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ORIGINAL PAPER

Involvement of PTEN/Akt signaling in capsular invasive carcinomas developed in a rat two-stage thyroid carcinogenesis model after promotion with sulfadimethoxine

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Abstract
Purpose Rat thyroid follicular cell carcinomas invading into the thyroid capsule are highly produced by promotion with sulfadimethoxine (SDM) in a rat two-stage thyroid carcinogenesis model. In this study, we investigated the participation of phosphoinositide 3-kinase (PI3K) signaling pathway that is associated with malignant phenotypes of many cancers on the development of SDM-induced capsular invasive carcinomas.
Methods Thyroid proliferative lesions developed 10 or 15 weeks after promotion with SDM in male F344 rats initiated with *N*-bis(2-hydroxypropyl)nitrosamine were immunohistochemically analyzed with regard to cellular distribution of phosphatase and tensin homolog (PTEN) and Akt isoforms, as well as their downstream molecules.
Results Increased expression of PI3K signaling molecules was evident in association with the development of lesion stages from the early focal hyperplasia to the late carcinomas. Capsular carcinomas, and the less frequent parenchymal carcinomas, exclusively expressed phosphorylated, inactive PTEN, and active Akt isoforms, as did their downstream molecules. Among the Akt isoforms, enhanced expression of Akt1 was more prominent than that of Akt2 in both capsular and parenchymal carcinomas.
Conclusions Activation of the PI3K pathway through phosphorylation of PTEN promotes the high production of capsular carcinomas as well as the development of less frequent parenchymal carcinomas.

Keywords Thyroid follicular cell carcinomas · Phosphoinositide 3-kinase pathway · Sulfadimethoxine · Rat

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Introduction

There are few appropriate in vivo models that characterize the whole process of carcinogenesis from the generation of neoplastic cells to their metastasis, including their direct invasion to surrounding tissues. Recently, Imai et al. (2004, 2005) reported a rapid-induction model of capsular invasive follicular cell carcinomas (associated with capsular thickening and inflammatory cell responses) using a rat two-stage thyroid carcinogenesis model. Rats were given a single injection of the thyroid carcinogen, *N*-bis(2-hydroxypropyl) nitrosamine (DHPN), and then treated with an anti-thyroid agent, sulfadimethoxine (SDM), during the promotion stage. Although the role of inflammatory cell responses in capsular invasion was investigated in this model (Imai et al.

2005, 2007), cellular signaling in the neoplastic cells responsible for this invasion was not.

The molecular basis of invasive growth in human malignant tumors has been intensively investigated in recent years. A key signaling pathway, phosphoinositide 3-kinase (PI3K), responsible for the mechanisms involved in invasive cellular growth, and which involves the inactivation of phosphatase and tensin homolog (PTEN) and activation of Akt, has attracted attention. Recent studies in both humans and experimental animals have indicated that aberrant PI3K-Akt signaling toward Akt activation may play a role in the pathogenesis and progression of thyroid tumors (Furuya et al. 2007; Ringel et al. 2001).

PI3K isoforms are known to be regulators of cellular growth and proliferation and are often altered in human malignancies (Vivanco and Sawyers 2002). Studies suggest an important role for PI3K signaling in the regulation of cell growth, cell migration, epithelial to mesenchymal transition, alternative splicing events, and maintenance of tissue morphology (Wang et al. 1998; Blaustein et al. 2004; Liu et al. 2004; Horowitz et al. 2004). Aberrant activation of the PI3K signaling pathway is associated with several human cancers, including thyroid cancers (Furuya et al. 2007). Mechanisms that activate this pathway include the loss of tumor suppressor PTEN function, amplification or mutation of PI3Ks and Akt, and activation of growth factor receptors (LoPiccolo et al. 2007).

Here, we attempted to clarify the association between the development of capsular invasive lesions in the thyroid and the activation of the PI3K signaling in a rat induction model, using DHPN and SDM. The cellular localization of PTEN/Akt signaling molecules, including the substrates for Akt kinase activity, was investigated using immunohistochemistry. The expression of related molecules was also analyzed by immunoblotting.

Materials and methods

Chemicals, animals, and experimental design

N-bis(2-hydroxypropyl)nitrosamine (DHPN) and sulfadimethoxine (SDM) were purchased from Nacalai Tesque (Kyoto, Japan) and Sigma (St. Louis, MO, United States), respectively.

