Role for loss of nuclear PTEN in a harbinger of brain metastases

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Abstract

Background: Earlier studies proposed phosphatase and tensin homolog (PTEN) acts as a 3'-specific phosphatidylinositol phosphatase and inhibits the PI3K pathway. Recent reports show that PTEN mRNA expression is significantly downregulated in brain metastases compared to primary breast cancer. We focused on the differential expression of nuclear and cytoplasmic PTEN between primary tumors and brain metastases.

Materials and Methods: We retrospectively studied 30 patients with histologically confirmed primary tumors and brain metastases. PTEN and PDK1 expression levels were examined by immunohistochemical staining and categorized as negative, positive, or strong positive expression. The difference in PTEN expression levels were compared, and the values with $P<0.05$ were considered statistically significant.

Results: Expression of cytoplasmic PTEN was 100% at primary site, and 70% at brain metastases. Expression of nuclear PTEN was 87% at primary site, and 20% at brain metastases. Study results demonstrated that PTEN expression levels in brain metastases are lower compared with that of primary tumors. Especially, nuclear PTEN expression was significantly downregulated in various brain metastases. Higher PDK1 expression at brain metastases also confirmed the down regulation of PTEN function.

Conclusions: Our findings indicate that decreased PTEN function by loss of nuclear PTEN expression may be associated with brain metastases.

Key words: brain metastasis, PTEN, subcellular localization, PDK1

Highlights

- The subcellular localization of PTEN in brain metastasis and primary cancers was examined.
- PTEN expression levels in brain metastases are lower compared with that of primary site
- Nuclear PTEN expression was significantly downregulated in various brain metastases.
- PDK1 expression was inversely associated with PTEN expression.

Introduction

Brain metastases are the most common type of brain tumors. With the improved life expectancy and increased survival of patients with systemic diseases, the incidence of the most common cancers (lung, breast, renal, colon and melanoma) is thought to be rising. Autopsy data show that the frequency of brain metastases varies from 20 to 50%. Improvement in the life expectancy of cancer patients with the development of cancer therapy, results in the increased incidence of brain metastases. This signifies the importance to improve intracranial therapy as well. Most brain metastases originate from lung cancer (40-50%), breast cancer (15-25%), malignant melanoma (5-20%) and renal cell carcinoma (5-10%). Surgery along with adjuvant radiation therapy and radiosurgery is the mainstay of treatment, while molecular target therapies, combined with advances in the evaluation of brain metastases for targetable mutations such as epidermal growth factor receptor (EGFR) [1] or anaplastic lymphoma kinase (ALK) [2] driver mutation positive non-small cell lung cancer (NSCLC), $BRAF^{V600E}$ expressing melanoma [3], and human epidermal growth factor receptor type2 (HER2) expressing breast cancer [4], are showing increased efficacy [5].

The role of activation of EGFR/HER2-phosphoinositide 3-kinase (PI3K)-Akt pathway in brain metastases has been reported [6]**.** Phosphatase and tensin homologue deleted on

chromosome 10 (PTEN), a lipid and protein phosphatase, is a well-known tumor suppressor acting through its ability to counter the activity of PI3K and reduce phosphorylation and activation of the kinase Akt. Recent report mentioned that the rate of PTEN mutations were higher in brain metastasis than primary cancer site in breast cancer [7].

Although some recent reports have shown the importance of PTEN function and its subcellular localization, definitive role of PTEN in brain metastasis has not yet been determined. In this study, we focused on the expression and subcellular localization of PTEN in brain metastasis and primary cancers. We found that PTEN inactivation via its nuclear localization was associated with brain metastasis.

Materials and methods

Clinical data and patient selection

This retrospective study was approved by our institutional review board. We included a total of 30 patients who were pathologically confirmed to have both primary cancers and brain metastases. These patients underwent surgical interventions at our institution between March 1997 and March 2015. Primary tumors comprised of gastric, esophageal, lung, colorectal, breast, ovarian, cervical, adrenocortical, liver, and prostate cancer. We

also included recurrence and relapse cases after radiation therapy (3 patients underwent stereotactic irradiation, 1 patient underwent whole-brain radiotherapy plus stereotactic irradiation). Patient records were reviewed for clinical events, including development and duration of brain metastases.

