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ヒト正常精巣と精巣腫瘍における癌タンパク質

ガンキリンとリン酸化 Rb タンパクの発現について

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Expression of the oncoprotein gankyrin and phosphorylated-retinoblastoma protein in human testis and testicular germ-cell tumor

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Short running title: Gankyrin expression in testicular cancer

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Abstract:

Objective: The oncoprotein, gankyrin, is known to facilitate cell proliferation through phosphorylation and degradation of retinoblastoma protein. In the present study, we evaluated the expression of gankyrin and phosphorylated retinoblastoma protein in human testis and testicular germ cell tumors.

Methods: The effects of suppression of gankyrin by locked nucleic acid on phosphorylation status of retinoblastoma and cell proliferation were analyzed using western blot analysis and testicular tumor cell line NEC8. The expressions of gankyrin, retinoblastoma and retinoblastoma protein were analyzed in 93 testicular germ cell tumor samples and five normal human testis by immunohistochemistry. The retinoblastoma protein expression was determined using an antibody to retinoblastoma protein, Ser795.

Results: Gankyrin was expressed in NEC8 cells as well as a normal human testis and testicular tumors. Suppression of gankyrin by locked nucleic acid led to suppression of retinoblastoma protein and cell proliferation in NEC8 cells. Immunohistochemistry of normal testis showed that gankyrin is expressed dominantly in spermatocytes. In testicular germ cell tumors, high expressions of gankyrin and phosphorylated-retinoblastoma protein were observed in seminoma and embryonal carcinoma, whereas the expressions of both proteins were weak in histological subtypes of non-seminoma. Growing teratoma and testicular malignant transformation tissues expressed phosphorylated-retinoblastoma protein strongly, but gankyrin faintly.

Conclusion: Gankyrin is dominantly expressed in normal spermatocytes and seminoma/ embryonal carcinoma, and its expression correlates well with retinoblastoma protein expression except in the growing teratoma and testicular malignant transformation cases. These data provide new insights into the molecular mechanisms of normal spermatogenesis and pathogenesis of testicular germ cell tumors.

Key words: gankyrin, human testis, neoplasm of testis, teratoma.

Introduction

Gankyrin was originally identified as a gene overexpressed in HCC.[1] Functionally, gankyrin regulates cell growth and apoptotic properties through binding to Rb and CDK-4.[1,2] Gankyrin also functions in regulation of p53 through binding to the ubiquitin ligase, murine double minute 2, and possesses chaperone activity for assembly of 19S proteasomes.[2,3] Overexpression of gankyrin was also observed in a variety of malignancies including esophageal squamous cell carcinoma,[4] pancreatic cancer,[5] colorectal cancer[6] and oral cancer,[7] although there was no report of its expression in normal human testis or TGCT.

In testis, spermatogenic cells actively proliferate by mitotic cell division and meiosis after puberty. TGCT is a common malignancy in young males, and arises from these spermatogenic cells. TGCT are histologically classified into two subtypes including seminoma and non-seminoma. Non-seminoma tumors are often composed of several mixed histological subtypes including embryonal carcinoma, choriocarcinoma, yolk sac tumor and also teratoma. Furthermore, teratomas derived from TGCT are known to possess potential for malignancy and to turn into a GT or TMT.[8–10] Generally, TGCT are highly sensitive to chemotherapy, and most patients are cured with systemic chemotherapy and surgery, even if they have metastatic disease. However, the 5-year overall survival rates of patients who belong to the poor-risk group of the IGCCC are

only approximately 50%.[11] The tumors of the poor-risk IGCCC group were exclusively non-seminomas.

One of the problems in the clinical setting is how to treat residual masses after chemotherapy. Residual masses sometimes contain viable tumor cells or teratoma components that are resistant to chemotherapy.[12] The prognoses of patients with viable cells in residual masses or TMT especially are highly discouraging.[8–10] Therefore, it is important to identify the molecular mechanisms underlying development or progression of TGCT to develop a novel targeted strategy. In the present study, we analyzed the expression of gankyrin in normal spermatogenesis and various histological subtypes of TGCT by IHC.

Methods

Patients and cell lines

In the present study, the immunohistochemistries of 93 surgical TGCT specimens and five normal human testes were analyzed (Table 1). All surgical procedures were carried out at the Department of Urology, University of Tsukuba Hospital, Tsukuba, Japan. Clinical information was obtained from the medical records of patients. A normal testicular tissue specimen was obtained from the orchiectomy sample of a prostate cancer patient before administering hormonal therapy, and normal spermatogenesis was confirmed by HE staining.

Among 93 TGCT specimens, 64 were from radical orchiectomy samples including 20 seminoma and 44 non-seminoma. The remaining 29 specimens were obtained from surgery for metastases, of which seven were residual viable tumor cells without teratomatous components and 22 were teratomatous tissues (19) or teratoma with malignant transformations (3). Among 19 teratomatous tissues, six cases showed a tendency to grow during or after chemotherapy and were defined as GT. In total, they were categorized into two groups, GT/TMT (nine specimens) and residual teratoma without a tendency to grow (13 specimens).

For western blot analysis, we used five testicular tumor cases and six cancer cell lines including NEC8, NEC14, NT2 (embryonal carcinoma derived from testis), NCCIT (embryonal carcinoma derived from mediastinum), JEG-3 (choriocarcinoma derived from placenta) and Hs38.T (ovarian teratoma), purchased from RIKEN BioResource Center (Ibaraki, Japan) or ATCC (Manassas, VA, USA). For RT-PCR, we used five testicular tumor cases and NEC8. We collected specimens from tumor sites and corresponding normal-appearing tissues. Samples were immediately frozen at -80°C by liquid nitrogen. Histological subtypes of tumors were two seminomas, and three mixed germ cell tumors including non-seminomas. These five cases were different from samples for tissue microarray. Comprehensive informed consent was obtained from all participants.

Construction of tissue microarray (TMA)

To construct TMA, all HE sections were independently reviewed by two researchers, SS and SA. Sections with representative histology were selected for IHC in each case. Three representative tumor-bearing core cylinders (diameter: 2 mm) of the same histological subtype were obtained from each donor paraffin block and transferred into the recipient paraffin block. Successful transfer of tumor tissue was confirmed microscopically using HE-stained sections. In non-seminoma cases containing mixed histological subtypes in radical orchiectomy specimens, three core cylinders were obtained from the area of the same histological subtype in each case. In summary, the TMA contained 92 TGCT components from 64 primary radical orchiectomy samples, seven residual tumor samples after chemotherapy without teratomatous components and one normal testis. The 92 TGCT components included 31 seminoma specimens (20 pure seminomas and 11 seminomas with non-seminomatous components), 26 embryonal carcinomas, 10 yolk sac tumors, six choriocarcinomas and 19 teratomas. Because six of the 19 teratomas were prepubertal cases, we excluded these six specimens from immunohistochemical analysis in the present study (Table S1). The pathological subtypes of seven residual tumor cells were three embryonal carcinomas, three choriocarcinomas and one yolk sac tumor. The ethics committee of the University of Tsukuba approved constructing TMA, and comprehensive informed consent was obtained from all participants.