For the main study, a total of 24 six-week-old male F344/NSlc rats (Japan SLC, Inc., Hamamatsu, Japan) were allowed access to a basal diet (CRF-1, Oriental Yeast Co. Ltd., Tokyo, Japan) and water ad libitum and were kept in polycarbonate cages, with white wood chips for bedding, in an air conditioned room (24 ± 1°C, 55 ± 5% relative humidity; 12-h light and dark cycle). They were divided into two groups, and animals in group 2 ($n = 16$) were initiated with a single subcutaneous injection of DHPN at a dose of 2,800 mg/kg body weight. Animals in group 1 (untreated controls; $n = 8$) received an equivalent volume of vehicle only (saline). One week after the initiation, distilled drinking water containing 0.1% SDM was provided to animals in group 2 for 10 or 15 weeks. Animals in group 1 were given distilled water only. At either 10 or 15 weeks after the commencement of SDM treatment, eight animals from group 2 were necropsied under ether anesthesia and thyroid samples taken. Thyroid lobes were excised and cut in half coronally. The caudal half of each thyroid lobe was snap-frozen with liquid nitrogen and preserved at −80°C until use. The other half of each thyroid lobe was fixed in phosphate-buffered 10% formalin (pH 7.4) and embedded in paraffin. At the end of the experiment, the thyroids were collected from the untreated controls; the left lobe was fixed for histological preparation and the right lobe snap-frozen.

As a satellite study, eight male F344/NSIc rats were similarly initiated with DHPN and then promoted with 0.1% SDM for 10 weeks. Thyroid tissues were processed as above.

The animal protocol was reviewed and approved by the Animal Care and Use Committee of the National Institute of Health Sciences, Japan.

Histopathology

One coronal slice each from the paraffin-embedded left and right thyroid lobes was sectioned (4–5 μm thick) and stained with hematoxylin and eosin (HE) for microscopic examination. Histopathologically, thyroid proliferative lesions were classified into focal follicular cell hyperplasias (FFCH), adenomas, and carcinomas within the thyroid parenchyma, as well as capsular invasive carcinomas, according to published criteria (Imai et al. 2004, 2005). Microscopic counts for each histological lesion were made using the sections from both lobes from each animal. The average number of proliferative lesions was then calculated for each animal.

Immunohistochemistry

Immunohistochemical analysis of the molecules involved in the PI3K signaling pathway was performed on the paraffin-embedded thyroid tissue sections using the following antibodies purchased from Cell Signaling Technology, Inc. (Danvers, MA, United States): PTEN (rabbit monoclonal antibody, clone 138G6; 1:100), phospho-PTEN (Ser380) (rabbit polyclonal antibody; 1:50), Akt1 (mouse monoclonal antibody, clone 2H10; 1:500), Akt2 (rabbit monoclonal antibody, clone 54G8; 1:150), phospho-Akt (Ser473) (rabbit monoclonal antibody, clone 736E11; 1:50), phospho-Akt

(Thr308) (rabbit monoclonal antibody, clone 244F9; 1:100), phospho-(Ser/Thr) Akt substrate (rabbit polyclonal antibody; 1:50), glycogen synthase kinase (GSK)-3β (rabbit monoclonal antibody, clone 27C10; 1:100), phospho-GSK-3β (Ser9) (rabbit polyclonal antibody; 1:25), and β-catenin (rabbit polyclonal antibody; 1:50). Signal detection was performed using the VECTASTAIN® Elite ABC kit for rabbit or mouse IgG (Vector Laboratories Inc., Burlingame, CA, United States) with 3,3'-diaminobenzidine/H_2O_2 as the chromogen. Antigen retrieval was carried out in an autoclave for 10 min at 120°C in 10 mM citrate buffer (pH 6.0) for Akt1 and phospho-Akt (Thr308), or in a microwave for either 1 or 5 min at 100°C in the same buffer for Akt2, phospho-Akt (Ser473), phospho-(Ser/Thr) Akt substrates, phospho-PTEN, GSK-3β, phospho-GSK-3β (Ser9), and β-catenin. Phospho-PTEN and phospho-GSK-3β are inactive forms of PTEN and GSK-3β, respectively, and phospho-Akt is the active form of Akt.

The intensity of immunohistochemical staining for each molecule in the follicular proliferative lesions was scored as follows: 0 = no signal, or the same signal intensity as the surrounding non-proliferating follicular cells; 1 = slightly increased signal intensity compared with the surrounding follicular cells; 2 = moderately increased signal intensity; 3 = strongly increased signal intensity.