Tissue specimens and immunohistochemical staining

All tumor specimens were fixed in 10% phosphate-buffered formalin before embedding in paraffin. Representative slides were stained with the hematoxylin–eosin reagent for standard histological diagnosis. Tumors were histologically confirmed by consensus of two authors (Y.T. and V.A.). Paraffin-embedded tumor specimens were used for immunohistochemical (IHC) staining as described previously [8]. Diluted mouse monoclonal (dilution 1:100) antibodies for PTEN (antibody PTEN; Dako, Glostrup, Denmark) and rabbit polyclonal (dilution 1:200) antibodies for PDK1 (antibody PDK1; OriGene Technologies, Inc., Rockville, USA) were employed as primary antibodies. Pathological specimens (thickness 4 μm) were mounted on gelatin-coated slides and deparaffinized by xylene treatment for 15-min. To prevent digestion by endogenous peroxidase, the slides were immersed for 30 min in hydrogen peroxidase (3%)/ phosphate-buffered saline (PBS; pH 7.5) mixture. Each specimen was rinsed three times

for a total of 15 min in PBS with gentle stirring before overnight incubation with the primary antibodies at 4℃. The streptavidin–biotin procedure was performed next using the histofine SAB® kit (Nichirei Co., Tokyo, Japan). After washing thoroughly in PBS, the sectioned specimens were exposed to tetrahydrochloride (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in hydrogen peroxidase (0.003%)-treated PBS (pH 7.6) for 7 min. To facilitate visualization of cytoplasmic immunostaining, the slides were counterstained with Mayer hematoxylin. Each series of experiments with controls was performed at least twice on different days.

Evaluation of PTEN &PDK1 expression

The specimens were scored by consensus of two other authors (F.Y. and T.T.), who had no knowledge of the pathological diagnosis or any clinical or radiological data. The positively identified tumor cells count (%) was recorded for each specimen. Grading of PTEN expression levels was performed for each cytoplasm and nucleus as follows: no or negative (no cells stained), positive (up to 50% of cells stained), and strong positive (50–100% of cells stained). Grading of cytoplasmic PDK1 expression levels was performed in the same manner. Examples of the PTEN and PDK-1 expression levels are shown in Fig. 1.

Statistical analysis

Statistical analyses were performed using SPSS 16.0 software (SPSS Inc., Chicago, IL) for Windows. Differences in level of expression of the PTEN and PDK-1 between primary tumors and brain metastases were compared using chi-square test. In all analyses, differences with $P<0.05$ were considered statistically significant.

Results

Clinical characteristics

Clinical characteristics of the 30-patients are shown in Table 1. The brain metastases were derived from lung (n=8), colorectal (n=7), breast (n=6), esophageal (n=3), gastric (n=1), ovarian (n=1), cervical (n=1), prostate (n=1), adrenocortical (n=1), and liver (n=1) cancers. Of the 30 patients, 19 had a single brain metastasis, whereas the rest of the cases had multiple brain metastases diagnosed radiographically, or surgically. The ratio of female (n=16) to male (n=14) patients was 1.1:1. The median age (in years) of patients at the time of diagnosis of primary cancer was 59 [range 35−80, standard deviation (SD) 10.6, average 57] years and at the time of brain metastasis was 60 [range 35−84, SD 10.8,

average 59]. The median duration of metastases (months) was 24 [range 0−115, SD 25.5, average 29].

Immunohistochemical results

The results of immunohistochemical staining are summarized in Table 2.

First we evaluated the expression level of cytoplasmic PTEN. All primary tumors expressed cytoplasmic PTEN at primary cancer site, while the expression was observed in 70% of brain metastases (Fig. 2a).

Second, we examined the expression level of nuclear PTEN. Expression of nuclear PTEN was observed in 87% at primary cancer site, while that was 20% at brain metastasis. Both cytoplasmic and nuclear PTEN expression levels were lower in brain metastases compared with primary tumors ($P<0.05$, Fig. 2a). Especially, nuclear PTEN expression level was significantly downregulated in brain metastasis ($P<0.0001$).

We then analyzed the expression level of PDK-1, a downstream molecule in PI3K-AKT pathway. In our results, PDK-1 expression was observed in 70% of primary cancer site, while it was detected in all of brain metastases ($P<0.05$). PDK1 expression was inversely associated with PTEN expression (Fig. 2a).

Finally, we evaluated the primary cancer site individually (Fig. 2b-e). We confirmed that at each primary cancer site, PTEN expression levels are lower in brain metastases compared with primary tumors. Representative case is presented in Fig. 3.