IHC

IHC analysis was carried out using a polyclonal rabbit antibody to gankyrin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), a monoclonal mouse antibody to Rb (Ab-5) clone LM95.1 (MERCK, Tokyo, Japan) and a polyclonal rabbit antibody to phospho-Rb Ser795 (Full Moon BioSystems, Sunnyvale, CA, USA) following the manufacturer's instructions. We chose phospho-Rb Ser795 among multiple phosphorylation sites because Higashitsuji et al. reported that transfection of gankyrin resulted in an increased amount of the phospho-Rb (Ser795) in osteosarcoma cell lines.[1] Briefly, formalin-fixed, paraffin-embedded specimens were cut into 4-μm thick sections. The sections were deparaffinized and rehydrated. For antigen retrieval, they were pretreated by autoclaving at 105ºC for 10 min in TE buffer (1×10^{-2} mol/L Tris-HCl with 1×10^{-3} mol/L ethylenediamine tetraacetic acid, pH 8.0) for Rb and phospho-Rb Ser795, and by microwaving at 100ºC for 10 min in sodium citrate buffer (1×10^{-2} mol/L sodium citrate monohydrate, pH 6.0) for gankyrin. After the antigen retrieval procedure, endogenous peroxidase activity was blocked and the slides were incubated with the primary antibodies for Rb (dilution 1:20) and phospho-Rb Ser795 (dilution 1:100) at room temperature for 30 min. The primary antibody to gankyrin was used at dilution 1:100 at 4°C overnight. The immunohistochemical reaction was visualized using DAKO Envision+ Dual Link for Rb and phospho-Rb Ser795 or Histofine Simple Stain MAX PO(R) (Nichirei

Biosciences, Tokyo, Japan) for gankyrin with diaminobenzidine as the chromogen. Staining levels were classified as strong or weak. For gankyrin cases, more than 60% of stained tumor cells were defined as strong staining. For Rb, cases were defined as strong staining when more than 10% of tumor cells were stained. For phospho-Rb Ser795, cases were defined as strong staining when more than 50% of tumor cells were stained. Scoring in all cases was carried out by two independent researchers, SS and SA.

Cell line and locked RNA

NEC8 and JEG-3 cells were grown in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum. LNA purchased from GeneDesign (Osaka, Japan) was used for suppression of gankyrin expression. Sequences for LNA were 5'-ACCCCtccatttcgcTGTCC for anti-gankyrin and 5'-GGACAgcgaaatggaGGGGT for the control. For western blot analysis and MTT assay, cells were transfected with 5×10^{-2} mol/L of LNA using Lipofectamine 2000, and cells were harvested 48 h after transfection. MTT assay was carried out following the manufacturer's instructions. Briefly, cells were plated on 96-well plates and 10 μL of WST-8 was added after 24, 48 or 72-h transfection with LNA. The OD were measured after 4-h incubation.

Western blot analysis

Western blot analysis was carried out as described previously.[1] Briefly,

proteins were extracted from tissues or cells with radioimmunoprecipitation assay buffer (1.5×10^{-2} mol/L Tris-HCl [pH 7.5], 0.1% sodium dodecyl sulfate, 2×10^{-3} mol/L NaCl, 2×10^{-2} mol/L ethylenediamine tetraacetic acid, 1% [v/v] TritonX-100 and 1% [w/v] sodium deoxycholate). The protein concentration was determined using Bio-Rad dye reagent (Bio-Rad, Hercules, CA, USA). Protein lysates (10 μg) from each sample were separated by sodium docecyl sulfate polyacrylamide gel electrophoresis and blotted onto a polyvinylidene difluoride membrane (Hybond-P; GE Healthcare Japan, Tokyo, Japan). Western blot analysis was carried out according to a standard protocol using TBS-T (2×10^{-2} mol/L Tris-HCl [pH 7.6], 1.37×10^{-1} mol/L NaCl, and 0.1% [w/v] Tween 20) containing 2% (w/v) ECL Prime Blocking Reagent as the blocking solution. Antibody concentrations used for western blot analysis were: gankyrin (sc-8991) at 1:500, phospho-Rb Ser795 (Cell Signaling Technology Japan, Tokyo, Japan) at 1:1000, and anti-rabbit immunoglobulin G, horseradish peroxidase-linked whole donkey Ab (GE Healthcare Japan) at 1:100000. Western blots were visualized with ECL Prime Western Blotting Detection System (GE Healthcare) using a Fujifilm LAS-4000 imager (Fujifilm, Tokyo, Japan). Beta-actin was used for an internal control.

RT-PCR

RNA was extracted from TGCT tissues, adjacent normal tissues and NEC8 cells using Trizol Reagent (Life Technologies, Tokyo, Japan). Total RNA was isolated according to the manufacturer's protocol, followed by ethanol precipitation. The extracted RNA was resuspended in RNase-free water. Subsequently, cDNA was synthesized from the extracted RNA using random primers and a cDNA synthesis kit (Applied Biosystems, Tokyo, Japan). RT-PCR was then carried out with a Taq PCR Core Kit (#201225 Qiagen, Tokyo, Japan). The following temperature program was used: one cycle at 94°C for 3 min, 35

cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 1 min, followed by a final extension at 72°C for 10 min. Primers used for amplification were a human gankyrin forward primer corresponding to nucleotides 562–582 (5’-AGCAGCCAAG GGTAACTTGAA-3’), and reverse primer complementary to nucleotides 697–716 (5’- TACTTGCTCCTTGGGACACC-3’).

Statistical analysis

Categorical variables were compared using Fisher’s exact test. The difference between ODs of the LNA-transfection group and control group were statistically compared by Student’s *t* test. A P value less than 0.05 was considered statistically significant. All statistical analyses were performed using the IBM SPSS Statistics program Version 21 (IBM Japan, Tokyo, Japan).

Results

Effects of gankyrin suppression on Rb status and cell proliferation

The expression levels of gankyrin were analyzed by western blot in six cancer cell lines (4 TGCT, 1 choriocarcinoma and 1 ovarian teratoma). Four TGCT cell lines and JEG-3 highly expressed gankyrin protein, but lesser expression was observed in Hs38.T, an ovarian teratoma cell line (Fig. 1a). Western blot analysis showed that the testicular tumor cell line NEC8 expressed Rb and phospho-Rb as well as gankyrin (Fig. 1b). Because gankyrin phosphorylates Rb at Ser795, which leads to degradation of Rb and drives the

cell cycle, we analyzed the effects of gankyrin expression on the Rb status and cell proliferation using LNA transfection. Transfection of the LNA for gankyrin in NEC8 cells reduced the mRNA expression levels of gankyrin to 57% (data not shown), and suppressed the protein expression of gankyrin to the same level (Fig. 1b,c). Western blot analysis showed that LNA suppressed the expression of phospho-Rb, but not the expression of Rb (Fig. 1b,c). The effects of gankyrin on cell proliferation were analyzed by MTT assay. The OD of the gankyrin-LNA transfection group was statistically lower than those of the control transfection group (Fig. 1e,f). These data show that gankyrin was functionally expressed in TGCT cells.

