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# Alternative Lengthening of Telomeres in Primary Pancreatic Neuroendocrine Tumors Is Associated with Aggressive Clinical Behavior and Poor Survival

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## Abstract

**Purpose:** Alternative lengthening of telomeres (ALT), a telomerase-independent telomere maintenance mechanism, is strongly associated with ATRX and DAXX alterations and occurs frequently in pancreatic neuroendocrine tumors (PanNET).

**Experimental Design:** In a Korean cohort of 269 surgically resected primary PanNETs and 19 sporadic microadenomas, ALT status and nuclear ATRX and DAXX protein expression were assessed and compared with clinicopathologic factors.

**Results:** In PanNETs, ALT or loss of ATRX/DAXX nuclear expression was observed in 20.8% and 19.3%, respectively, whereas microadenomas were not altered. ALT-positive PanNETs displayed a significantly higher grade, size, and pT classification (all, $P < 0.001$). ALT also strongly correlated with lymphovascular ($P < 0.001$) and perineural invasion ($P = 0.001$) and the presence of lymph node ($P < 0.001$) and distant metastases ($P = 0.002$). Furthermore, patients with ALT-positive primary PanNETs had a shorter recurrence-free survival [HR = 3.38; 95% confidence interval (CI), 1.83–6.27; $P < 0.001$]. Interestingly, when limiting to patients with distant metastases, those with ALT-positive primary tumors had significantly better overall survival (HR = 0.23; 95% CI, 0.08–0.68; $P = 0.008$). Similarly, tumors with loss of ATRX/DAXX expression were significantly associated with ALT ($P < 0.001$), aggressive clinical behavior, and reduced recurrence-free survival ($P < 0.001$). However, similar to ALT, when limiting to patients with distant metastases, loss of ATRX/DAXX expression was associated with better overall survival ($P = 0.003$).

**Conclusions:** Both primary ALT-positive and ATRX/DAXX-negative PanNETs are independently associated with aggressive clinicopathologic behavior and displayed reduced recurrence-free survival. In contrast, ALT activation and loss of ATRX/DAXX are both associated with better overall survival in patients with metastases. Therefore, these biomarkers may be used as prognostic markers depending on the context of the disease. *Clin Cancer Res; 23(6); 1598–606. ©2016 AACR.*

## Introduction

Pancreatic neuroendocrine tumors (PanNET) are rare and comprise 1% to 3% of all pancreatic tumors (1, 2). Patients with PanNETs usually display a better survival than those with pancreatic ductal adenocarcinomas (3–6). However, PanNETs are still malignant, as evidenced by the fact that the 10-year survival rate for PanNET patients is only 40% to 50% (7–10). Although surgical resection is the only curative treatment option for patients diagnosed with a PanNET (11, 12), a growing number of medical therapies have been utilized in recent years for unresectable or metastatic PanNET patients, including somatostatin analogues, cytotoxic chemotherapies, and molecular targeted therapies, such as mTOR inhibitors (12, 13). To better predict clinical outcomes and to identify patients who may ultimately benefit from targeted therapies, a better understanding of the molecular mechanisms driving PanNETs is warranted.

Whole-exome sequencing studies of PanNETs have demonstrated prevalent mutations in several genes, including *ATRX*, *DAXX*, *MEN1*, and mTOR pathway genes (14). In particular, Jiao and colleagues reported that 43% of PanNETs harbored

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## Translational Relevance

A better understanding of the molecular mechanisms driving pancreatic neuroendocrine tumors (PanNET) can help identify patients who will benefit from targeted therapies. We performed telomere-specific FISH to determine the alternative lengthening of telomeres (ALT) status of 269 primary PanNETs and compared ALT status with nuclear ATRX and DAXX protein expression and clinicopathologic factors. In primary PanNETs, the ALT-positive tumors behaved aggressively, and these patients had a shorter recurrence-free survival. Conversely, when limited to patients with distant metastases, ALT positivity conferred significantly better survival. One possible explanation for this observation is that although ALT-positive primary PanNET clones seem to progress and metastasize readily to other organ sites, these metastases may grow more slowly in the new microenvironment.

inactivating mutations in either the *ATRX* or *DAXX* gene; the presence of these mutations was mutually exclusive (14). Consequently, alterations in these two proteins are tightly associated with alternative lengthening of telomeres (ALT) in PanNETs (15, 16) and other tumor types (17–19).

ALT is a telomerase-independent mechanism of telomere maintenance, and ALT-positive tumors are thought to coopt the homologous recombination machinery for telomere maintenance (20). Characteristics of ALT-positive tumors include the presence of large, ultra-bright telomeric DNA FISH signals, dramatic telomere length heterogeneity, the presence of extrachromosomal telomeric DNA, and increased chromosomal instability (21, 22). In previous studies, mostly including Caucasian patients, ALT was identified in a substantial fraction of PanNETs (48%–61%), although a lower prevalence (15%) was observed in a small Korean cohort (15, 17, 21, 23). These studies have consistently observed a significant correlation between the presence of ALT and alterations in ATRX or DAXX. However, the prognostic effect of ALT and ATRX or DAXX alterations in PanNETs remains inconclusive. Previous studies reported that ALT activation is associated with better prognosis (14, 24), whereas another reported ALT is associated with an aggressive phenotype and a poor prognosis (21). In addition, recent studies have suggested that ATRX loss and/or ALT positivity confer *in vitro* sensitivity to inhibition of the DNA damage mediator, ATR, as well as topoisomerase inhibitors and radiation (25, 26). Therefore, the study of ALT and ATRX or DAXX loss in PanNETs is warranted from a prognostic, as well as a potential therapeutic, standpoint.

Hence, we evaluated a well-characterized, large Korean cohort of primary PanNETs associated with or without synchronous or metachronous metastases. Using this cohort, we evaluated the presence of ALT and alterations in ATRX or DAXX expression and correlated these findings with clinicopathologic factors.

