunohistochemically with the following antibodies: ATRX (Sigma-Aldrich, St. Louis, MO, USA; HPA001906), DAXX (Sigma-Aldrich, HPA008736), MENMenin (Bethy1 Laboratories, Montgomery, TX, USA; Clone A300-105A), TSC2, Tuberin (Santa Cruz Biotechnology, Satna Cruze, CA, USA; C-20), PTEN (Dako, Carpinteria, CA, USA; Clone 6H2.1), p53 (Dako, DO-7), Rb (QED Bioscience, San Diego, CA, USA; 3C8), p16 (MTM Laboratories, Tucson, AZ, USA; E6H4).

IHC results were interpreted by a pathologist (KK) and a surgeon (JU) who were experienced with IHC assessment. They were completely blinded to all clinical data and the tumor status of each patient's slides at the time of analysis. ATRX and DAXX expressions were classified as either positive, defined as unequivocal nuclear staining in tumor cells; or negative, defined as complete absence of nuclear staining in the presence

of an unequivocal internal positive control provided by non-neoplastic cells with retained nuclear expression (for example lymphocytes, endothelial cells, or stromal cells)[16, 19]. MEN1 (Menin) was evaluated as follows: results were scored as positive if the intensity of cytoplasmic staining was stronger than the intensity of nuclear staining. Immunohistochemical staining for TSC2 (Tuberin) and PTEN were evaluated by an individual immunoreactivity score (IRS). Staining intensity was scored as 0 = negative, 1 = weak, 2 = moderate, or 3 = strong. Two parameters, the intensity and the percentage of cells stained, were obtained and multiplied. IRS ≥ 100 was regarded as positive and IRS < 100 was regarded as negative. P53 immunolabeling was classified as follows: normal if 5–30% of tumor cells were stained positively on p53 immunolabeling; and abnormal if either <5% of tumor cells were stained positively on p53 immunolabeling (suggesting the presence of an intragenic deletion or nonsense mutation) or if >30% of tumor cells were stained positively on p53 immunolabeling (suggesting the presence of a missense mutation)[5, 17, 20].

Statistics

All statistical analyses were performed using JMP13 (SAS Institute Inc., Cary, NC,

USA). For the investigated clinicopathologic parameters, between-group comparisons were performed using the chi-square test or Fisher's exact test. Survival curves were estimated using the Kaplan-Meier method, and differences in survival were compared using the log-rank test. A two-sided p-value <0.05 was considered statistically significant. Variables that were found to be significant in univariate analysis at the $p<0.05$ level were included in the multivariate analysis in a backward stepwise fashion. Cox proportional hazards models were used for the multivariate analysis.

RESULTS

Clinicopathological characteristics and outcome

The study included a total of 100 patients who underwent surgical resections for G1 and G2 PNETs. Their clinical and pathological characteristics are compared with clinical outcomes in Table 1. Briefly, the median age at operation was 63 years (range, 23-88), whereas the median post-operative duration of follow-up was 43.5 months (range, 1-147). The cohort of 100 patients (Table 1) consisted of 49 women (49%) and 51 men (51%). The 3- and 5-year overall survival (OS) rates were 94.1% and 89.2%. Of the 100

re-evaluated tumors, 57 were NET G1 and 43 were NET G2.

The IHC results are also shown in Table 1. A single formalin-fixed paraffin-embedded sample did not react with any immunohistochemical labeling, and was excluded from the IHC evaluation. Among the 99 remaining tumors, loss of ATRX was detected in 41 (41.4%) (Figure 1A, B), loss of DAXX was detected in 64 (64.6%), loss of MEN1 was detected in 25 (25.3%), loss of TSC2 was detected in 58 (58.6%) (Figure 2A, B), and loss of PTEN was detected in 37 (37.8%) (Figure 3A, B).

Kaplan-Meier survival analysis (Table 1) indicated that nodal metastasis ($p=0.0513$), liver metastasis ($p<0.0001$), vascular invasion ($p=0.0142$), perineural infiltration ($p=0.0002$), loss of TSC2 ($p=0.007$) (Figure 2C), and loss PTEN ($p=0.0486$) (Figure 3C) were significantly associated with shorter OS. Kaplan-Meier recurrence analysis indicated that WHO classification ($p=0.0005$), tumor size ($p<0.0001$), liver metastasis ($p=0.008$), vascular invasion ($p=0.0003$), perineural infiltration ($p=0.00124$), loss of ATRX ($p=0.0117$) (Figure 1D), and loss of TSC2 ($p=0.009$) (Figure 2D) were significantly associated with shorter recurrence-free survival (RFS).

Taking advantage of the large number of tumors that were profiled, we also analyzed

the associations between pairs of protein expression statuses and clinical outcomes (Figure 4, Supplementary figures 1, and 2). Interestingly, combined negative expression of any 2 of the 3 proteins ATRX, TSC2, and PTEN had strong associations with clinical outcome. Loss of both TSC2 and ATRX (n=31) was associated with significantly shorter RFS (Figure 4B). Loss of both TSC2 and PTEN (n=30) was also associated with significantly shorter RFS. (Supplementary figure 1B) Loss of both ATRX and PTEN (n=45) was also associated with significantly shorter RFS. (Supplementary figure 2B) In contrast, OS or RFS did not differ significantly between single protein expression loss and positive (intact) protein expression. Thus, loss of pairs in ATRX, TSC2, or PTEN would be clinically important than just single loss of individual genes.