Gankyrin expression in normal spermatogenesis

Next, we analyzed the expression of gankyrin in normal human testis and human TGCT, as well as NEC8 cells. Interestingly, western blot analysis showed the gankyrin expression at 28 KDa in normal testis, as well as TGCT samples and NEC8 cells (Fig. 2a). Similarly, RT-PCR showed that gankyrin mRNA was expressed in normal testis (Fig. 2a). IHC for gankyrin in normal human testis showed strong signals for gankyrin in spermatocytes, but only faint signals in spermatogonia and round/elongated spermatids in normal human testis (Fig. 2b). Signals for Rb and phospho-Rb were identified in spermatocytes, as well as in spermatogonia and round spermatids (Fig. 2c,d).

IHC of gankyrin, Rb, and phospho-Rb in TGCTs

IHC of gankyrin, phospho-Rb, and Rb was carried out in various TGCT at primary and metastatic sites. The results are summarized in Supplementary Table S1 (Table S1). Figure 3 shows the proportions of tumor cells strongly stained with gankyrin and phospho-Rb antibodies. The proportion of strong gankyrin staining in seminomas (95%) was significantly higher than that in other subtypes of non-seminomas (yolk sac tumor P = 0.009 and teratoma P = 0.003; Fig. 3). Similar proportions of strong staining were also found in embryonal carcinoma and seminoma components in non-seminomatous tumors. A high proportion of strong phospho-Rb staining was shown in seminomas (85%), which was significantly higher than that in yolk sac tumors (P = 0.041) and teratomas (P = 0.026).

In specimens from metastatic sites, residual viable tumor cells without teratomatous components were highly positive both for gankyrin and phospho-Rb irrespective of histological subtype (Fig. 3). In contrast, expression patterns of gankyrin and phospho-Rb were quite different in teratomatous tumors and metastatic sites. The proportion of cells strongly staining for gankyrin was significantly higher in residual teratomas (69.2%) than in GT + TMT (0%; P = 0.001). There was no strong staining for gankyrin in any GT/TMT sample. However, unexpectedly, the proportion of strong staining of phospho-Rb was significantly higher in GT + TMT (88.9%) rather than in residual teratoma (30.8%; P = 0.001). The representative staining of gankyrin, phospho-Rb, and

Rb of primary TGCT and teratomatous tumor at metastatic sites are shown in Figures 4 and 5, respectively. To assess their possible role as clinical biomarkers in non-seminoma, we examined the correlations between gankyrin expression and recurrence. In seminoma, most of them showed strong gankyrin staining. We categorized non-seminoma patients into two groups according to gankyrin expression pattern. In the case of more than two types of histology, cases in which at least one type of histology showed negative staining were defined as negative. Interestingly, the recurrence rate tended to be higher in patients with gankyrin-positive staining (3/12, 25%) than those with negative staining (1/9, 11%), although it was not a statistically significant difference (P = 0.603).

Discussion

In the present study, we showed the expression profiles of gankyrin during normal spermatogenesis and TGCT. In normal tissues, gankyrin mRNA is reported to be expressed dominantly in the brain and heart, and weakly in the lung, spleen and muscle,[13] but to our knowledge, there is no report describing its expression in normal testis. Our data clearly showed that gankyrin is dominantly expressed in spermatocytes during spermatogenesis. Spermatogenesis comprises three steps: mitosis of spermatogenic stem cells, meiosis from spermatocytes to spermatids and differentiation of round spermatids to sperm. Rb and phosphor-Rb was reported to be expressed during spermatogenesis,[14]

and was confirmed in the present study. In combination with the evidence that gankyrin regulates the cell cycle through phosphorylation of Rb, the present findings suggested that gankyrin might play several roles in regulation of Rb during meiosis. Further studies using transgenic or knock-out mice models should be carried out to show the function of gankyrin in spermatogenesis.

It is clinically important to elucidate the underlying mechanisms of how TGCT growth is regulated, because TGCT proliferate rapidly and are highly chemosensitive. In contrast, a typical teratoma shows slow growth, but is highly resistant to chemotherapy. Despite the benign histological findings, teratomatous components of TGCT are believed to possess malignant potential, although a teratoma derived from other organs is generally benign. In fact, some populations of teratomatous components of TGCT turn into GT or TMT.

In the present study, we showed that gankyrin was functionally expressed in TGCT using NEC8 cells, and also showed several interesting findings through investigation of its expression profiles among histological subtypes of TGCT. First, gankyrin is highly expressed in seminoma and embryonal carcinoma, and positively stained to a lesser degree in other non-seminomatous tumors. The pattern of gankyrin expression correlated well with expression of phospho-Rb in each histological subtype of primary TGCT. These results are consistent with our previous report, which showed that expression levels of Ki-67 were higher in seminoma and embryonal carcinoma among all tumor histology.[15] These results suggest that gankyrin might play a role in tumor proliferation. This is supported

by our in vitro observation, which showed suppression of gankyrin by LNA resulted in suppression of phospho-Rb expression and cell proliferation of NEC8 cells. Because gankyrin can interact with MDM2 and facilitate the ubiquitination of p53, we carried out immunostaining of p53 and CDK4 in TMA tissues, but there are few cases with p53 staining of more than 10% of tumor cells or positive staining of CDK4 (Figs S1,S2). As generally positive staining of p53 indicated mutation of p53, however, we believe that negative staining of p53 did not deny the interaction of gankyrin and p53 in TGCT. Overexpression of gankyrin was reported to be observed in a variety of malignancies including HCC, esophageal squamous cell cancer and others.[4–7] As for the roles of gankyrin in oncogenesis, Umemura et al. suggested that it might play oncogenic roles mainly in the early stages of human hepatocarcinogenesis.[16] The present findings suggest the role of gankyrin in deregulation of the Rb pathway, but further study is required to clarify the oncogenic roles of gankyrin in TGCT.

Another interesting finding was the expression profiles of gankyrin and Rb/phospho-Rb among teratomatous components. The expression of Rb was high, but the positive rate of phospho-Rb was low in residual teratoma (Fig. 3 and Table S1) when compared with other histological subtypes of TGCT. These observations are compatible with previous studies,[15] and are in agreement with the limited proliferative activity of most residual teratomas. Despite the low phosphorylation status of Rb, relatively high gankyrin expression was unexpectedly observed in residual teratoma. In contrast, the positive rates of

gankyrin were low, but those of phospho-Rb were high in all GT/TMT cases, which showed rapid proliferation. Although the mechanism underlying dissociation of the expression patterns of gankyrin and phospho-Rb is unclear, this suggested that the molecular mechanisms underlying regulation of phosphorylation of Rb were different between teratomatous components and other histological subtypes of TGCT. Although the clinical data is preliminary, a case report suggested the therapeutic benefit of CDK inhibitor (PD 0332991) for patients with inoperable growing teratoma.[17] It will be interesting to investigate the molecular mechanisms regulating cell proliferation of teratomatous components of TGCT.