Discussion

In this study, we showed that PTEN expression levels are lower in brain metastases compared with that of primary tumors not only in breast cancer as reported previously [7], but also in other kind of tumors. Furthermore, we revealed that nuclear PTEN expression level was significantly downregulated in various brain metastases compared to primary tumors. Moreover, PDK1 was upregulated in brain metastases compared to primary tumors, which could be associated with downregulation of PTEN function. This is the first study that focused on the subcellular localization of PTEN in both brain metastases and primary tumors, and our results imply that PTEN inactivation via its cytoplasmic sequestration is important for brain metastasis.

PTEN, a tumor suppressor, is mainly involved in the homeostatic maintenance of the phosphatidylinositol 3 kinase (PI3K)/AKT cascade. PI3K converts the lipid second messenger phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-trisphosphate (PIP3). PIP3 activates PDK1 which in turn activates AKT via

phosphorylation at Thr308. PTEN blocks the PI3K-AKT pathway by dephosphorylating PIP3 to PIP2. PTEN function is commonly lost or decrease in many kinds of human cancers through somatic mutations, gene silencing, or epigenetic mechanisms including methylation, micro-RNA, or pseudo gene expression, and/or protein phosphorylation [9-12].

PTEN was isolated by mapping homozygous deletions on human chromosome 10q23 from glioblastoma, prostate, and breast cancer cell lines [13-15]. Subsequently, a series of mutations in PTEN were identified in sporadic tumors and cancer cell lines from various tissues including brain, endometrium, prostate, breast, kidney, liver, and melanoma [10, 16-19]. In all these tumors, PTEN was mutated with a higher frequency in glioblastoma, advanced stages of prostate cancers, and in all stages of endometrial cancers [16, 17, 20]. Furthermore, PTEN loss or mutation in inherited cancer predisposing syndromes (e.g. Cowden disease [21], Bannayan-Zonana syndrome [22], and Proteus syndrome [23]) has been reported.

Loss of PTEN was observed in 25-71% of breast cancer brain metastases compared to unmatched primary breast cancers. Genetic alterations of PTEN were especially common in brain metastases from triple-negative and HER2-negative breast cancers. The discrepancy in detection rate of overall loss of heterozygosity (LOH) (50%) and PTEN

mutation (14%) in metastatic cancer suggests the presence of one or more additional tumor suppressor genes [24]. Recent study with whole exome sequencing of tumor tissues from matched brain metastases and primary tumors could detect clinically common alterations of matched primary tumor samples in only 47% cases of brain metastases. This suggests that the tumor cells of brain metastases are genetically and mutationally different from primary tumor cells [25]. The same study reported that the rate of PTEN mutation is 8% in primary tumors, 9% in brain metastases, and other common alterations are 66%, thus implying that the dysfunction of PTEN is predominantly due to loss of PTEN function rather than PTEN mutation. In addition, emerging evidence shows that PTEN gene/protein is quantitatively related to tumorigenesis as partial loss of PTEN function is sufficient to promote malignancy and worsen tumor grade. Our data confirmed that loss of nuclear PTEN, which is indicative of decrease in PTEN function, worsens tumor grade in the form of metastasis to brain.

Studies have reported that nuclear PTEN maintains genomic stability and DNA repair, mediated at least in part by PI3K-independent mechanisms, and depends on physical interaction with nuclear target proteins, such as the tumor suppressor p53 or the oncoprotein MSP58/MCRS1 [26]. Cells lacking nuclear PTEN were hypersensitive to DNA damage, whereas PTEN-deficient cells were susceptible to killing by a combination

of genotoxic stress and a small-molecule PI3K inhibitor, both in vitro and in vivo [27]. Various molecular mechanisms of PTEN nuclear localization have been described; acetylation/deacetylation by histone acetyltransferases (HATs; such as PCAF) and deacetylases (HDACs), ubiquitination by neural precursor cell expressed developmentally down-regulated protein 4-1 (NEDD4-1), or other post-translational modifications regulating specific functions of nuclear PTEN [28-30]. The absence of nuclear PTEN is associated with more aggressive cancers, and our data demonstrating loss of nuclear PTEN in brain metastases is consistent with these findings.