## Materials and Methods

### Case selection

A total of 269 primary PanNETs and 19 sporadic neuroendocrine microadenomas resected between January 1995 and March 2015 were retrieved from the Department of Pathology at the Asan Medical Center (Seoul, Korea). Neuroendocrine microadenomas were defined as well-differentiated, nonfunctional neuroendocrine tumors <0.5 cm in diameter, whereas PanNETs were neuroendocrine neoplasms $\geq$ 0.5 cm in diameter (27, 28). All PanNETs were classified according to the 2010 World Health Organization (WHO) classification using mitotic activity and the Ki67 labeling index (28). Poorly differentiated neuroendocrine carcinomas, including small-cell carcinomas and large-cell carcinomas, were not included in this study. Clinical data, such as patients' age, gender, symptoms, survival status, and survival time, were reviewed. Pathologic data were extracted from the pathology reports, including tumor size, extension, lymph node and distant metastases, and perineural and lymphovascular tumor invasion. The protein expression profiles of specific peptide hormones, including insulin, glucagon-like peptide 1, glucagon, gastrin, serotonin, and somatostatin, were determined previously (29). The extent of tumor was evaluated according to the T classification of the 7th edition of the American Joint Committee on Cancer cancer staging system (30).

### Tissue microarray construction

Tissue microarrays (TMA) were constructed from archived, formalin-fixed, paraffin-embedded tissue blocks with a Manual Tissue Microarrayer (Uni TMA Co. Ltd.). Three cores from tumors and one core from normal pancreatic parenchyma with a diameter of 2.0 mm were punched from each PanNET or neuroendocrine microadenomas and placed into recipient blocks. From the TMA, 4-μm thick sections were cut.

### ATRX and DAXX IHC

Immunohistochemical labeling for ATRX and DAXX was performed as described previously (15). Anti-ATRX (HPA001906, Sigma-Aldrich, 1:300) and anti-DAXX (HPA008736, Sigma-Aldrich, 1:100) antibodies were used. Briefly, 4-μm thick tissue sections were deparaffinized and hydrated in xylene and serially diluted in ethanol. For antigen retrieval, sections were steamed with EDTA for anti-ATRX and citrate buffer for anti-DAXX for 50 and 30 minutes, respectively. After cooling for 5 minutes, sections were blocked against endogenous peroxidase activity with Dual Endogenous Enzyme-Blocking Agent (Dako) for 10 minutes. Sections were incubated with primary antibodies for 1 hour at room temperature, followed by secondary antibody (Leica Microsystems) for 30 minutes and detected with 3,30-diaminobenzidine (Sigma-Aldrich) after 10 minutes. Wash steps were performed with PBS containing 0.1% Tween-20 for 5 minutes. Sections were counterstained with hematoxylin, rehydrated, and mounted. Immunohistochemical labeling was successfully performed on 264 of the 269 (98.1%) PanNETs on the TMAs. Some cases (4 cases of ATRX and 5 cases of DAXX) were not immunolabeled successfully due to loss of tissue cores during the sectioning or labeling process.

Immunolabeling for ATRX and DAXX was considered positive if >5% of neoplastic cells had nuclear staining, as described previously (31). Neoplasms were scored as negative for ATRX or DAXX if the pattern was that of cytoplasmic accumulation with nuclear clearing, as long as the adequate internal controls (i.e., nuclear labeling of adjacent endothelial cells, lymphocytes, and/or islets of Langerhans) were present (14, 21). Cases with inadequate internal controls were validated by relabeling whole sections of each case.

### Telomere FISH

Briefly, deparaffinized slides were hydrated, steamed for 25 minutes in citrate buffer (Vector Laboratories), dehydrated, and hybridized with a Cy3-labeled peptide nucleic acid (PNA) probe complementary to the mammalian telomere repeat sequence [(N-terminus to C-terminus) CCCTAACCCTAACCCTAA]. As a positive control for hybridization efficiency, an Alexa Fluor-488–labeled PNA probe specific to human centromeric DNA repeats (ATTCGTTGGAAACGGGA; CENP-B–binding sequence) was included in the hybridization solution. Following posthybridization washes, the slides were counterstained with DAPI. Slides were imaged with a Nikon 50i epifluorescence microscope equipped with X-Cite series 120 illuminator (EXFO Photonics Solutions Inc.) and appropriate fluorescence excitation/emission filters. Grayscale images were captured using Nikon NIS-Elements software and an attached Photometrics CoolsnapEZ digital camera, pseudo-colored, and merged.

Characteristics of ALT-positive tumors in fixed tissue specimens are dramatic cell-to-cell telomere length heterogeneity and the presence of large, ultra-bright nuclear foci of telomere FISH signals marking ALT-associated telomeric DNA in interphase nuclei. As such, cases were visually assessed and classified as ALT positive if they met the following criteria: (i) the presence of very bright nuclear foci of telomere FISH signals (e.g., total intensities for individual foci have been previously measured to be >10-fold that of the mean integrated signal intensities for individual non-neoplastic stromal cells in the same cases; ref. 15); and (ii) $\geq$1% of tumor cells with ALT-associated telomeric foci. ALT negativity was defined by the lack of ALT-associated telomeric foci in $\geq$1% of tumor cells, with at least 500 tumor cells evaluated (17). Areas exhibiting necrosis were excluded from consideration.

### Statistical analysis

Statistical analysis was performed with $\chi^2$ and Fisher exact tests to identify correlations between ALT and other clinicopathologic factors. The overall survival and recurrence-free survival rate was analyzed by the Kaplan–Meier method, and significance was evaluated with the log-rank test. The Cox proportional hazards regression model was used to investigate the significance of ALT as a prognostic factor. $P < 0.05$ was considered statistically significant. All statistical analyses were performed with SPSS version 18.0 (SPSS Inc.).