Although there were few samples, we investigated the expression of Rb and gankyrin in surgical specimens from the chemorefractory cases. Gankyrin and phospho-Rb Ser795 are highly expressed in viable cells in post-chemotherapy metastasectomy specimens. Expressions of both gankyrin and phospho-Rb Ser795 in choriocarcinoma and yolk sac tumor were higher in metastasectomy specimens compared with those with chemotherapy-naïve status. In esophageal cancer and colorectal cancer, gankyrin overexpression is known to be associated with a lower survival rate or malignant phenotype.[4,6] It might be interesting to investigate the roles of gankyrin in chemoresistance of TGCT. The present study had several limitations. First, the results were mainly carried out by immunohistochemistry. Further functional experiments are required to elucidate the role of gankyrin in normal testis and TGCT. Another

limitation of the present study was the limited number of cases. We could not elucidate the clinical impact of gankyrin expression because of the small number of cases.

There were several findings of the present study. First, gankyrin was expressed in normal testis, especially in spermatocytes during spermatogenesis. Second, high expressions of gankyrin and phospho-Rb were observed in seminoma and embryonal carcinoma. Finally, suppression of gankyrin resulted in suppression of phospho-Rb expression and cell proliferation in TGCT cell lines. In conclusion, we showed that gankyrin could be involved in the regulation and deregulation of the Rb pathway during normal spermatogenesis and development of TGCT. For a better understanding of the biological basis of the response to novel targeted drugs, a better understanding of the gankyrin-Rb pathway in the pathogenesis of TGCT is required.

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Conflicts of Interest

The authors have no conflict of interest to declare**.**

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Figure legends

Figure 1　Effects of gankyrin suppression on Rb and cell proliferation status

(a) The expression of gankyrin were analyzed by western blot in six cancer cell lines. NEC8, NEC14 and NT2: embryonal carcinoma derived from testis; NCCIT: embryonal carcinoma derived from mediastinum; JEG-3: choriocarcinoma derived from placenta; Hs38.T: Ovarian teratoma.

(b) The expressions of gankyrin, phospho-Rb, and Rb were analyzed by western blot at 48 h after transfection of NEC8 cells with anti-gankyrin LNA relative to control LNA and (c) the quantitative data of western blot was calculated by Image Quant TL software (Fujifilm, Tokyo, Japan). (d) NEC8 cells transfected with anti-gankyrin LNA showed significantly lower cell proliferation activity than those transfected with control LNA. OD values of cell growth were assayed at (e) 48 and 72 h in NEC8 cells, and at (f) 24h and 48h in JEG-3 cells after transfection with anti-gankyrin LNA relative to control LNA.

Figure 2　Protein and mRNA expressions of gankyrin in human normal testis, tumor tissue and a TGCT cell line (NEC8).

(a) Western blot and RT–PCR of gankyrin. Western blot and RT–PCR showed gankyrin expression in normal testis as well as tumor tissue and the NEC8 cell line. Lane 3: normal testis corresponding to lane 1 (seminoma), lane 4: normal

testis corresponding to lane 2 (non-seminoma). Immunohistochemical staining of (b) gankyrin, (c) Rb and (d) phospho-Rb in normal testis. Rs, round spermatid; Sc, spermatocyte; Sg, spermatogonia.

Figure 3 Rates of strong gankyrin and phospho-Rb staining in various TGCT at primary and metastatic sites.

The proportions of cases with strong staining are presented. Statistical analysis was carried out in comparison with seminoma in primary sites, and also between teratoma and growing teratoma/teratoma with malignant transformation (GT/TMT). Primary sites are compared with seminoma. Secondary sites are compared with teratoma without growth. *P<0.5, **P < 0.01.

Figure 4 Immunohistochemical staining of gankyrin, phospho-Rb S795 and Rb in primary TGCT

Representative stainings of seminoma, embryonal carcinoma and yolk sac tumor are presented.

Figure 5 Immunohistochemical staining of gankyrin, phospho-Rb Ser795 and Rb in teratomatous tumors at metastatic sites

Representative stainings of teratoma, growing teratoma and teratoma with malignant transformation are shown.