PDK1 is a cytoplasmic kinase that functions as a component of the PI3K pathway which is one of the most important pathways in cancer metabolism and growth [31]. Loss of PTEN function results in unregulated activation of PI3K signaling, including PDK1 [32]. High expression of PDK1 has been detected in various invasive cancers, e.g. breast cancer [33], lung cancer [34] and, esophageal cancer [35]. In breast cancer and esophageal squamous cell cancer, role of increased PDK1 expression in the metastatic process has been mentioned [36, 37]. Our results showed that PDK1 is detected at a higher frequency in brain metastases than in matched primary cancers, and is inversely correlated with PTEN expression. Overexpression of PDK1 in brain metastases, therefore, confirms the

inactivation of PTEN function via its cytoplasmic sequestration which is an important harbinger for brain metastasis.

This study has the following limitations: (1) the sample comprised of 30 patients with brain metastases warranting further study with larger patient populations (2) our data did not include PTEN genome sequence (3) detailed mechanism of cytoplasmic subcellular localization of PTEN was not investigated and requires future studies to elucidate its mechanisms (4) this study being a retrospective one, entails a prospective study to confirm our present findings. Future larger study inclusive of clinical data about prognosis and time to brain metastasis may be necessary.

Conclusions

We have demonstrated that loss of nuclear PTEN expression and activation of PDK1 were observed in brain metastases compared to primary cancer sites. Our findings imply that decreased PTEN function by loss of nuclear PTEN expression may be associated with brain metastases.

Acknowledgement

This study was partly supported by the Japan Society for the Promotion of Science Grant-in-Aid for Scientific Research (C) number 16K10757.

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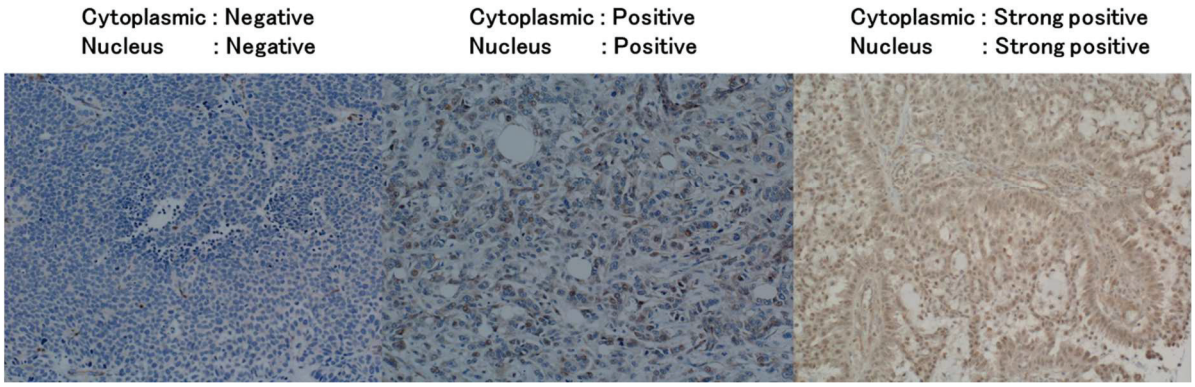
Fig. 1. Examples of the PTEN and PDK-1 expression levels

PTEN



PDK-1

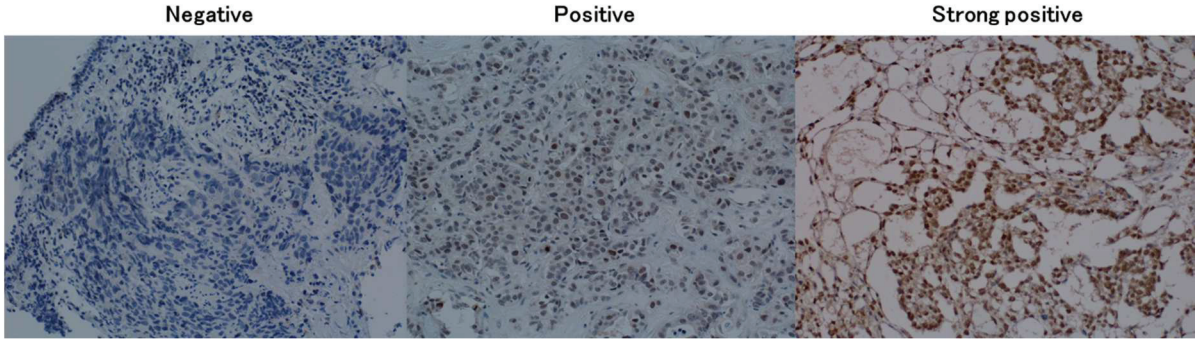


Table 1. Clinical characteristics of patients case series.