Immunohistochemical analysis of PTEN, phospho-PTEN (Ser380), Akt1, Akt2, phospho-Akt (Ser473), phospho-Akt (Thr308), and the phospho-(Ser/Thr) Akt substrates was performed using the main study samples. Satellite study samples were used for analysis of GSK-3β, phospho-GSK-3β (Ser9), and β-catenin.

Expression analysis of polypeptide signals

Western blotting was performed using thyroid tissues after 15 weeks of tumor promotion with SDM (n = 3/group). Tissue extraction and estimation of protein concentration were performed as described previously (Lee et al. 2006), and the tissue extract was loaded onto a 10% SDS–PAGE gel. The resolved polypeptides were then transferred to a PVDF membrane (Millipore, Billerica, MA, United States). After blocking with 0.2% casein, the blots were incubated with antibodies against β-actin (mouse monoclonal antibody, clone AC-15; Sigma; 1:20,000), Akt1 (1:1000), PTEN (1:1000), phospho-PTEN (Ser380; 1:1000), p70-S6 kinase (rabbit monoclonal antibody, clone 49D7; Cell Signaling Technology, Inc.; 1:1000), and GSK-3β (1:1000). The amount of protein extract applied to the SDS–PAGE gels was 1 μg for β-actin and 4 μg for the other antigens. Protein signals were detected using the ECL Plus Western Blotting Detection Reagent (GE Healthcare UK Ltd., Little Chalfont, United Kingdom).

Statistical analysis

The number of follicular proliferative lesions per animal was compared between 10 and 15 weeks after SDM-promotion using the Student's t-test when the variance was proven to be homogeneous among the groups by the test for equal variance. If a significant difference in the variance was observed, Welch's t-test was performed. Scores for immunostaining intensity were compared between the pre-neoplastic FFCH and each type of follicular neoplastic lesion using the Mann–Whitney's U-test. The incidence of immunoreactive lesions of Akt1 and Akt2 was tested by the Fisher's exact probability test between the FFCH and each type of follicular neoplastic lesion. Differences were considered significant when the P value was <0.05.

Results

Histological types of proliferative lesions

The number of proliferative lesions was examined in the main study. After 10 or 15 weeks of SDM-promotion, all animals developed follicular cell adenomas, as well as FFCH, in the thyroidal parenchyma. The mean number of FFCH lesions seen at Week 15 was half the number seen at Week 10 (Table 1). Cases of carcinoma generated in the thyroidal parenchyma (named "parenchymal carcinomas") were not frequent at either time point. Within the capsular tissue, invasive carcinomas (named as "capsular carcinomas") were frequently observed in all cases at both time points. Although not statistically significant, the number of capsular carcinomas increased with time. Although we did not perform any morphometric measurements, the growth of neoplastic lesions, particularly the parenchymal carcinomas and capsular carcinomas, was evident from Weeks 10 to 15. Similarly, the thickness of the capsular tissue increased with time.

Table 1 Number of thyroid proliferative lesions developed at 10 or 15 weeks after promotion with sulfadimethoxine (main study)

Proliferative lesion	Week 10	Week 15
No. of animals examined	8	8
FFCH (No./animal)	9.75 ± 4.20	4.75 ± 1.90**
Adenoma (No./animal)	8.50 ± 5.29	9.75 ± 4.10
Carcinoma (No./animal)	1.13 ± 1.36	1.25 ± 1.28
Capsular Carcinoma (No./animal)	5.00 ± 1.69	6.75 ± 3.85

Abbreviation: *FFCH* focal follicular cell hyperplasia

** $P < 0.01$ vs. Week 10 (Mann–Whitney's U-test)

Distribution of PTEN/Akt signaling molecules

As shown in Fig. 1, increased staining intensity of both PTEN and phospho-PTEN (Ser380) was observed in the proliferative lesions. At Week 10, a statistically significant increase in PTEN staining intensity was evident for both adenomas and capsular carcinomas compared with FFCH. An increased staining intensity for phospho-PTEN was also seen in capsular carcinomas compared with FFCH. One parenchymal carcinoma that was evaluated immunohistochemically at Week 10 had a grade 3 score for both PTEN and phospho-PTEN. At Week 15, significant increases in both PTEN and phospho-PTEN staining intensity were observed for both parenchymal and capsular carcinomas compared with FFCH.