## Results

### Characteristics of cases

Patient characteristics and molecular features of the tumors are shown in Table 1. The mean age of the patients (127 men and 142 women) was 52.3 $\pm$ 0.8 years. According to the 2010 WHO classification scheme, 181 PanNETs were G1, 80 were G2, and 8 were G3. Overall, the mean tumor size was 3.0 $\pm$ 0.1 cm. Although 113 cases were classified as pT1, the others were classified at a higher pT stage (109 as pT2, 44 as pT3, and 3 as pT4). Seventy cases (26.0%) had lymphovascular invasion, and 35 cases (13.0%) had perineural invasion. Lymph node metastasis occurred in 35 cases (13.0%), synchronous distant metastasis occurred in 10 cases (3.7%), and metachronous distant metastasis occurred in 32 cases (11.9%). Of the 269 total PanNETs, 265 were sporadic, and 2 were associated with multiple endocrine neoplasia type 1 (MEN-1) syndrome and 2 with the von Hippel–Lindau syndrome. Across the entire cohort, the median follow-up time was 28 months (range, 1–188 months).

**Table 1.** Comparison of ALT and ATRX/DAXX expression status with clinicopathologic factors of PanNETs

| | | | ALT status | | | ATRX or DAXX expression | | |
|---|---|---|---|---|---|---|---|---|
| **Clinicopathologic factors** | | **Number** | **Negative** | **Positive** | ***P*** | **Intact** | **Loss** | ***P*** |
| Age (years) | ≤60 | 189 | 152 (80.4%) | 37 (19.6%) | 0.441 | 152 (82.2%) | 33 (17.8%) | 0.351 |
| | >60 | 80 | 61 (76.2%) | 19 (23.8%) | | 61 (77.2%) | 18 (22.8%) | |
| Sex | Male | 127 | 94 (74.0%) | 33 (26.0%) | 0.048[a] | 97 (77.6%) | 28 (22.4%) | 0.229 |
| | Female | 142 | 119 (83.8%) | 23 (16.2%) | | 116 (83.5%) | 23 (16.5%) | |
| Grade | Grade 1 | 181 | 158 (87.3%) | 23 (12.7%) | <0.001[a] | 157 (88.7%) | 20 (11.3%) | <0.001[a] |
| | Grade 2 | 80 | 49 (61.2%) | 31 (38.8%) | | 50 (63.3%) | 29 (36.7%) | |
| | Grade 3 | 8 | 6 (75.0%) | 2 (25.0%) | | 6 (75.0%) | 2 (25.0%) | |
| Ki67 index | <3% | 195 | 167 (85.6%) | 28 (14.4%) | <0.001[a] | 166 (86.9%) | 25 (13.1%) | <0.001[a] |
| | 3%–20% | 66 | 40 (60.6%) | 26 (39.4%) | | 41 (63.1%) | 24 (36.9%) | |
| | >20% | 8 | 6 (75.0%) | 2 (25.0%) | | 6 (75.0%) | 2 (25.0%) | |
| Size | ≤2 cm | 119 | 112 (94.1%) | 7 (5.9%) | <0.001[a] | 114 (96.6%) | 4 (3.4%) | <0.001[a] |
| | >2 cm | 150 | 101 (67.3%) | 49 (32.7%) | | 101 (68.2%) | 47 (31.8%) | |
| pT classification | pT1 | 113 | 109 (96.5%) | 4 (3.5%) | <0.001[a] | 108 (98.2%) | 2 (1.8%) | <0.001[a] |
| | pT2–pT4 | 156 | 104 (66.7%) | 52 (33.3%) | | 105 (68.2%) | 49 (31.8%) | |
| Lymphovascular invasion | Absence | 199 | 173 (86.9%) | 26 (13.1%) | <0.001[a] | 173 (88.7%) | 22 (11.3%) | <0.001[a] |
| | Presence | 70 | 40 (57.1%) | 30 (42.9%) | | 40 (58.0%) | 29 (42.0%) | |
| Perineural invasion | Absence | 234 | 193 (82.5%) | 41 (17.5%) | 0.001[a] | 191 (83.4%) | 38 (16.6%) | 0.004[a] |
| | Presence | 35 | 20 (57.1%) | 15 (42.9%) | | 22 (62.9%) | 13 (37.1%) | |
| Lymph node metastasis | Absence | 234 | 194 (82.9%) | 40 (17.1%) | <0.001[a] | 193 (84.3%) | 36 (15.7%) | <0.001[a] |
| | Presence | 35 | 19 (54.3%) | 16 (45.7%) | | 20 (57.1%) | 15 (42.9%) | |
| Distant metastasis | Absence | 259 | 209 (80.7%) | 50 (19.3%) | 0.002[a] | 209 (82.3%) | 45 (17.7%) | 0.001[a] |
| | Presence | 10 | 4 (40.0%) | 6 (60.0%) | | 4 (40.0%) | 6 (60.0%) | |
| Insulin expression | Absence | 124 | 86 (69.4%) | 38 (30.6%) | <0.001[a] | 82 (68.3%) | 38 (31.7%) | <0.001[a] |
| | Presence | 43 | 43 (100.0%) | 0 (0.0%) | | 42 (100.0%) | 0 (0.0%) | |
| GLP-1 expression | Absence | 141 | 105 (74.5%) | 36 (25.5%) | 0.046[a] | 100 (73.5%) | 36 (26.5%) | 0.038[a] |
| | Presence | 26 | 24 (92.3%) | 2 (7.7%) | | 24 (92.3%) | 2 (7.7%) | |
| Hormone expression | Absence | 94 | 59 (62.8%) | 35 (37.2%) | <0.001[a] | 55 (61.1%) | 35 (38.9%) | <0.001[a] |
| | Presence | 73 | 70 (95.9%) | 3 (4.1%) | | 69 (95.8%) | 3 (4.2%) | |

[a]Statistically significant at $P < 0.05$.

The mean size of the neuroendocrine microadenomas was 0.3 ± 0.1 cm, and the mean age of these patients was 63.0 ± 6.4 years. Pancreatic lesions associated with microadenomas consisted of 8 ductal adenocarcinomas, 3 sporadic PanNETs, 3 chronic pancreatitis, 2 intraductal papillary mucinous neoplasms, 1 mucinous cystic neoplasm, 1 serous cystadenoma, and 1 type I autoimmune pancreatitis.