	Gender		Age (y) ; range (mean ± SD)		Duration from primary to first detection of METs (m) ; range (mean ± SD)	Number of METs	
	male	female	Origin	METs		Single	Multiple
All (n=30)	14 (46.7%)	16 (53.3%)	35-80 (57 ± 10.6)	35-84 (59 ± 10.8)	0-115 (29 ± 25.5)	19 (63.3%)	11 (36.7%)
Lung (n=8)	3 (37.5%)	5 (62.5%)	48-72 (62 ± 7.1)	48-73 (63 ± 7.5)	0-68 (20 ± 21.6)	6 (75%)	2 (25%)
Breast (n=6)	0 (0%)	6 (100%)	48-80 (56 ± 11.4)	51-84 (60 ± 11.3)	22-115 (52 ± 30.2)	2 (33.3%)	4 (66.7%)
Colorectum (n=7)	5 (71.4%)	2 (28.6%)	47-73 (58 ± 8.6)	48-74 (60 ± 7.9)	4-74 (29 ± 22.0)	5 (71.4%)	2 (28.6%)
Others (n=9)	6 (66.7%)	3 (33.3%)	35-75 (53 ± 12.2)	35-77 (54 ± 12.9)	0-50 (21 ± 17.4)	6 (66.7%)	3 (33.3%)

Table 2. Pathological results of patients case series.

		Cytoplasmic PTEN			Nuclear PTEN			PDK-1		
		Negative	Positive	Strong positive	Negative	Positive	Strong positive	Negative	Positive	Strong positive
All (n=30)	Origin	0 (0%)	14 (47%)	16 (53%)	4 (13%)	17 (57%)	9 (30%)	9 (30%)	11 (37%)	10 (33%)
	METs	9 (30%)	17 (57%)	4 (13%)	24 (80%)	4 (13%)	2 (7%)	0 (0%)	11 (37%)	19 (63%)
Lung (n=8)	Origin	0 (0%)	3 (37%)	5 (63%)	1 (13%)	4 (50%)	3 (37%)	2 (25%)	3 (37%)	3 (37%)
	METs	2 (25%)	4 (50%)	2 (25%)	7 (87%)	0 (0%)	1 (13%)	0 (0%)	2 (25%)	6 (75%)
Breast (n=6)	Origin	0 (0%)	2 (33%)	4 (67%)	1 (17%)	3 (50%)	2 (33%)	2 (33%)	2 (33%)	2 (33%)
	METs	2 (33%)	4 (67%)	0 (0%)	5 (83%)	1 (17%)	0 (0%)	0 (0%)	2 (33%)	4 (67%)
Colorectum (n=7)	Origin	0 (0%)	4 (57%)	3 (43%)	1 (14%)	4 (57%)	2 (29%)	1 (14%)	3 (43%)	3 (43%)
	METs	3 (43%)	4 (57%)	0 (0%)	6 (86%)	1 (14%)	0 (0%)	0 (0%)	2 (29%)	5 (71%)
Others (n=9)	Origin	0 (0%)	5 (56%)	4 (44%)	1 (11%)	6 (67%)	2 (22%)	4 (44%)	3 (33%)	2 (22%)
	METs	2 (22%)	5 (56%)	2 (22%)	6 (67%)	2 (22%)	1 (11%)	0 (0%)	5 (56%)	4 (44%)

Fig. 2a. Results of the PTEN and PDK-1 expression levels in all cancers.