WHO 2017 grade is based on Ki-67 or mitosis assessment, and has been accepted as the most important prognostic factor for PNETs. Among the 57 patients with G1 PNETs, only 1 developed recurrence (time to recurrence: 20 months after surgery). Considering the indolent nature of G1 PNETs, we limited the multivariate RFS analysis to the 43 patients who had G2 PNETs. The multivariate analysis demonstrated that vascular invasion (hazard ratio 13.5, p=0.03) and nodal metastasis (hazard ratio 6.8, p=0.02) were

independent prognostic factors for RFS. Single losses of ATRX, TSC2, are PTEN were not independent prognostic factors for RFS. However, multivariate analysis of combined protein expressions demonstrated that loss of TSC2 and ATRX (hazard ratio 10.1, p=0.003) was an independent prognostic factor for RFS (Table 2).

Associations between protein expression and clinicopathological factors

Table 3 summarizes the associations of ATRX, DAXX, MEN1, TSC2, and PTEN negativity with clinicopathological parameters (details are shown in the supplementary table). The loss of ATRX was significantly associated with tumor size (p=0.0215), nodal metastasis (p=0.0145), recurrence (p=0.0412), vascular invasion (p=0.0047), and perineural infiltration (p=0.0024). The loss of MEN1 was significantly associated with WHO classification grade (p=0.0205), tumor size (p=0.0299), liver metastasis (p=0.0231), hormone production (p=0.0033), and vascular invasion (p=0.024). The loss of TSC2 was significantly associated with tumor size (p=0.0240), nodal metastasis (p=0.0016), liver metastasis (p=0.0194), recurrence (p=0.0109), hormone production (p=0.0008), and vascular invasion (p=0.0302). The loss of PTEN was significantly associated with tumor size (p=0.0202), recurrence (p=0.0446), and vascular invasion (p=0.0078).

DISCUSSION

In this study of 100 patients with G1 or G2 PNETs who received curative-intent surgery, loss of ATRX, TSC2, and PTEN was immunohistochemically observed in 41%, 58%, and 37% of the patients and was associated with shorter OS or RFS in univariate analyses. Furthermore, ATRX, MEN1, TSC2, and PTEN status were identified as biologic traits that had associations with different sets of clinicopathologic features. Interestingly, clinical outcomes had strong associations with combined negative expression for pairs of the proteins ATRX, TSC2, and PTEN. For patients with G2 PNETs, multivariate Cox proportional hazards regressions demonstrated that combined loss of TSC2 and ATRX was an independent prognostic factor for RFS (hazard ratio 10.1, $P = 0.003$). In contrast, loss of single protein expression had no significant association with OS or RFS in multivariate analysis.

Altogether, our results for ATRX, TSC2, and PTEN suggest that, as compared with assessments of single genetic alterations, assessments of combined genetic alterations are more predictive of survival outcomes. Previous sequencing studies and

studies of genetically engineered mouse models have established that the accumulation of genetic alterations contributes to clonal evolution and thereby has a considerable influence on the biology of malignant tumors[21-23]. In an earlier study of pancreatic ductal adenocarcinoma (PDAC), we reported comparable consequences related to combined gene status for *TP53*, *CDKN2A/p16*, and *SMAD4/DPC4*: increasing numbers of alterations were associated with shorter survival.[20] Curiously, single-gene alterations of *ATRX*, *TSC2*, and *PTEN* had no significant associations with OS or RFS in our multivariate analyses of PNETs from the present study. In our earlier study of PDAC, alterations to 1 of the 3 investigated genes had a significant association with OS when assessed individually. The differences between the nature of PNETs and PDACs may reflect the role of single-gene alterations. Further studies of the relationship between clinical outcomes and the cooperative interaction of essential genes in PNETs appear warranted.

Mutations in *DAXX* or *ATRX* have been detected in about 40% of PNETs. Jiao et al.[13] showed that *DAXX* and *ATRX* mutations were associated with prolonged survival in patients with PNETs. In contrast, Marinoni et al.[16] showed that the loss of DAXX and

ATRX expression was correlated with significantly shortened RFS. More recently, Chou et. al.[19] reported that single loss of expression of ATRX as determined by IHC is a useful independent predictor of shorter overall survival, while DAXX has no impact on clinical outcomes. The present study also confirmed that loss of ATRX protein expression in PNETs was associated with worse outcomes, as evaluated using univariate analysis. The discrepant findings regarding DAXX and ATRX may be explained by associations with differences in disease stages. Specifically, patients with advanced stage disease (stages III and IV) were dominant in the study by Jiao et al.[13], and all patients with ATRX- and DAXX-negative tumors presented with metastatic disease. ATRX and DAXX loss may be associated with shorter survival in early stage cases, but results may be different for advanced stage or metastatic cases. Because the detailed roles of the genes in each stage are unclear, further studies would be required to confirm whether this is a true association and to investigate potential mechanisms.

Among the down-regulated genes found in PNETs, *TSC2* is a tumor suppressor of the Akt/mTOR pathway with GTPase activating function[24]. *TSC2* mutation leads to tuberous sclerosis complex. Chromosome arm 16p, which contains *TSC2*, has been

found to be lost in 37% of PNETs[25, 26]. *PTEN* is another important tumor suppressor gene that is involved in the same pathway. *PTEN* is also frequently mutated or lost in several forms of sporadic or familiar cancers. In PNETs, however, the frequency of loss is between 10% and 29%[25-27]. Missiaglia et al.[24] showed that TSC2 cytoplasmic protein level was down-regulated in 35% of patients with PNETs, while staining of PTEN was altered in nuclear, cytoplasmic, or both cell compartments in around 60% of cases. TSC2 staining correlated with both overall survival and disease-free survival in patients with assumed complete tumor excision. Our findings support their results in that neither PTEN nor TSC2 was an independent prognostic predictor in our multivariate analysis. However, we also found that combined loss of these proteins had a strong association with clinical outcomes. Several pieces of evidence associated with our TSC2 and PTEN findings support the hypothesis that the Akt/mTOR pathway is involved in PNETs progression[24, 28, 29]. Deficiency of both TSC2 and PTEN may reduce the inhibition of mTOR activity more strongly than either single alteration. The current study's finding related to the accumulation of genetic alterations in the Akt/mTOR pathway may present some clues for selecting treatment targets for mTOR inhibition.