### ALT activation in PanNETs

ALT was assessed in our cohort by telomere-specific FISH, and representative images are depicted in Fig. 1A and B. None of the 19 neuroendocrine microadenomas from sporadic PanNETs were ALT positive (0%, 0/19 cases), whereas 20.8% (56/269 cases) of PanNETs were ALT positive. All 56 ALT-positive tumors were sporadic PanNETs; none of the 4 hereditary PanNETs showed activation of ALT. As shown in Table 1, ALT-positive PanNETs were more commonly observed in tumors with higher grade, higher Ki67 index, larger size, and higher pT classification ($P < 0.001$ for all). ALT-positive PanNETs were also associated with the presence of lymphovascular ($P < 0.001$) and perineural ($P = 0.001$) invasion. In addition, ALT-positive PanNETs were strongly associated with lymph node metastasis ($P < 0.001$), distant metastasis ($P = 0.002$), absence of insulin expression ($P < 0.001$), absence of GLP-1 expression ($P = 0.046$), and without any hormone expression ($P < 0.001$). Strikingly, the presence of ALT increased along with tumor size; larger tumors were more likely to display the ALT phenotype than smaller tumors (Fig. 2A; $P < 0.001$).

### ATRX and DAXX expression and correlation with ALT activation

Representative images of ATRX and DAXX immunostaining are depicted in Fig. 1C–F. None of the 19 neuroendocrine microadenomas from sporadic PanNET cases showed loss of nuclear ATRX or DAXX expression (0%, 0/19 cases), whereas 8.6% (23/274) and 10.4% (28/273) of PanNETs showed loss of nuclear ATRX or DAXX expression, respectively. Altogether, 51 cases (19.0%) showed loss of nuclear expression of either ATRX or DAXX; this loss of protein expression was mutually exclusive. ALT was observed in 50 of 51 cases (98.0%) with loss of nuclear ATRX or DAXX expression, in addition to 6

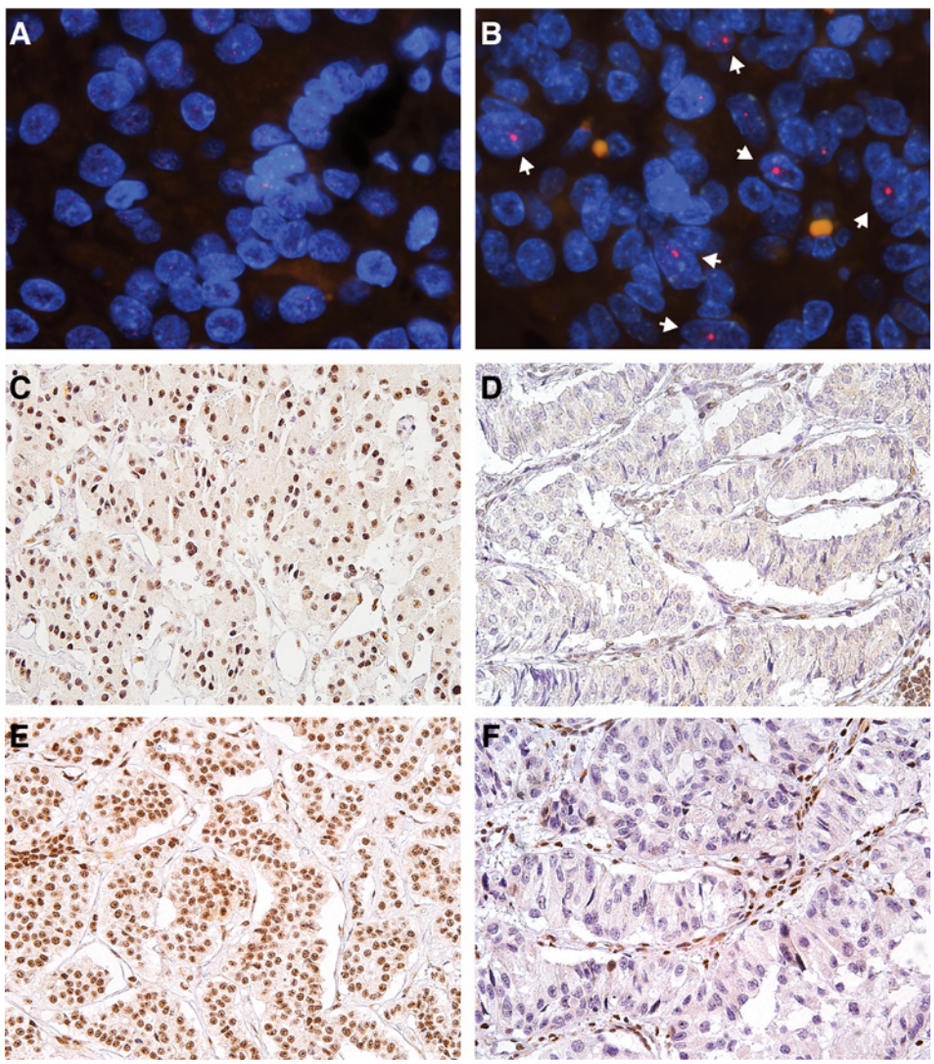


**Figure 1.**
Representative images of telomere-specific FISH and ATRX and DAXX immunostaining. **A** and **B,** A PanNET that is ALT negative (**A**) and a PanNET that is ALT positive (**B**). The telomere foci (arrows) are indicative of ALT. For **A** and **B,** telomeres are depicted in red. **C** and **D,** A PanNET with intact ATRX expression (**C**; 40×) and a PanNET with loss of nuclear ATRX expression (**D**). Tumor cells show loss of ATRX expression, whereas lymphocytes and endothelial cells demonstrate intact ATRX (40×). **E** and **F,** A PanNET with intact DAXX expression (**E**; 40×) and a PanNET with loss of nuclear DAXX expression (**F**). Tumor cells show loss of nuclear DAXX expression, whereas lymphocytes and endothelial cells demonstrate intact DAXX expression (40×).