As shown in Figs. 2, 3, 4, increased staining intensity of both Akt1 and Akt2 was also observed in the proliferative lesions, similar to that of PTEN, and the staining intensity was rather high for Akt1. In each type of proliferative lesion, total incidence showing positive immunoreactivity was rather high with Akt1 when compared with Akt2 (Fig. 4). At Week 10, statistically significant increases in

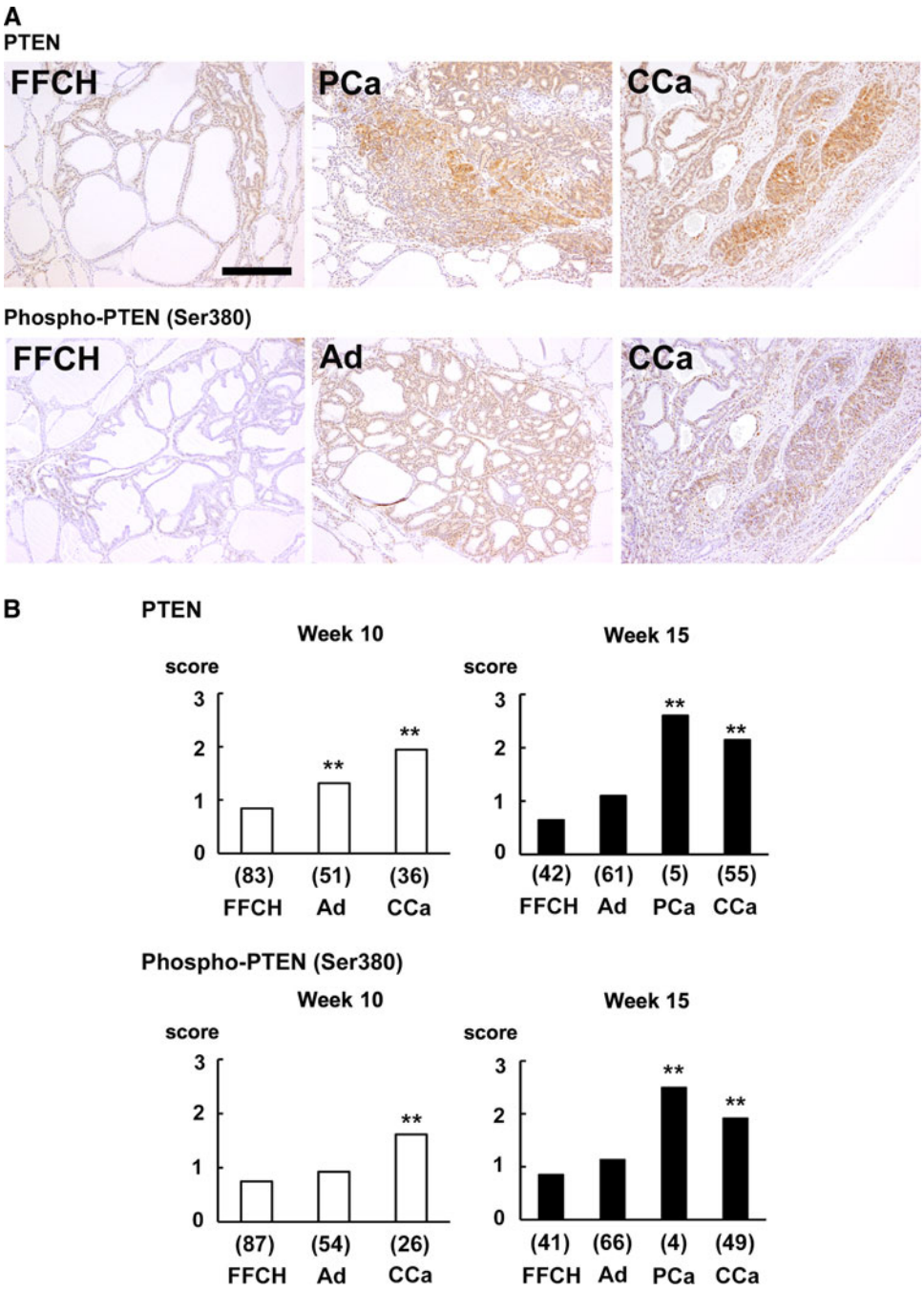


Fig. 1 Immunohistochemical distribution of phosphatase tensin homolog (PTEN), and phospho-PTEN (Ser380) in the thyroid proliferative lesions induced by promotion with sulfadimethoxine. **a** Photomicrographs of representative immunoreactivity patterns for focal follicular cell hyperplasias (FFCH), adenomas (Ad), parenchymal carcinomas (PCa), or capsular carcinomas (CCa) induced after 15 weeks of promotion are shown (×200, *Bar* 200 μm). **b** Immunohistochemical staining intensity scores for PTEN and phospho-PTEN (Ser380) in FFCH, Ad, PCa, and CCa at Week 10 (*open column*) and Week 15 (*black column*). Values in parentheses represent the number of lesions examined. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. FFCH (Mann–Whitney's *U*-test)