Abbreviations & Acronyms

CDK= Cyclin-dependent kinase

GT= Growing teratoma

HCC= Hepatocellular carcinoma

H&E= Hematoxylin and eosin

IGCCC= International Germ Cell Cancer Classification

IHC= Immunohistochemistry

LNA= Locked nucleic acid

ODs= Optical densities

Phospho-Rb= Phosphorylated retinoblastoma

Rb= Retinoblastoma

RT-PCR= Reverse transcriptase-polymerase chain reaction

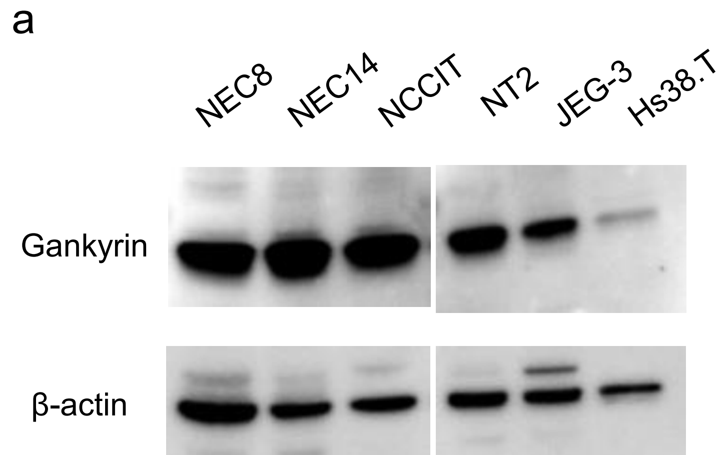
TGCT= Testicular Germ Cell Tumor

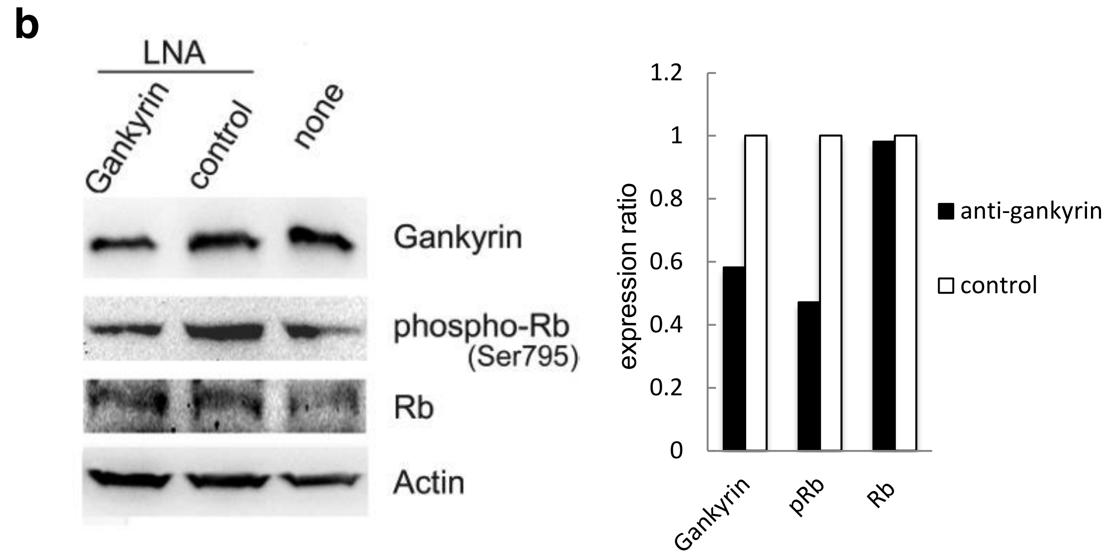
TGCTs= Testicular Germ Cell Tumors

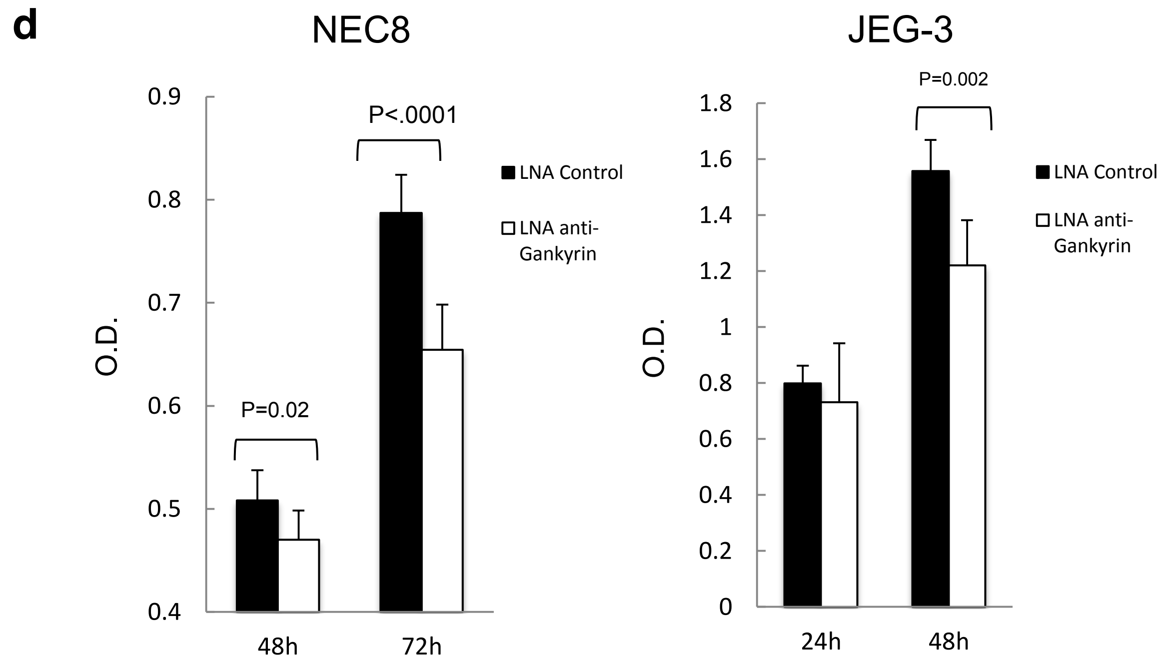
TMT= Teratoma with malignant transformation

UTH= University of Tsukuba Hospital

Figure 1







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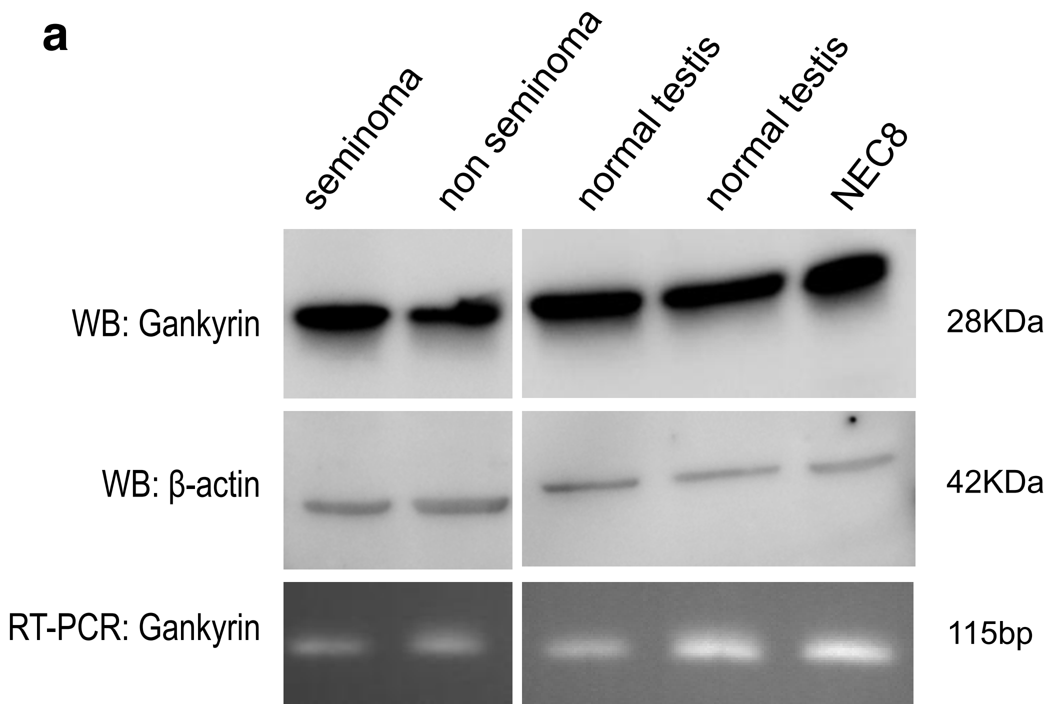
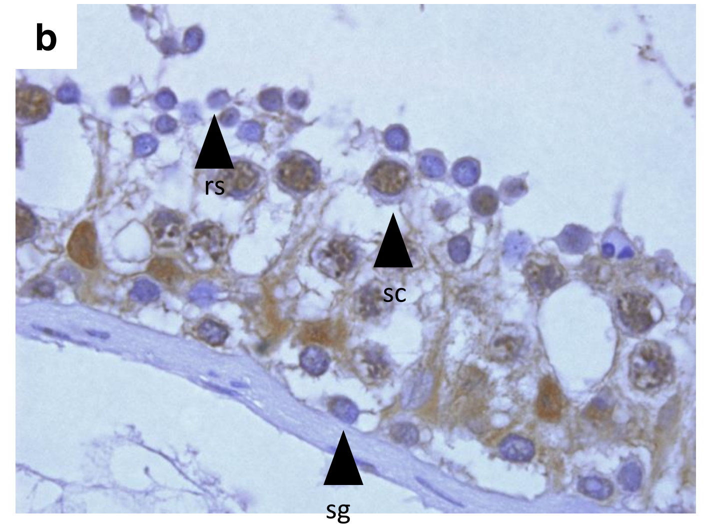
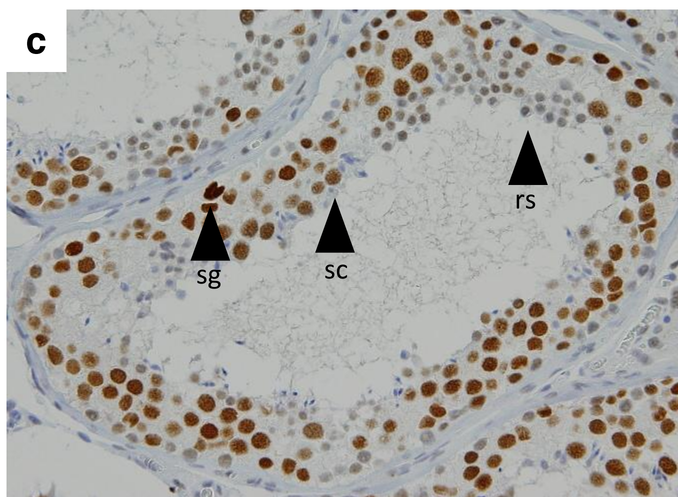