The IHC-based results of the current study should be clinically valuable and might have links to treatment options. In routine clinical practice, IHC results for ATRX, TSC2, and PTEN could be obtained promptly from surgical specimens or biopsy samples. For example, combined negative expression for pairs of ATRX, TSC2, and PTEN could provide rapidly available predictions of high-risk status for postoperative recurrence, which might apply to about one-third of the patients. The group of patients with high-risk status might be suitable candidates for adjuvant treatment.

Endoscopic ultrasound fine needle aspiration (EUS-FNA) has altered the diagnostic strategy for pancreatic tumor[20, 30, 31]. Genetic variants can be preoperatively assessed immunohistochemically using EUS-FNA materials. Surgical approaches that include standard lymph node dissection may be more beneficial in patients who are at elevated risk of nodal metastasis (as potentially identifiable from ATRX or TSC2 loss). Additionally, when considering entry into upcoming clinical trials, it would be reasonable to stratify patients based on genetic status. Such stratification may help to accurately define the role of currently available molecular targeted agents.

The present study has several limitations. First, the study design is retrospective.

Furthermore, the patients did not all receive the same treatment (e.g., type of operation and lymph node dissection). To validate the individual and combined expressions of ATRX, TSC2, and PTEN as prognostic biomarkers, a prospective study with a large series of patients is clearly warranted.

In conclusion, we found that IHC-demonstrated loss of ATRX, TSC2, and PTEN occurred in 41%, 58%, and 37% of the patients, and was associated with worse OS and/or RFS in univariate analyses. Furthermore, ATRX, MEN1, TSC2, and PTEN were identified as biologic traits that were associated with different sets of clinicopathologic features. Combined negative expression of pairs of ATRX, TSC2, and PTEN had strong associations with clinical outcome in G2 PNETs. IHC-identified loss of ATRX, TSC2, and PTEN protein expressions might be clinically valuable and should be linked with treatment options. Loss of pairs in ATRX, TSC2, or PTEN would be clinically useful for selecting the candidate for postoperative adjuvant therapy in patients with Grade 1 and 2 PNETs.

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Figure Legends

Figure 1

ATRX protein expression and its relationships with the overall and recurrence-free survival of patients with pancreatic endocrine tumors (PNETs).

Immunohistochemistry with anti-ATRX antibody (Sigma-Aldrich, HPA001906) (original magnification X40): (A) PNET tissue with positive staining and (B) PNET tissue with negative staining. Kaplan-Meier curves showing the correlations between ATRX immunostaining status and (C) overall survival and (D) recurrence-free survival.

Figure 2

Tuberous sclerosis 2 (TSC2) protein expression and its relationships with the overall and recurrence-free survival of patients with pancreatic endocrine tumors (PNETs).

Immunohistochemistry with anti-tuberin antibody (Santa Cruz, C-20) (original magnification X40): (A) PNET tissue with positive staining and (B) PNET tissue with negative staining. Kaplan-Meier curves showing the correlations between tuberin immunostaining and (C) overall survival and (D) recurrence-free survival.

Figure 3

PTEN protein expression and its relationships with the overall and recurrence-free survival of patients with pancreatic endocrine tumors (PNETs). Immunohistochemistry with anti- PTEN antibody (Dako, Clone 6H2.1) (original magnification X40): (A) PNETs tissue with positive staining and (B) PNETs tissue with negative staining. Kaplan-Meier curves showing the correlations between PTEN immunostaining status and (C) overall survival and (D) recurrence-free survival.

Figure 4

Kaplan-Meier survival curves stratified by combined TSC2 and ATRX protein expression status. (A) Correlation between combined TSC2 and ATRX protein expression status and overall survival. (B) Correlation between combined TSC2 and ATRX protein expression status and recurrence-free survival.

Supplementary Figure 1

Kaplan-Meier survival curves stratified by combined TSC2 and PTEN protein expression status. (A) Correlation between combined TSC2 and PTEN protein expression status and overall survival. (B) Correlation between combined TSC2 and PTEN protein expression

status and recurrence-free survival.

Supplementary Figure 2

Kaplan-Meier survival curves stratified by combined ATRX and PTEN protein expression status. (A) Correlation between combined ATRX and PTEN protein expression status and overall survival. (B) Correlation between combined ATRX and PTEN protein expression status and recurrence-free survival.

References

1. Oberg K, Eriksson B. Endocrine tumours of the pancreas. *Best Pract Res Clin Gastroenterol* 2005; 19(5):753-81.

2. Halfdanarson TR, Rabe KG, Rubin J, et al. Pancreatic neuroendocrine tumors (PNETs): incidence, prognosis and recent trend toward improved survival. *Ann Oncol* 2008; 19(10):1727-33.

3. Lloyd RV OR, Kloppel G, Rosai J, editors. WHO Classification of Tumours of Endocrine Organs 4th edition. . *IARC* 2017.

4. Gao H, Liu L, Wang W, et al. Novel recurrence risk stratification of resected

pancreatic neuroendocrine tumor. *Cancer Lett* 2018; 412:188-193.

5. Yachida S, Vakiani E, White CM, et al. Small cell and large cell neuroendocrine carcinomas of the pancreas are genetically similar and distinct from well-differentiated pancreatic neuroendocrine tumors. *Am J Surg Pathol* 2012; 36(2):173-84.

6. Benetatos N, Hodson J, Marudanayagam R, et al. Prognostic factors and survival after surgical resection of pancreatic neuroendocrine tumor with validation of established and modified staging systems. *Hepatobiliary Pancreat Dis Int* 2018; 17(2):169-175.

7. Fesinmeyer MD, Austin MA, Li CI, et al. Differences in Survival by Histologic Type of Pancreatic Cancer. *Cancer Epidemiology Biomarkers & Prevention* 2005; 14(7):1766-1773.