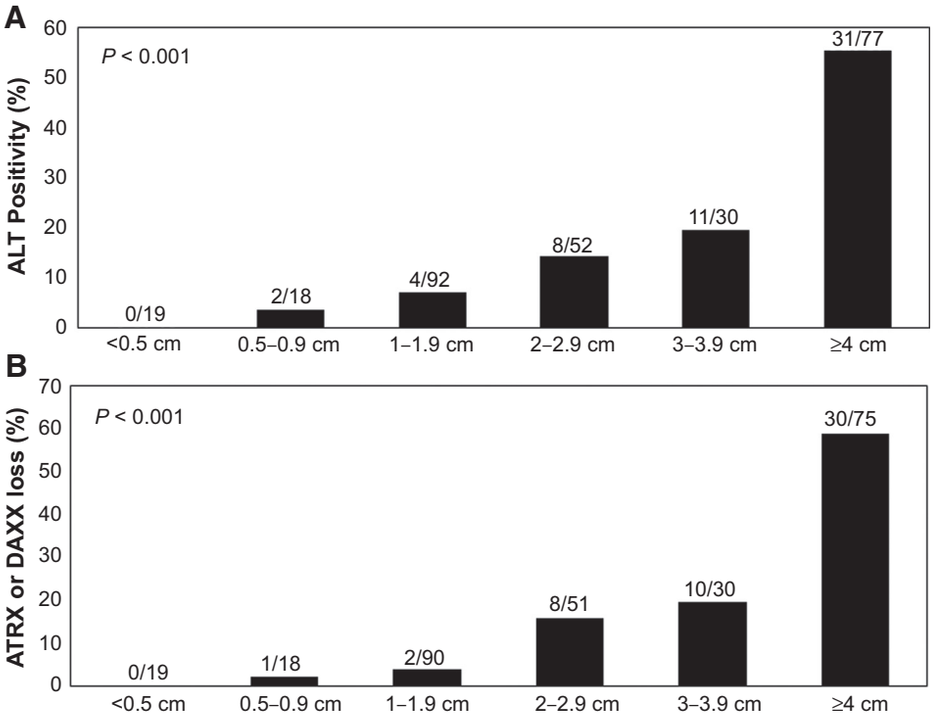


**Figure 2.**
The correlation of ALT and ATRX and DAXX immunostaining with tumor size. The proportion of ALT-positive (**A**) or ATRX- or DAXX-negative tumors (**B**) increases with the size of the tumor ($P < 0.001$).

cases (2.8%) with intact nuclear ATRX and DAXX expression (Supplementary Table S1). One case showed loss of nuclear ATRX expression, but was ALT negative. Overall, ALT activation was significantly correlated with loss of nuclear ATRX or DAXX expression ($P < 0.001$). Given the near perfect association between ALT positivity and the loss of nuclear ATRX or DAXX observed, all correlations between ALT-positive PanNETs and clinicopathologic variables reported here are nearly identical when considering loss of ATRX or DAXX rather than ALT status (Table 1). Thus, similar to the ALT results, loss of nuclear ATRX or DAXX expression increased with tumor size (Fig. 2B; $P < 0.001$).

### Recurrence-free survival analysis based on ALT status in PanNETs without metastasis

After excluding cases with R1 ($n = 14$) resection or synchronous ($n = 10$) distant metastases, the recurrence-free survival was analyzed for 245 primary PanNETs. The recurrence-free survival in patients with ALT-positive primary tumors was significantly worse than those with ALT-negative primary tumors [HR = 3.38; 95% confidence interval (CI), 1.83–6.27; $P < 0.001$; Fig. 3A]. Similarly, patients with tumors demonstrated to have nuclear ATRX or DAXX loss had worse recurrence-free survival than those with intact ATRX and DAXX protein expression (HR = 4.01; 95% CI, 2.14–7.50; $P < 0.001$; Fig. 3B).

Other clinicopathologic factors associated with poor recurrence-free survival were older age (>60 years), higher grade, higher Ki67 index, large size, high T classification, presence of lymphovascular and perineural invasion, and lymph node metastasis (Table 2). In a multivariate analysis, presence of ALT (HR = 1.97; 95% CI, 1.02–3.78; $P = 0.043$), older age (HR = 2.28; 95% CI, 1.18–4.41; $P = 0.014$), and higher grade (HR = 5.49; 95% CI, 2.46–12.24; $P < 0.001$) were independent poor prognostic factors (Table 2). Next, due to the robust correlation between the presence of ALT and loss of ATRX/DAXX expression, we substituted loss of ATRX/DAXX for ALT status in another model. In this multivariate analysis, loss of ATRX/DAXX expression (HR = 2.26; 95% CI, 1.17–4.39; $P = 0.016$), older age (HR = 2.18; 95% CI, 1.12–4.27; $P = 0.022$), and higher grade (HR = 5.06; 95% CI, 2.26–11.33; $P < 0.001$) remained independent poor prognostic factors.

In the entire cohort ($N = 269$), the overall survival in patients with ALT-positive tumors was not significantly different from those with ALT-negative tumors (HR = 1.01; 95% CI, 0.47–2.20; $P = 0.973$; Fig. 3C). Similarly, overall survival did not differ between patients with ATRX or DAXX loss and intact ATRX and DAXX expression (HR = 1.01; 95% CI, 0.46–2.21; $P = 0.988$; Fig. 3D).

### Survival analysis of ALT in PanNETs with distant metastasis

Synchronous ($n = 10$) or metachronous ($n = 32$) metastatic PanNETs were observed, and within this subgroup, the patients with ALT-positive primary tumors had significantly better overall survival than those with ALT-negative tumors (HR = 0.23; 95% CI, 0.08–0.68; $P = 0.008$; Fig. 3E). Also, the patients with loss of ATRX or DAXX expression had better overall survival than those with intact ATRX and DAXX expression (HR = 0.20; 95% CI, 0.07–0.59; $P = 0.003$). In addition, when limiting to only the metachronous metastatic PanNETs, the overall survival in patients with ALT-positive tumors remained significantly longer than those with ALT-negative tumors (HR = 0.24; 95% CI, 0.07–0.82; $P = 0.023$; Fig. 3F). Other clinicopathologic factors, including age, sex, grade, Ki67 index, size, pT classification, lymphovascular invasion, perineural invasion, or lymph node metastasis, were not significantly associated with overall survival of metastatic PanNETs (Table 3).