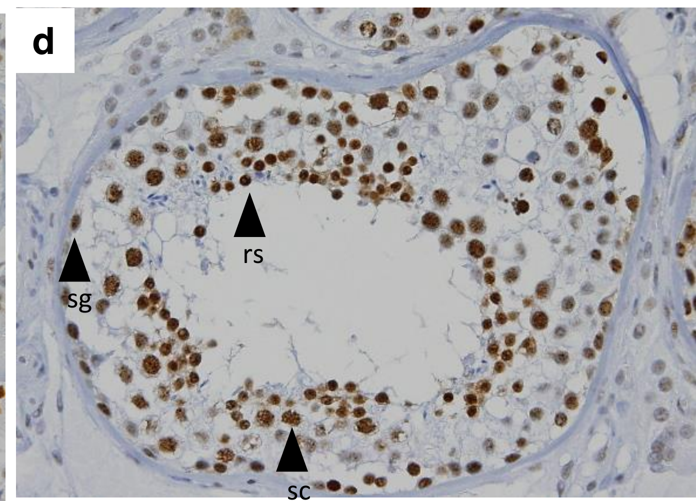
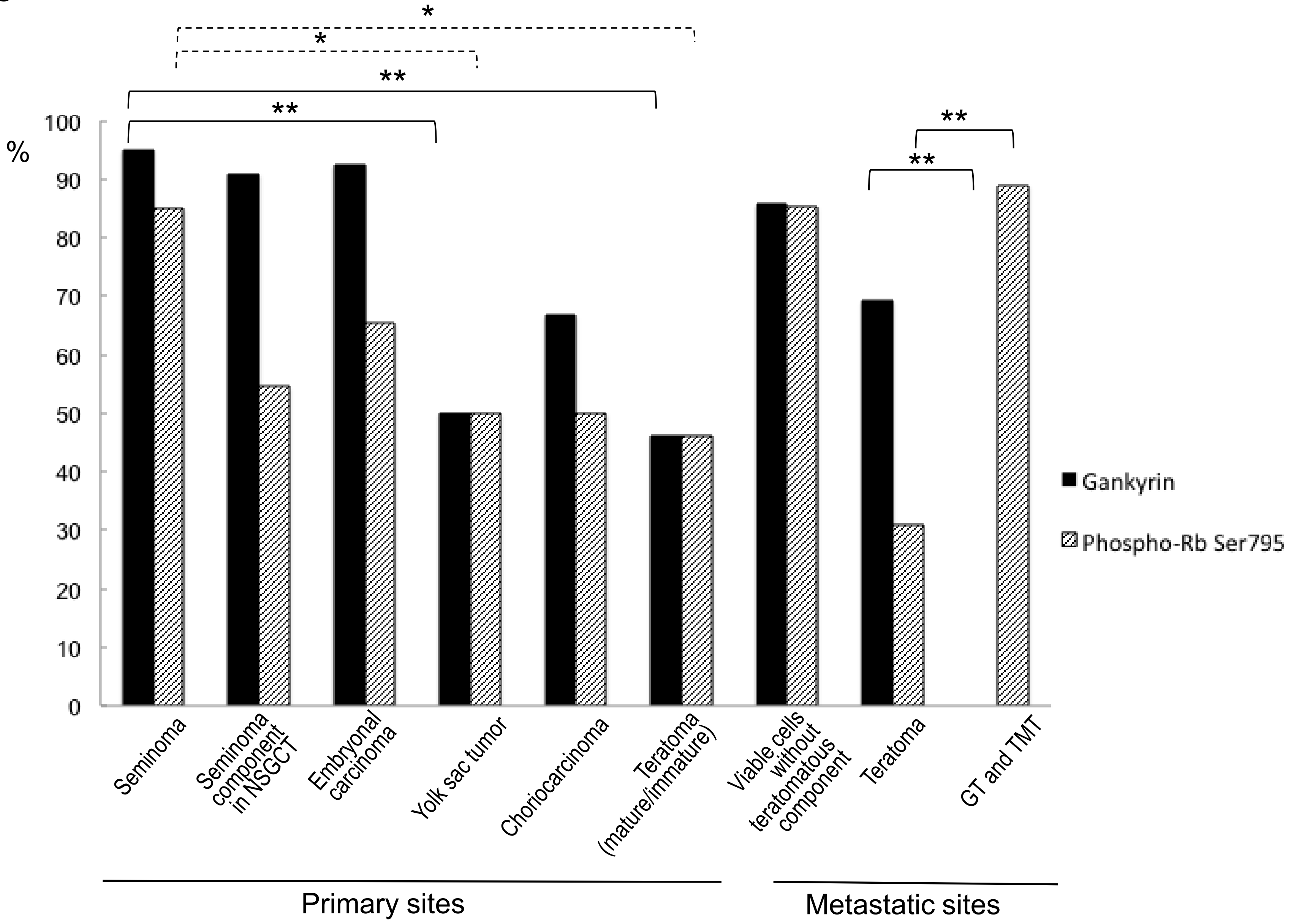


Figure 3



* p<0.05, ** p<0.01
Primary sites; compared with seminoma
Metastatic sides; compared with teratoma without growth