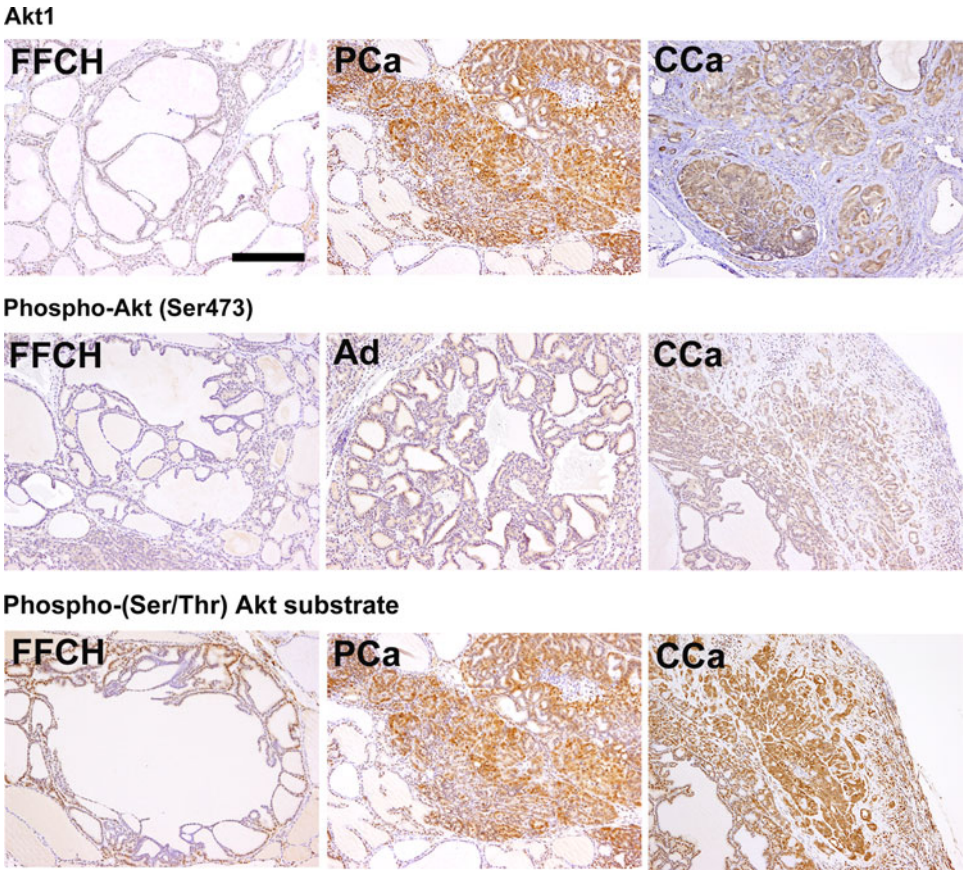


Fig. 2 Immunohistochemical distribution of Akt1, phospho-Akt (Ser473), and phospho-(Ser/Thr) Akt substrates in thyroid proliferative lesions induced by promotion with sulfadimethoxine. Photomicrographs of representative immunoreactivity in focal follicular cell hyperplasias (FFCH), adenomas (Ad), parenchymal carcinomas (PCa), or capsular carcinomas (CCa) induced after 15 weeks of tumor promotion are shown (×200, *Bar* 200 μm)