8. Gibril F, Doppman JL, Reynolds JC, et al. Bone metastases in patients with gastrinomas: a prospective study of bone scanning, somatostatin receptor scanning, and magnetic resonance image in their detection, frequency, location, and effect of their detection on management. *J Clin Oncol* 1998; 16(3):1040-53.

9. Hashim YM, Trinkaus KM, Linehan DC, et al. Regional lymphadenectomy is indicated in the surgical treatment of pancreatic neuroendocrine tumors (PNETs). *Ann Surg* 2014; 259(2):197-203.

10. Krampitz GW, Norton JA, Poultsides GA, et al. Lymph nodes and survival in pancreatic neuroendocrine tumors. *Arch Surg* 2012; 147(9):820-7.

11. Metz DC, Jensen RT. Gastrointestinal neuroendocrine tumors: pancreatic endocrine tumors. *Gastroenterology* 2008; 135(5):1469-92.

12. Nanno Y, Matsumoto I, Zen Y, et al. Pancreatic Duct Involvement in Well-Differentiated Neuroendocrine Tumors is an Independent Poor Prognostic Factor. *Ann Surg Oncol* 2017; 24(4):1127-1133.

13. Jiao Y, Shi C, Edil BH, et al. DAXX/ATRX, MEN1, and mTOR pathway genes are frequently altered in pancreatic neuroendocrine tumors. *Science* 2011; 331(6021):1199-203.

14. Scarpa A, Chang DK, Nones K, et al. Whole-genome landscape of pancreatic neuroendocrine tumours. *Nature* 2017; 543:65.

15. Heaphy CM, de Wilde RF, Jiao Y, et al. Altered telomeres in tumors with ATRX

and DAXX mutations. *Science* 2011; 333(6041):425.

16. Marinoni I, Kurrer AS, Vassella E, et al. Loss of DAXX and ATRX are associated with chromosome instability and reduced survival of patients with pancreatic neuroendocrine tumors. *Gastroenterology* 2014; 146(2):453-60.e5.

17. Asano E, Okano K, Oshima M, et al. Phenotypic characterization and clinical outcome in ampullary adenocarcinoma. *J Surg Oncol* 2016; 114(1):119-27.

18. Edge S BD, Compton CC, et al. AJCC cancer staging manual, 7 edition. *Springer* 2010.

19. Chou A, Itchins M, de Reuver PR, et al. ATRX loss is an independent predictor of poor survival in pancreatic neuroendocrine tumours. *Hum Pathol* 2018.

20. Oshima M, Okano K, Muraki S, et al. Immunohistochemically detected expression of 3 major genes (CDKN2A/p16, TP53, and SMAD4/DPC4) strongly predicts survival in patients with resectable pancreatic cancer. *Ann Surg* 2013; 258(2):336-46.

21. Frese KK, Tuveson DA. Maximizing mouse cancer models. *Nat Rev Cancer* 2007; 7(9):645-58.

22. Izeradjene K, Combs C, Best M, et al. Kras(G12D) and Smad4/Dpc4 haploinsufficiency cooperate to induce mucinous cystic neoplasms and invasive adenocarcinoma of the pancreas. *Cancer Cell* 2007; 11(3):229-43.

23. Tuveson D, Hanahan D. Translational medicine: Cancer lessons from mice to humans. *Nature* 2011; 471(7338):316-7.

24. Missiaglia E, Dalai I, Barbi S, et al. Pancreatic endocrine tumors: expression profiling evidences a role for AKT-mTOR pathway. *J Clin Oncol* 2010; 28(2):245-55.

25. Chung DC, Brown SB, Graeme-Cook F, et al. Localization of putative tumor suppressor loci by genome-wide allelotyping in human pancreatic endocrine tumors. *Cancer Res* 1998; 58(16):3706-11.

26. Rigaud G, Missiaglia E, Moore PS, et al. High resolution allelotype of nonfunctional pancreatic endocrine tumors: identification of two molecular subgroups with clinical implications. *Cancer Res* 2001; 61(1):285-92.

27. Perren A, Komminoth P, Saremaslani P, et al. Mutation and expression analyses reveal differential subcellular compartmentalization of PTEN in endocrine

pancreatic tumors compared to normal islet cells. *Am J Pathol* 2000; 157(4):1097-103.

28. Chou WC, Lin PH, Yeh YC, et al. Genes involved in angiogenesis and mTOR pathways are frequently mutated in Asian patients with pancreatic neuroendocrine tumors. *Int J Biol Sci* 2016; 12(12):1523-1532.

29. Qian ZR, Ter-Minassian M, Chan JA, et al. Prognostic significance of MTOR pathway component expression in neuroendocrine tumors. *J Clin Oncol* 2013; 31(27):3418-25.

30. Chatzipantelis P, Konstantinou P, Kaklamanos M, et al. The role of cytomorphology and proliferative activity in predicting biologic behavior of pancreatic neuroendocrine tumors: a study by endoscopic ultrasound-guided fine-needle aspiration cytology. *Cancer* 2009; 117(3):211-6.

31. Piani C, Franchi GM, Cappelletti C, et al. Cytological Ki-67 in pancreatic endocrine tumours: an opportunity for pre-operative grading. *Endocr Relat Cancer* 2008; 15(1):175-81.