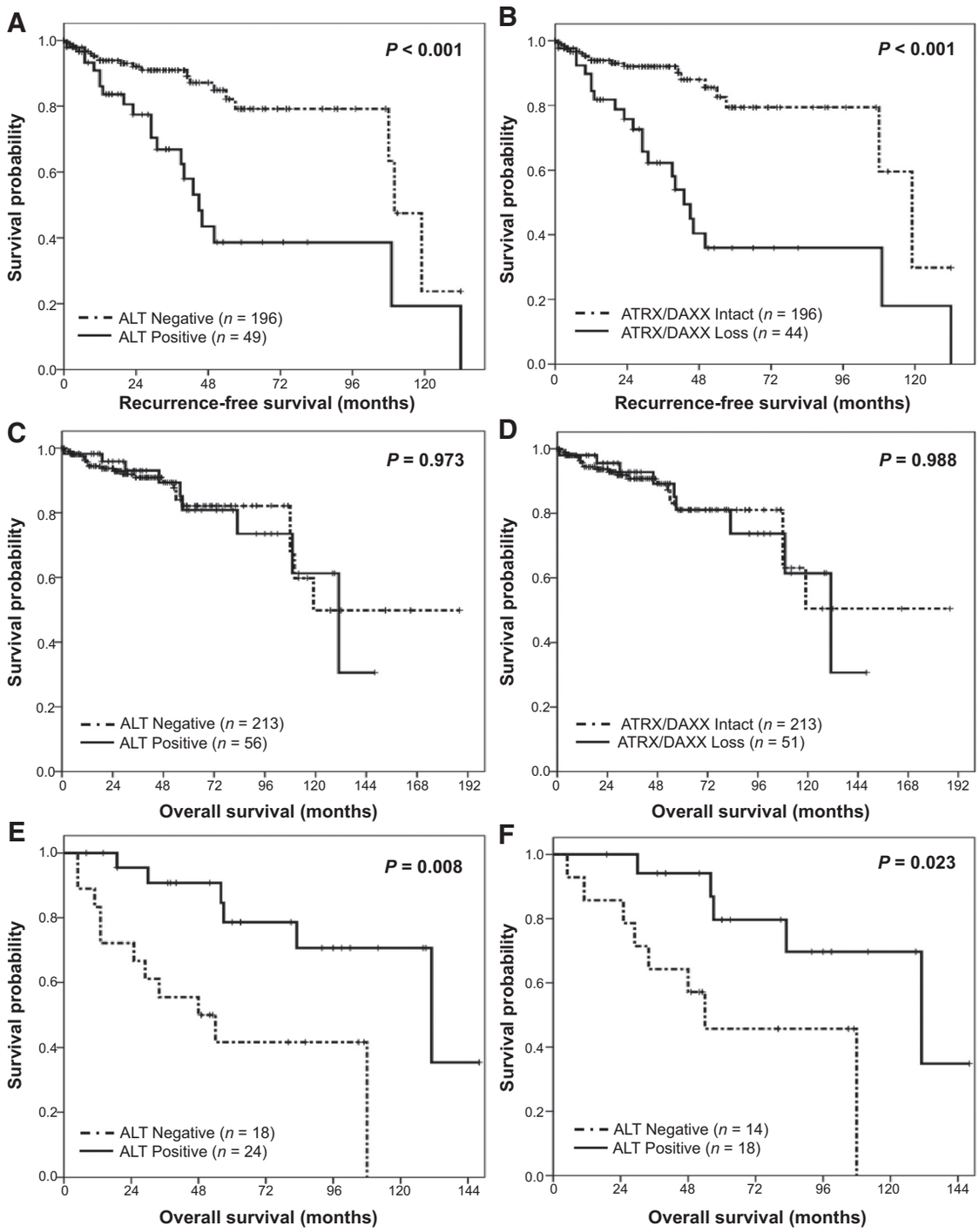


**Figure 3.**

Kaplan–Meier survival analyses. **A,** The recurrence-free survival of ALT-positive primary PanNET patients was significantly worse than those with ALT negative (5-year survival rate, 38.7% vs. 79.2%; $P < 0.001$). **B,** Primary PanNET patients with nuclear ATRX or DAXX loss had worse recurrence-free survival than those with intact ATRX and DAXX expression (5-year survival rate, 36.0% vs. 79.4%; $P < 0.001$). **C,** The overall survival in patients with ALT-positive primary PanNETs was not significantly different from those with ALT-negative tumors (5-year survival rate, 80.8% vs. 82.1%; $P = 0.973$). **D,** The overall survival in primary PanNET patients with ATRX or DAXX loss was not significantly different from those with ALT-negative tumors (5-year survival rate, 81.1% vs. 81.1%; $P = 0.988$). **E,** Synchronous and metachronous metastatic PanNET patients with ALT-positive tumors had significantly better overall survival time than those with ALT-negative tumors (5-year survival rate, 78.6% vs. 41.7%; $P = 0.008$). **F,** In metachronous metastatic PanNETs, the overall survival rate in metastatic PanNET patients with ALT positivity was significantly better than those with ALT negativity (5-year survival rate 79.6% vs. 45.7%; $P = 0.023$).