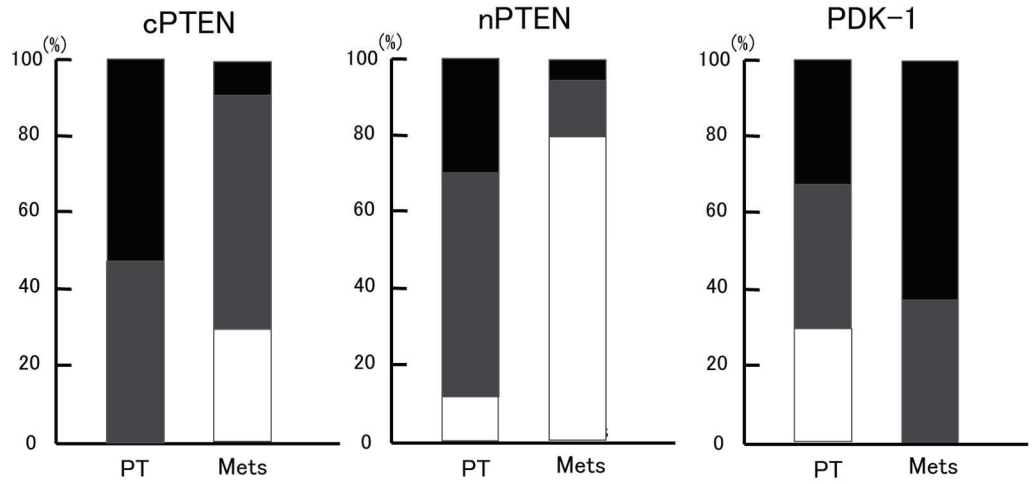


Fig. 2b. Results of the PTEN and PDK-1 expression levels in lung cancer.

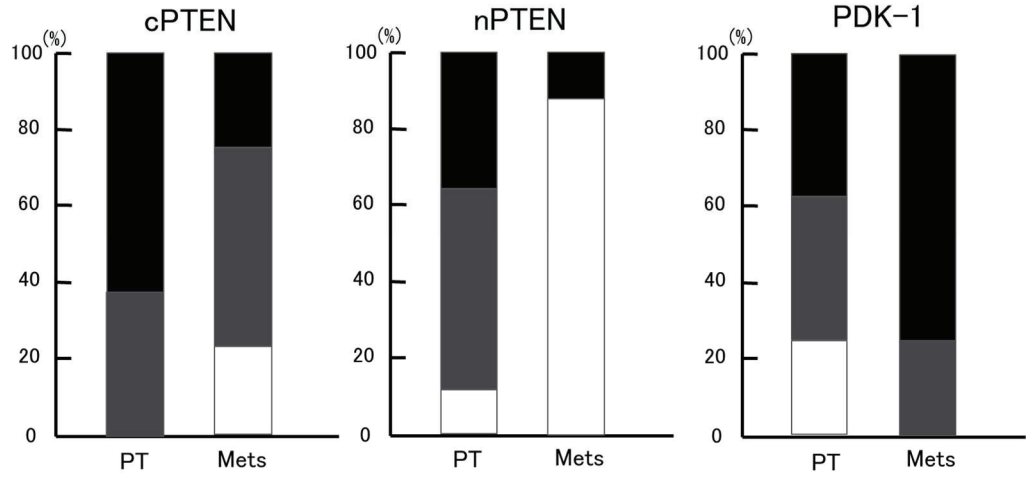


Fig. 2c. Results of the PTEN and PDK-1 expression levels in breast cancer.

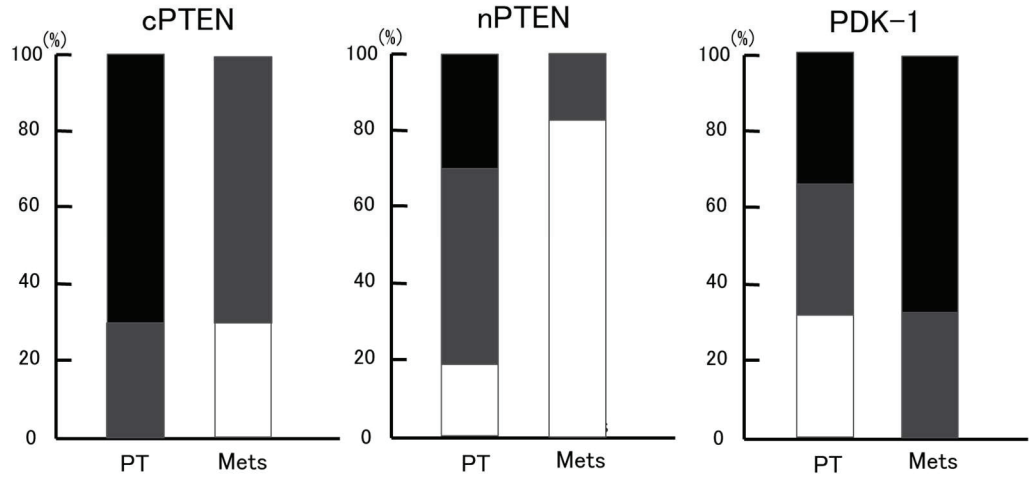


Fig. 2d. Results of the PTEN and PDK-1 expression levels in colorectal cancer.