Table 1. Clinicopathological Parameters and Outcome (n=100)							
Variable	No. of Patients (%)	Overall Survival (%)			Recurrence-Free Survival (%)		
		3 years	5 years	Log-rank (P Value)	3years	5 years	Log-rank (P Value)
Overall	100	94.1	89.2		87.2	85.2	
Gender				0.1245			0.4577
Female	49 (49.0)	97.1	97.1		93.0	89.1	
Male	51 (51.0)	91.4	80.8		82.3	82.4	
Age, years				0.749			0.708
Mean	60.8 (SD, 15.1)						
Median	63						
Range	23-88						
<65	55(55.0)	93.8	85.6		87.7	87.7	
.≥65	45(45.0)	94.5	94.5		86.9	82.6	
Outcome							
Follow-up, months							
Median	43.5						
Range	1-147						
WHO classification (2010)				0.0974			**0.0005**
NET G1	57 (57.0)	95.7	95.7		97.6	97.6	
NET G2	43 (43.0)	92.1	82.4		74.1	70.0	
Tumor Location				0.5429			0.1632

Head	56 (56.0)	92.1	85.5		79.5	79.5	
Body/tail	44 (44.0)	95.7	91.8		92.9	89.6	
Tumor size, mm				0.1293			**<0.0001**
Mean	24.3 (SD, 20.8)						
Median	20						
Range	0.8-156						
≤20 mm	51 (52.6)	97.6	97.6		100	100	
.>20 mm	46 (47.4)	94.9	86.4		72.6	68.1	
Nodal metastasis				**0.0513**			0.1286
Negative	77 (77.0)	96.9	94.1		89.5	87.1	
Positive	23 (23.0)	84.4	72.4		87.2	74.7	
Liver metastasis				**<.0001**			**0.0080**
Negative	92 (92.0)	97.5	94.9		88.7	86.5	
Positive	8 (8.0)	57.1	42.9		50.0	50.0	
Type of hormone production				0.2424			0.2010
Non-functioning	75(75.0)	92.1	84.2		85.9	82.3	
Functioning	25(25.0)	100	100		90.7	90.7	
Lymphatic invasion				0.1963			0.2342
Negative	71(78.0)	94.8	91.5		86.3	83.7	
Positive	20(22.0)	94.7	81.2		84.4	84.4	
Vascular invasion				**0.0142**			**0.0003**
Negative	55(60.4)	97.7	97.7		97.6	94.1	

Positive	36(39.6)	90.3	77.3		67.1	67.1	
Perineural infiltration				**0.0002**			**0.00124**
Negative	67(75.3)	98.2	98.2		91.8	89.0	
positive	22(24.7)	84.0	44.8		60.7	60.7	
Immunohistochemistry							
ATRX				**0.0558**			**0.0117**
Negative (loss)	41(41.4)	88.6	84.0		78.9	74.3	
Positive (intact)	58(58.6)	97.9	93.0		92.8	92.8	
DAXX				0.7908			0.2882
Negative (loss)	64(64.6)	94.5	88.1		83.0	80.1	
Positive (intact)	35(35.4)	93.7	93.7		95.7	95.7	
MEN1 (Menin)				0.5940			0.3759
Negative (loss)	25(25.3)	90.4	84.0		83.7	83.7	
Positive (intact)	74(74.7)	95.3	91.2		88.4	85.5	
TSC2 (tuberin)				**0.007**			**0.009**
Negative (loss)	58(58.6)	89.1	79.7		79.3	74.9	
Positive (intact)	41(41.4)	100	100		94.2	94.2	
PTEN				**0.0486**			0.0721
Negative (loss)	37(37.8)	90.1	84.1		84.5	78.4	
Positive (intact)	61(62.2)	98.3	94.0		88.6	88.6	
p53				0.1975			0.4697
abnormal (loss)	65(67.0)	92.9	89.0		87.0	83.8	

normal (intact)	32(33.0)	100	93.4		85.2	85.2	
Rb1				0.4927			0.5786
Negative (loss)	45(45.4)	92.1	92.1		88.2	84.2	
Positive (intact)	54(54.6)	95.9	85.2		85.6	85.6	
P16				0.3067			0.7769
Negative (loss)	29(29.3)	96.0	87.3		85.1	85.1	
Positive (intact)	70(70.7)	95.0	91.6		88.5	85.6	

Table 2. Multivariate Analysis of Relapse-Free Survival for patients with G2 PNETS including Loss of Combined Gene Protein Expression			
Variables	Hazard Ratio	95% CI	p
TSC2 and ATRX negative	10.1	2.1-66.9	0.003
Vascular invasion	10.9	1.3-10.3	0.02
Nodal metastasis	5.8	1.2-29.3	0.02
Perineural invasion	2.1	0.5-10.3	0.46

Table 3. Summary of significant association between phenotypes and clinicopathological parameters (n=100)

		N	Clinicopathological Parameters	P value
	ATRX negative (loss)	35	Perineural invasion	0.002
			Venous invasion	0.004
			Nodal metastasis	0.014
			Tumor size (>20 mm)	0.021
			Recurrence	0.041
	MEN1 negative (loss)	25	Hormone production	0.003
			Venous invasion	0.012
			Grade	0.02
			Liver metastasis	0.023
			Tumor size (>20 mm)	0.029
	TSC2 negative (loss)	58	Hormone production	0.0008
			Nodal metastasis	0.001
			Recurrence	0.01
			Liver metastasis	0.019
			Tumor size (>20 mm)	0.024
			Venous invasion	0.03
	PTEN negative (loss)	32	Venous invasion	0.007
			Tumor size (>20 mm)	0.02
			Recurrence	0.044