**Table 2.** Recurrence-free survival in M0 PanNET patients

| | | | Univariate analysis ($n = 245$) | | Multivariate analysis | |
|---|---|---|---|---|---|---|
| **Clinicopathologic factors** | | **No.** | **HR (95% CI)** | ***P*** | **HR (95% CI)** | ***P*** |
| ALT | Negative | 196 | 1.00 | | 1.00 | |
| | Positive | 49 | 3.38 (1.83–6.27) | <0.001[a] | 1.97 (1.02–3.78) | 0.043[a] |
| ATRX/DAXX | Intact | 196 | 1.00 | | | |
| | Loss | 44 | 4.01 (2.14–7.50) | <0.001[a] | | |
| Age | ≤60 | 173 | 1.00 | | 1.00 | |
| | >60 | 72 | 1.95 (1.03–3.72) | 0.042[a] | 2.28 (1.18–4.41) | 0.014[a] |
| Sex | Male | 114 | 1.00 | | | |
| | Female | 131 | 0.93 (0.50–1.73) | 0.814 | | |
| Grade | G1 | 173 | 1.00 | | 1.00 | |
| | G2 or G3 | 72 | 7.47 (3.60–15.50) | <0.001[a] | 5.49 (2.46–12.24) | <0.001[a] |
| Ki67 index | <3% | 183 | 1.00 | | | |
| | ≥3% | 62 | 5.47 (2.72–10.99) | <0.001[a] | | |
| Size | ≤2 cm | 114 | 1.00 | | 1.00 | |
| | >2 cm | 131 | 2.37 (1.16–4.86) | 0.018[a] | 1.29 (0.55–3.04) | 0.558 |
| pT classification | pT1 | 108 | 1.00 | | 1.00 | |
| | pT2–4 | 137 | 2.25 (1.10–4.62) | 0.026[a] | 0.38 (0.05–2.75) | 0.339 |
| Lymphovascular invasion | Absence | 187 | 1.00 | | 1.00 | |
| | Presence | 58 | 2.38 (1.26–4.49) | 0.007[a] | 1.51 (0.74–3.07) | 0.255 |
| Perineural invasion | Absence | 219 | 1.00 | | 1.00 | |
| | Presence | 26 | 2.86 (1.30–6.28) | 0.009[a] | 1.42 (0.49–4.07) | 0.518 |
| Lymph node metastasis | Absence | 220 | 1.00 | | 1.00 | |
| | Presence | 25 | 4.86 (2.37–9.95) | <0.001[a] | 2.04 (0.94–4.43) | 0.071 |

[a]Statistically significant at $P < 0.05$.

### Subgroup survival analysis according to clinicopathologic factors

Next, subgroup analyses based on clinicopathologic factors were performed. ALT-positive PanNET patients with lymph node metastasis had a tendency of better survival than ALT-negative PanNET patients with nodal metastasis (HR = 0.31; 95% CI, 0.08–1.19; $P = 0.088$; Supplementary Fig. S1A). In addition, ALT-positive PanNET patients with distant metastasis (overall 5-year survival rate, 75.0%) trended toward better survival than ALT-negative patients (25.0%), but this association was not statistically significant ($P = 0.118$; Supplementary Fig. S1B).

**Table 3.** Overall survival in PanNET patients with synchronous and metachronous metastasis

| **Clinicopathologic factors** | | **No.** | **HR (95% CI)** | ***P*** |
|---|---|---|---|---|
| ALT | Negative | 18 | 1.00 | |
| | Positive | 24 | 0.23 (0.08–0.68) | 0.008[a] |
| ATRX/DAXX | Intact | 17 | 1.00 | |
| | Loss | 25 | 0.20 (0.07–0.59) | 0.003[a] |
| Age (years) | ≤60 | 29 | 1.00 | |
| | >60 | 13 | 2.14 (0.82–5.60) | 0.121 |
| Sex | Male | 24 | 1.00 | |
| | Female | 18 | 1.38 (0.53–3.59) | 0.506 |
| Grade | Grade 1 | 14 | 1.00 | |
| | Grade 2 or 3 | 28 | 2.28 (0.72–7.17) | 0.160 |
| Ki67 index | <3% | 20 | 1.00 | |
| | ≥3% | 22 | 2.41 (0.86–6.72) | 0.094 |
| Size | ≤2 cm | 3 | 1.00 | |
| | >2 cm | 39 | 0.85 (0.11–6.54) | 0.880 |
| pT classification | pT1 | 4 | 1.00 | |
| | pT2–pT4 | 38 | 1.37 (0.18–10.48) | 0.762 |
| Lymphovascular invasion | Absence | 17 | 1.00 | |
| | Presence | 25 | 1.10 (0.41–2.99) | 0.850 |
| Perineural invasion | Absence | 29 | 1.00 | |
| | Presence | 13 | 1.47 (0.50–4.28) | 0.484 |
| Lymph node metastasis | Absence | 23 | 1.00 | |
| | Presence | 19 | 1.89 (0.72–4.97) | 0.200 |

[a]Statistically significant at $P < 0.05$.

## Discussion

In most human cancers, neoplastic cells can proliferate unlimitedly by maintaining their telomere length via activating the enzyme telomerase; however, 5% to 10% of human malignancies activate the ALT pathway to maintain telomere lengths in neoplastic cells (24). ALT is a telomerase-independent mechanism to maintain telomere lengths that is thought to be mediated through homologous recombination (20). This process gives rise to dramatic nuclear foci containing large amounts of telomere DNA and significant telomere length heterogeneity within the neoplastic cells, features easily detected by telomere-specific FISH in archived specimens. ALT activation is closely associated with inactivating mutations in the *ATRX* and *DAXX* genes, which encode chromatin remodeling proteins required for incorporation of histone H3.3 at the telomeres (14, 15, 21).

Overall, in this Korean population, ALT activation was identified in 20.8% of primary PanNETs. Interestingly, this prevalence is considerably lower than that of the previous studies in Western populations (48%–61%; refs. 15, 21, 24) and in a Chinese population (54.1%; ref. 32). However, the prevalence of ALT in PanNETs in the current study was similar to that of the previous Korean study assessing a smaller number of PanNETs (15%, 7/47 cases; ref. 23). Combining the results of the current study with those from the previous reports, we hypothesize that differences in ALT prevalence exist among Western, Chinese, and Korean populations.