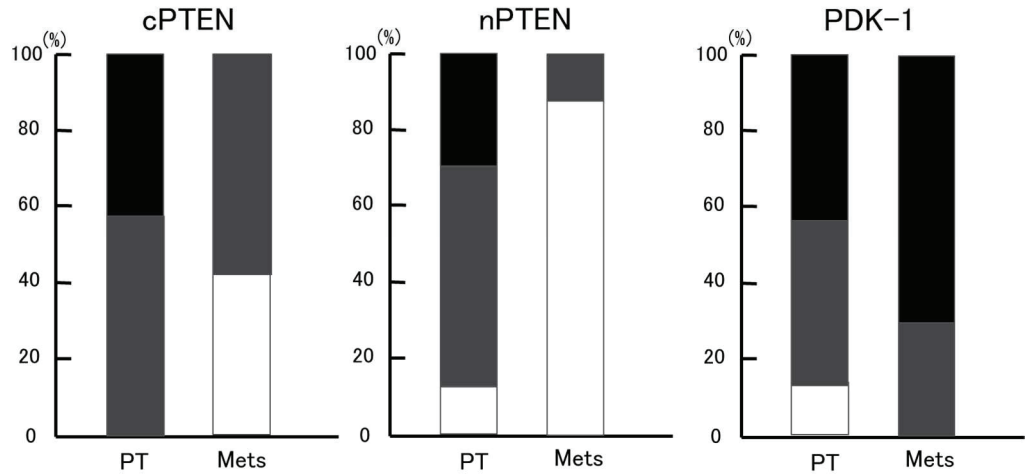


Fig. 2e. Results of the PTEN and PDK-1 expression levels in other cancers.