Figure 1

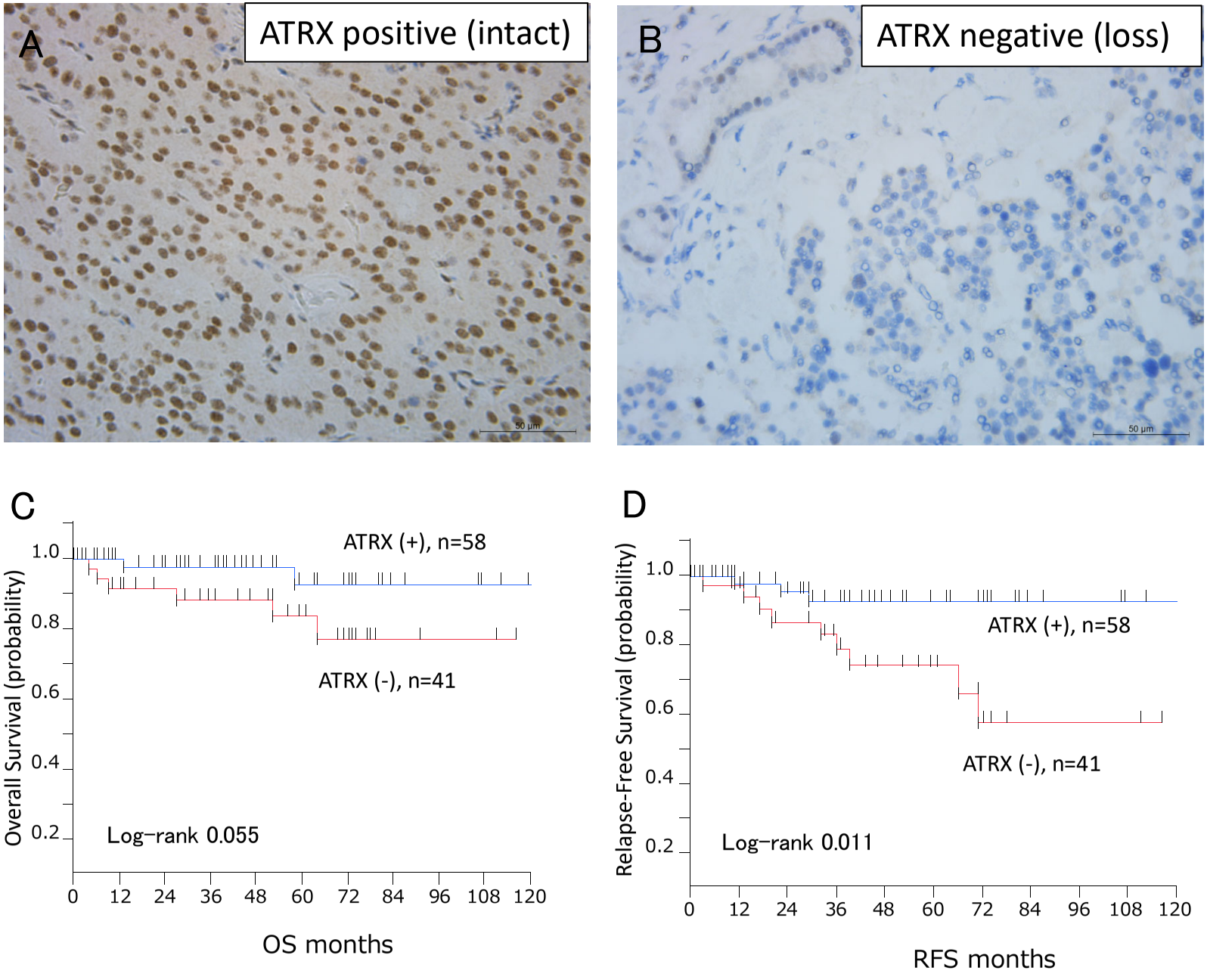

A
ATRX positive (intact)
B
ATRX negative (loss)
C
ATRX (+), n=58
ATRX (-), n=41
Log−rank 0.055
Overall Survival (probability)
1.0
0.8
0.6
0.4
0.2
0 12 24 36 48 60 72 84 96 108 120
OS months
D
ATRX (+), n=58
ATRX (-), n=41
Log−rank 0.011
Relapse-Free Survival (probability)
1.0
0.8
0.6
0.4
0.2
0 12 24 36 48 60 72 84 96 108 120
RFS months