In the current study, ALT-positive primary PanNETs were associated with larger tumor size and higher pT classification, and ALT was not observed in any of the 19 neuroendocrine microadenomas. Strikingly, the prevalence of ALT dramatically increased with tumor size, suggesting that ALT activation occurs as a late event in PanNET tumorigenesis. These results are in concordance with those from de Wilde and colleagues who assessed neuroendocrine microadenomas and PanNETs from MEN-1 syndrome patients.

The authors reported intact nuclear ATRX and DAXX expression and no ALT positivity in all 47 neuroendocrine microadenomas; however, loss of nuclear ATRX or DAXX expression was only observed in a subset of large (>3 cm) PanNETs (31). Similarly, Marinoni and colleagues reported only 14.2% of small tumors (<2 cm) with loss of ATRX or DAXX expression were ALT positive, whereas larger tumors (>2 cm) or metastatic PanNETs showed an almost perfect correlation between loss of ATRX or DAXX expression and ALT positivity (21). In addition to these findings, we also observed that 6 ALT-positive PanNETs retained nuclear ATRX and DAXX expression. These findings may result from genetic alterations in *ATRX* or *DAXX* that confer a loss of function, but not loss of nuclear protein expression. Conversely, it is possible that additional drivers of ALT in PanNETs have not yet been identified. Taken together, we conclude that loss of ATRX or DAXX expression and activation of ALT occur as a relatively late event in PanNET tumorigenesis.

We also observed that ALT-positive PanNETs displayed aggressive behavior, such as higher grade, presence of lymphovascular and perineural invasion, and lymph node and distant metastasis. Previously, PanNET patients with insulin, GLP-1, and multiple hormone expression were reported to have a better survival than those without insulin, GLP-1, and multiple hormonal expression (29). In the current study, ALT-positive PanNETs correlated with a lack of insulin, GLP-1, and multiple hormone expression, which were previously associated as poor prognostic factors in PanNET patients.

Previous studies evaluating the prognostic effect of ALT and ATRX or DAXX alterations in PanNETs are contradictory. Jiao and colleagues and Dogeas and colleagues both reported that PanNET patients with ALT-positive or ATRX- or DAXX-altered tumors had better clinical outcomes (14, 24). In contrast, in a larger study, Marinoni and colleagues concluded that PanNET patients with ALT activation and loss of ATRX or DAXX displayed a worse prognosis (21). The discrepancy between previous studies could be explained by different study populations, as was indicated in Marinoni and colleagues' study (21). In the previous studies of Jiao and colleagues and Dogeas and colleagues, all patients were metastatic, and most of them had higher pT classification (pT3 or pT4; refs. 14, 24). In contrast, most of the patients included in Marinoni and colleagues' study were pT1 and pT2 and nonmetastatic primary PanNETs (21). Our observations herein may help explain these apparent discrepancies. First, we observed a worse recurrence-free survival in patients with ALT-positive primary PanNETs, which confirms the findings by Marinoni and colleagues. Second, we also observed that ALT activation in primary PanNETs was significantly correlated with better overall survival when patients developed metastasis. This result is in agreement with those described by Jiao and colleagues and Dogeas and colleagues, who both observed that PanNET patients with ALT-positive or *ATRX*- or *DAXX*-mutated liver metastases had a longer survival time (14, 24). Our observations confirm the prognostic significance of ALT and ATRX or DAXX status with those of the previous studies. The presence of ALT was independently predictive of a worse prognosis in primary PanNETs and of a better prognosis in metastatic PanNETs. Thus, these results suggest that ALT may be used as a potential prognostic marker depending on the context of the disease. Possible explanations for this observation include potential differences in treatment response or that once ALT-positive clones establish as distant metastatic foci, these clones may grow more slowly in the new microenvironment. However, the exact molecular mechanism underlying ALT activation has not been elucidated and warrants further study.

Given the relatively large proportion of PanNETs that display the ALT phenotype and have ATRX or DAXX loss, the identification of therapies that target ALT-positive (or ATRX/DAXX-null) cancers would be tremendous for the field. Previous *in vitro* work has indicated that ALT-positive cancer cells are sensitive to inhibition of the DNA damage mediator, ATR (25), whereas recent data suggest that ATRX loss sensitizes mouse-derived glioma cells to radiation and topoisomerase inhibitors (26). The data presented herein indicate that both ATRX or DAXX loss and ALT are effective prognostic biomarkers. Further study will elucidate whether ATRX or DAXX loss and/or the presence of ALT can predict response to therapy in PanNETs.

The current study has several strengths. We were able to analyze a large, well-characterized cohort of primary PanNET patients with detailed clinical and pathologic information. Recurrence-free survival was calculated for PanNETs without metastasis, thus making a more precise estimation of the effect of ALT on PanNET patients without metastatic disease. In addition, the telomere-specific FISH assay used to detect ALT was performed with the same methodology as some of the previous studies, thus facilitating direct comparison of the prevalence of ALT between different populations. However, this study also has some limitations. We report the ALT status in primary PanNET tissues only; clinical data provided the status of synchronous or metachronous metastases. We did not directly compare matched primary and metastatic PanNET tissues. In addition, the median follow-up period of 28 months and the limited number of G3 tumors may not be adequate for complete evaluation of the biology of patients with PanNETs; thus, it would be informative to investigate these observations in cohorts with more extended follow-up information. Furthermore, the use of TMAs for our studies limits our ability to assess potential intratumoral heterogeneity. Finally, our cohort represents a population from a single center with a homogeneous ethnic background.

In conclusion, ALT activation and loss of ATRX or DAXX expression occurs at a relatively late stage of tumor progression. In our cohort, we have demonstrated that ALT-positive primary PanNETs display aggressive clinicopathologic behavior and have poor recurrence-free survival compared with their ALT-negative counterparts. In contrast, ALT activation is associated with better survival in patients with metastatic disease. In summary, ALT or loss of ATRX or DAXX expression may be used as potential prognostic markers depending on the context of the disease and may be eventually used to identify patients who may benefit from targeted therapies.

## Disclosure of Potential Conflicts of Interest

R.H. Hruban reports receiving authorship compensation from UpToDate. No potential conflicts of interest were disclosed by the other authors.

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