Figure 4

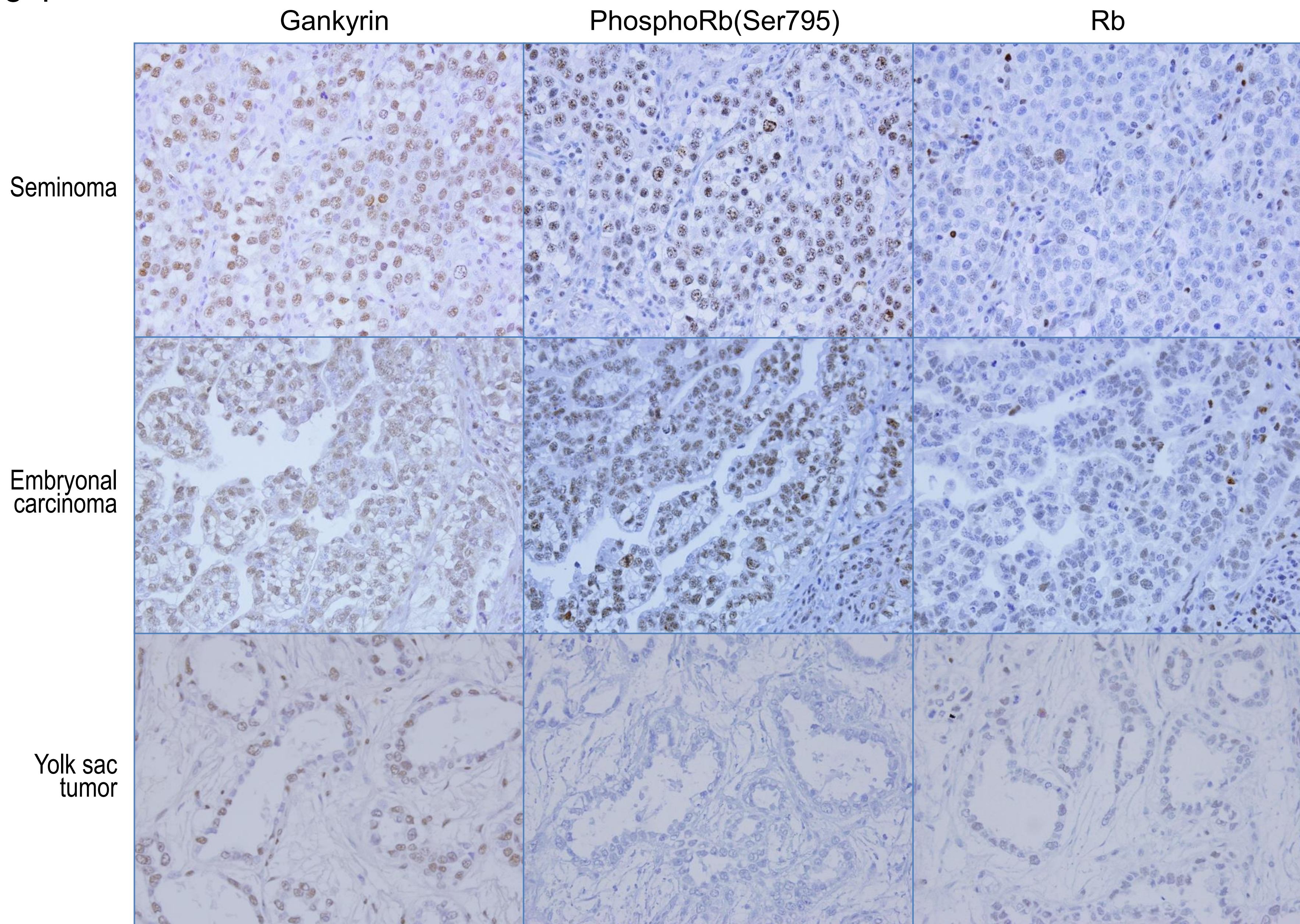
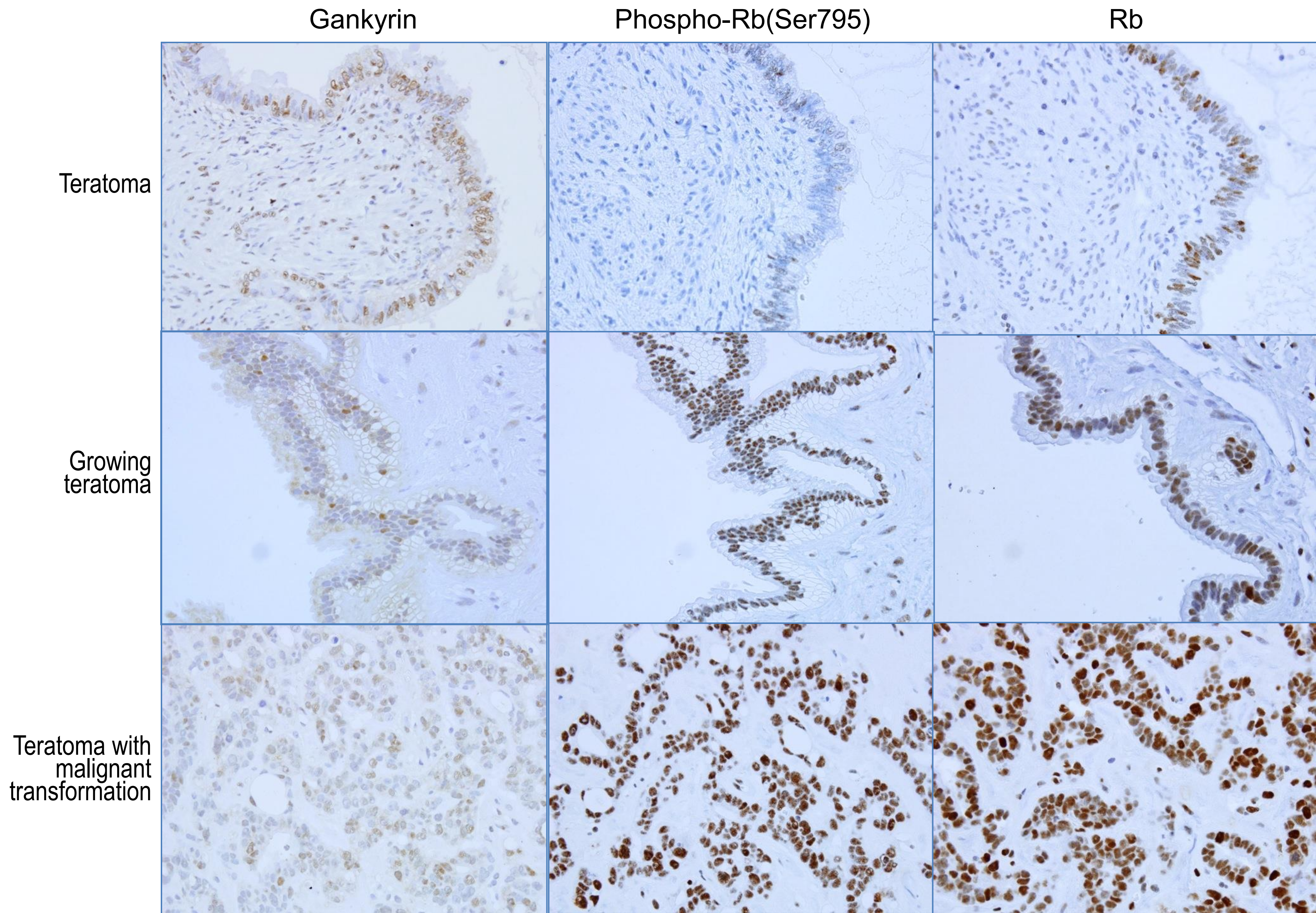
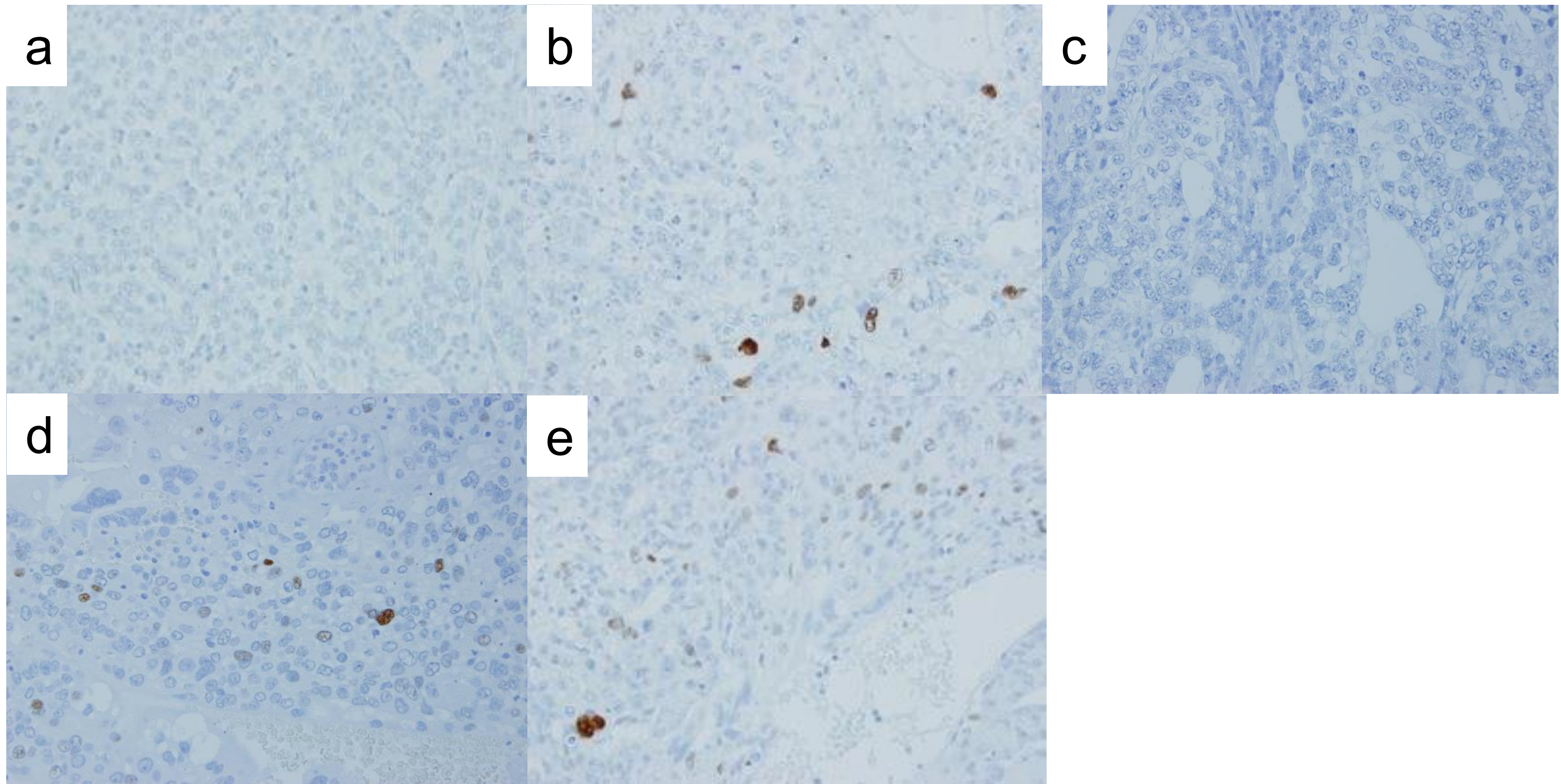