Figure 2

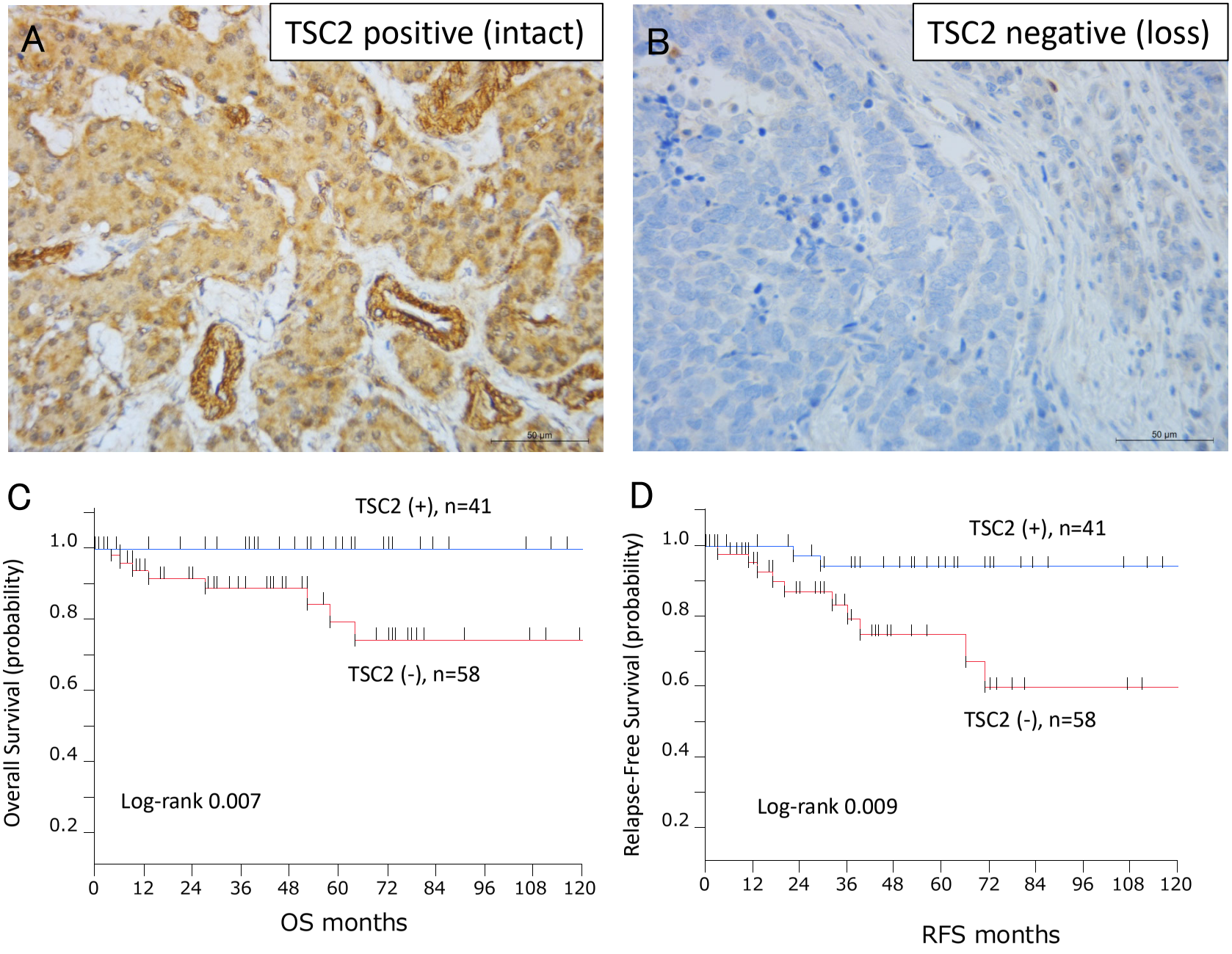

A
TSC2 positive (intact)
50 µm
B
TSC2 negative (loss)
50 µm
C
TSC2 (+), n=41
TSC2 (-), n=58
Log-rank 0.007
Overall Survival (probability)
1.0
0.8
0.6
0.4
0.2
0
12
24
36
48
60
72
84
96
108
120
OS months
D
TSC2 (+), n=41
TSC2 (-), n=58
Log-rank 0.009
Relapse-Free Survival (probability)
1.0
0.8
0.6
0.4
0.2
0
12
24
36
48
60
72
84
96
108
120
RFS months