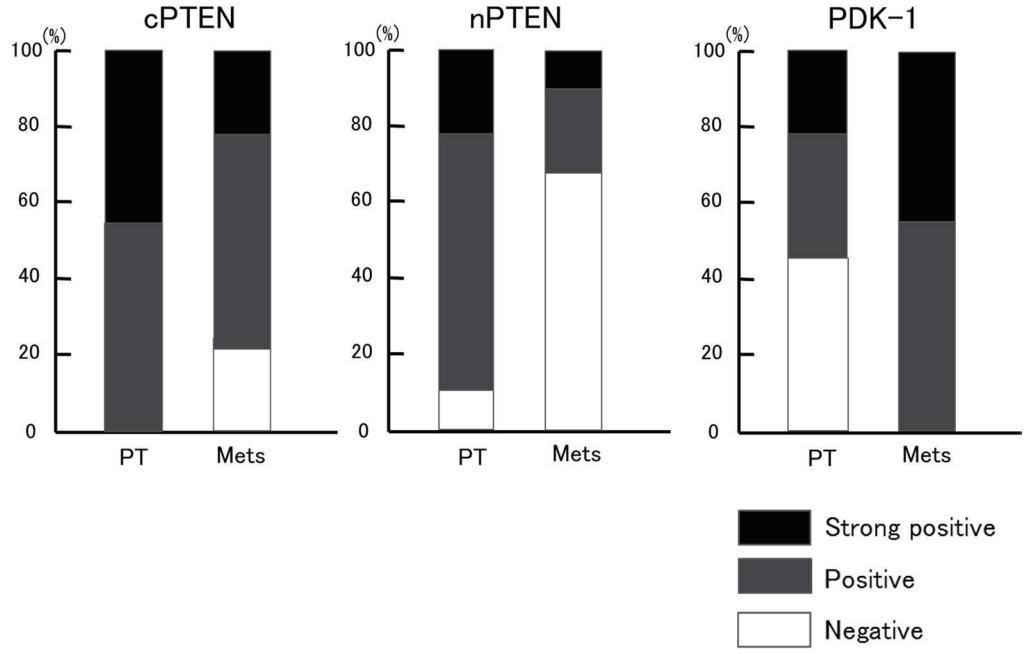
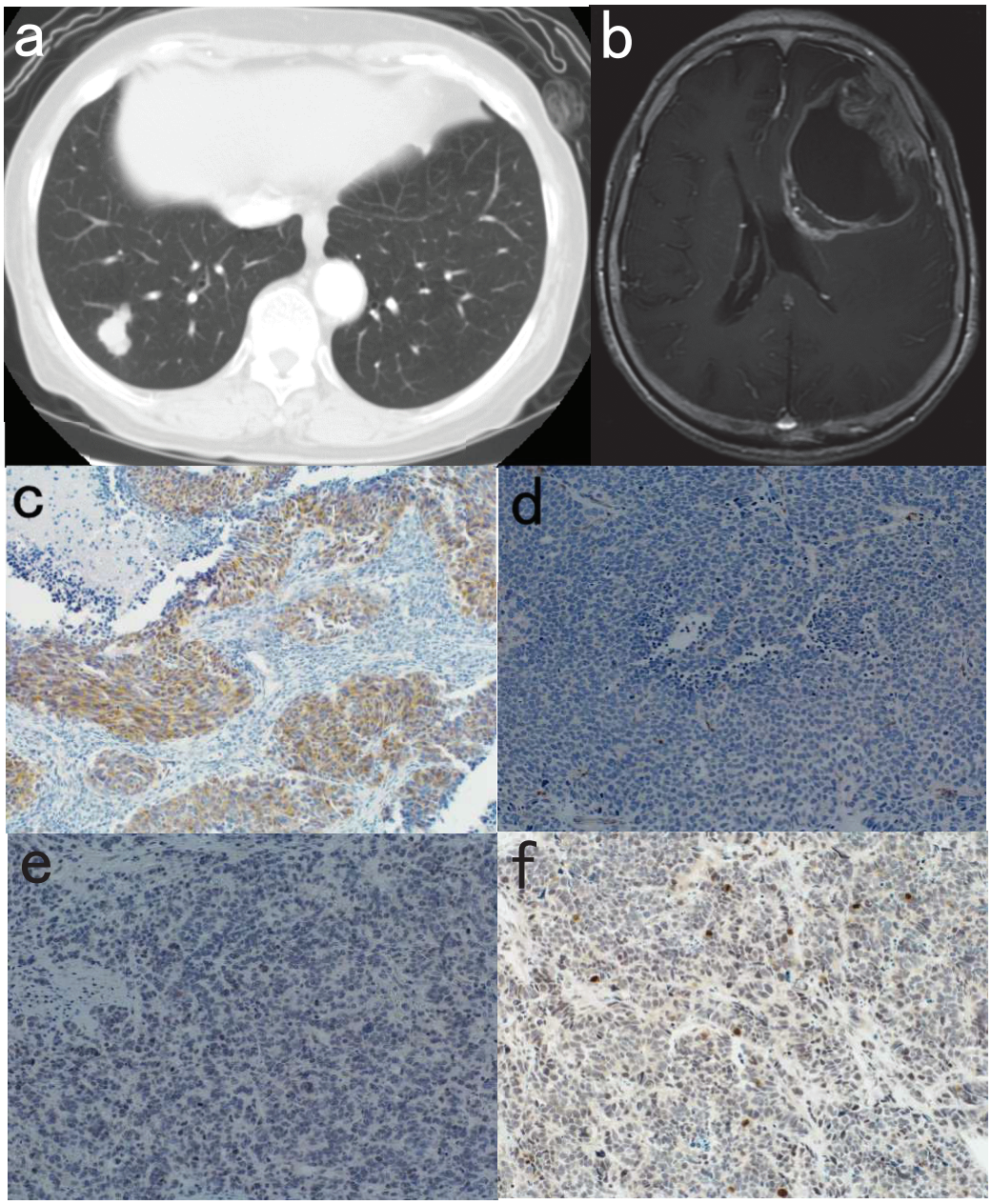


Fig. 3. Examples of the PTEN and PDK-1 expression levels in lung cancer.



72 years old female. Images of lung small cell carcinoma and left frontal lobe metastases and immunohistochemical staining of PTEN & PDK-1 (original magnification: ×200)

a. Thoracic CT image

b. MRI, T1-weighted gadolinium-enhanced image

c. origin; cytoplasmic PTEN: strong positive, nuclear PTEN: strong positive

d. METs; cytoplasmic PTEN: positive, nuclear PTEN: negative

e. origin; PDK-1: positive

f. METs; PDK-1: strong positive