Figure 5

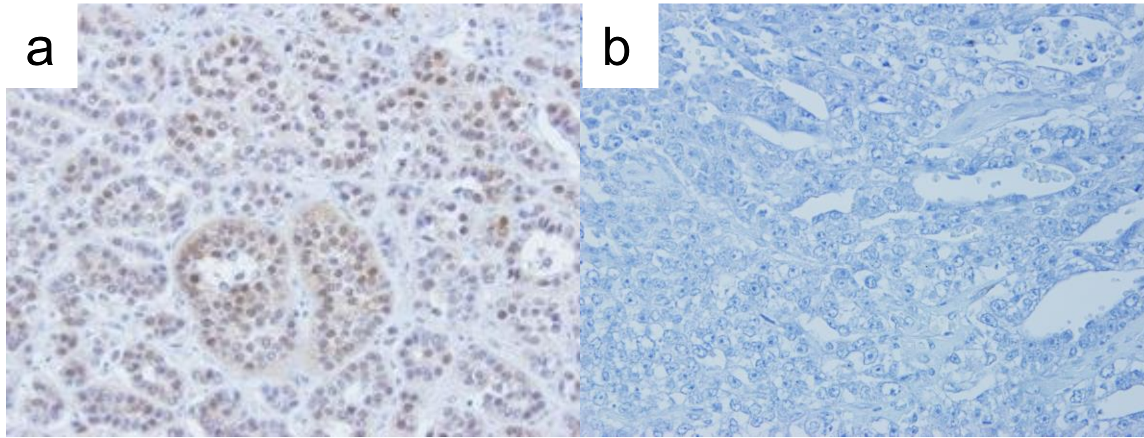


Supplementary Figure S1



Supplementary Figure S1. Immunohistochemical staining of p53 in primary TGCTs. Representative stainings of seminoma (a), embryonal carcinoma (b), yolk sac tumor (c), choriocarcinoma (d) and immature teratoma (e) are presented.

Supplementary Figure S2



Supplementary Figure S2. Immunohistochemical staining of CDK4 in primary choriocarcinoma.
(a) dedifferentiated liposarcoma (positive control for CDK4)
(b) Representative stainings of choriocarcinoma.