Figure 3

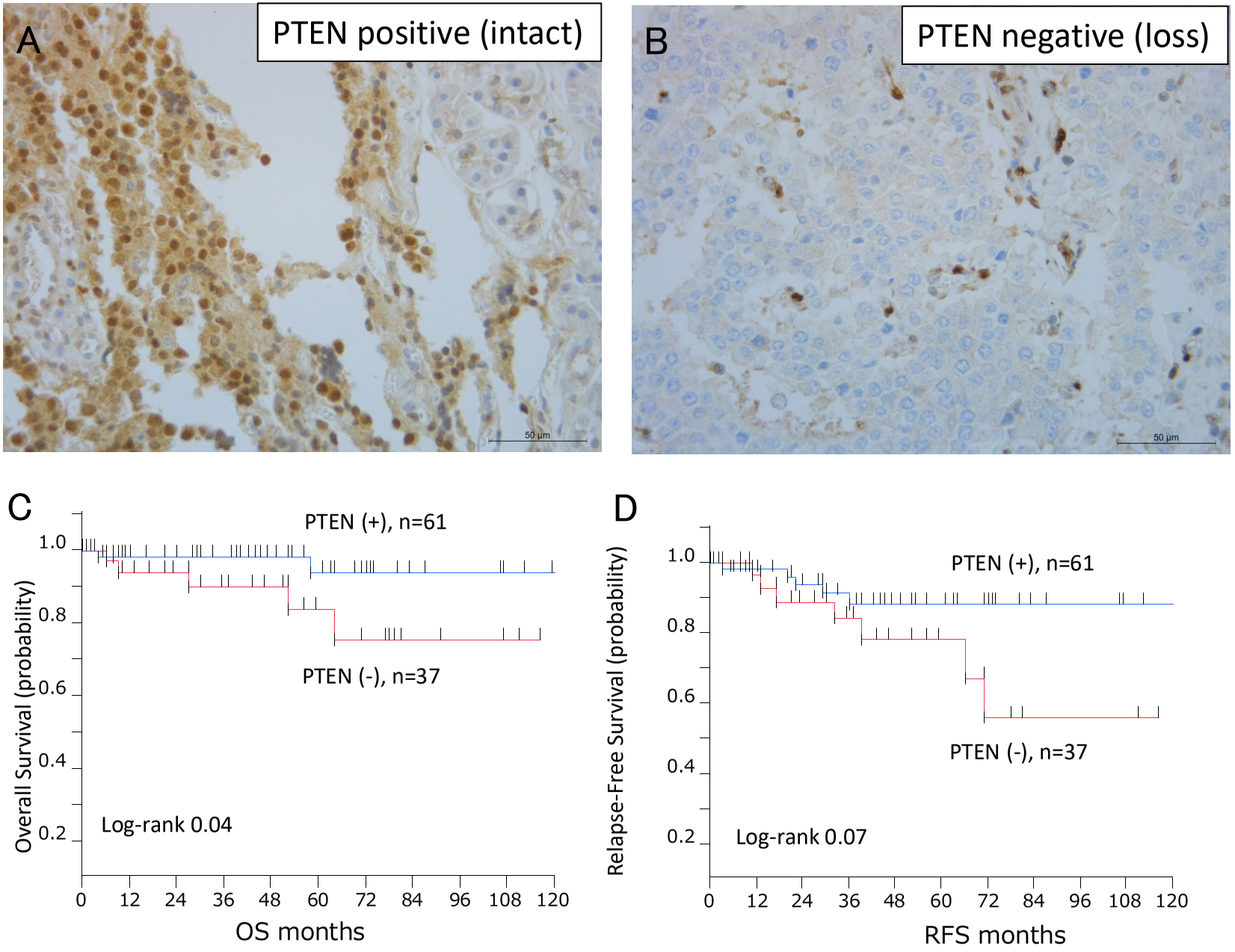
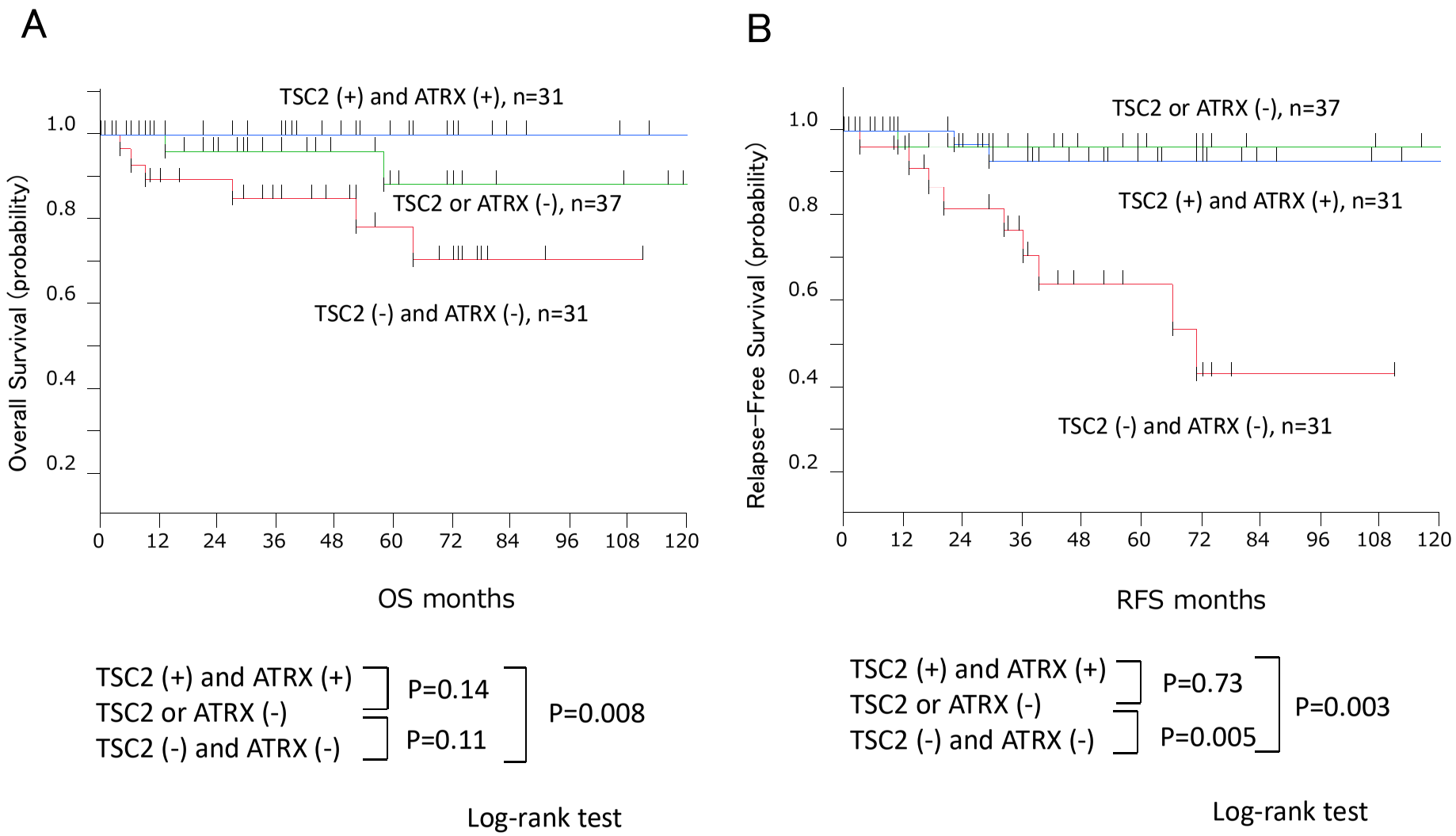

A
PTEN positive (intact)
50 µm
B
PTEN negative (loss)
50 µm
C
PTEN (+), n=61
PTEN (-), n=37
Log-rank 0.04
Overall Survival (probability)
1.0
0.8
0.6
0.4
0.2
0
12
24
36
48
60
72
84
96
108
120
OS months
D
PTEN (+), n=61
PTEN (-), n=37
Log-rank 0.07
Relapse-Free Survival (probability)
1.0
0.8
0.6
0.4
0.2
0
12
24
36
48
60
72
84
96
108
120
RFS months

Figure 4


A
TSC2 (+) and ATRX (+), n=31
TSC2 or ATRX (-), n=37
TSC2 (-) and ATRX (-), n=31
Overall Survival (probability)
1.0
0.8
0.6
0.4
0.2
0
12
24
36
48
60
72
84
96
108
120
OS months
TSC2 (+) and ATRX (+)
TSC2 or ATRX (-)
TSC2 (-) and ATRX (-)
P=0.14
P=0.11
P=0.008
Log-rank test
B
TSC2 or ATRX (-), n=37
TSC2 (+) and ATRX (+), n=31
TSC2 (-) and ATRX (-), n=31
Relapse−Free Survival (probability)
1.0
0.8
0.6
0.4
0.2
0
12
24
36
48
60
72
84
96
108
120
RFS months
TSC2 (+) and ATRX (+)
TSC2 or ATRX (-)
TSC2 (-) and ATRX (-)
P=0.73
P=0.005
P=0.003
Log-rank test