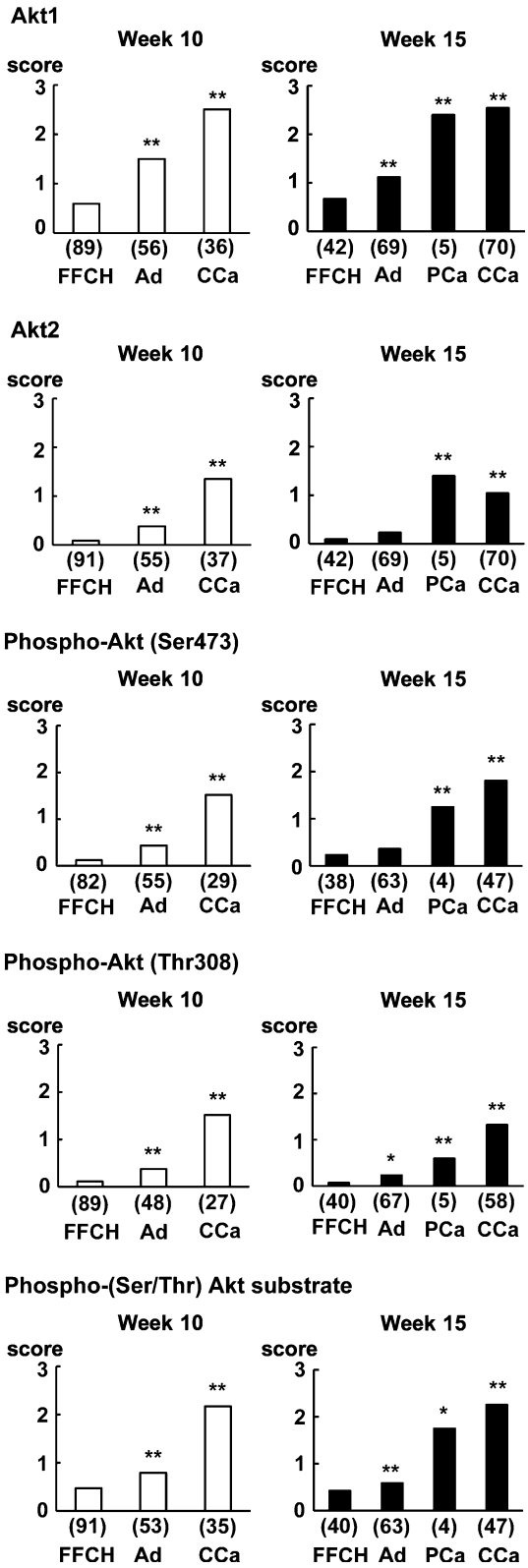
the total incidence showing positive immunoreactivity and the grade of staining intensity were evident for both Akt1 and Akt2 in the adenomas and capsular carcinomas compared with FFCH. One parenchymal carcinoma that was evaluated immunohistochemically for Akt1-immunoreactivity at Week 10 had a grade 3 score. At Week 15, a significant increase in Akt1 staining intensity was observed in adenomas and both parenchymal and capsular carcinomas compared with FFCH. The total incidence of Akt1-positive cases in adenomas and in capsular carcinomas was significantly higher than that in FFCH. For Akt2, significantly increased immunoreactivity in terms of the total incidence of positive cases and the grade of staining intensity was observed in both parenchymal and capsular carcinomas.

Increased staining intensity of both phospho-Akt (Ser473) and phospho-Akt (Thr308) was also seen in the proliferative lesions, similar to that for PTEN (Figs. 2, 3). At Week 10, the staining intensity of phospho-Akt (Ser473) was the same as that for phospho-Akt (Thr308), whereas at Week 15, the intensity of phospho-Akt (Ser473) in the proliferative lesions was higher than that of phospho-Akt (Thr308). At Week 10, statistically significant increases in both phospho-Akt (Ser473) and phospho-Akt (Thr308) staining intensity were evident in adenomas and capsular carcinomas. At Week 15, significantly increased immunoreactivity of phospho-Akt (Ser473) was observed in both parenchymal and capsular carcinomas. In the case of phospho-Akt (Thr308), a significant increase in staining intensity was observed in adenomas and both parenchymal and capsular carcinomas compared with FFCH.

Molecules immunoreactive with the anti-phospho-(Ser/Thr) Akt substrate antibody also showed similar localization patterns to PTEN/Akt molecules (Figs. 2, 3). At Week 10, statistically significant increases in staining intensity were evident in adenomas and capsular carcinomas compared with FFCH; and at Week 15, significant increases in staining intensity were observed in adenomas and both of parenchymal and capsular carcinomas compared with FFCH.

As shown in Fig. 5, increased staining intensity of both GSK-3β and phospho-GSK-3β (Ser9) was also observed in proliferative lesions, similar to that of PTEN at Week 10. Significant increases in staining intensity were observed for both GSK-3β and phospho-GSK-3β (Ser9) in adenomas and both the parenchymal and capsular carcinomas compared with FFCH.

The subcellular distribution of β-catenin was mainly observed at the cellular membrane in most of the proliferative lesions (Fig. 5). There were significantly more cases



showing both cytoplasmic and membranous immunoreactivity in adenomas and capsular carcinomas compared with FFCH (capsular carcinomas having more cases).

◀ **Fig. 3** Immunohistochemical staining intensity scores for Akt1, Akt2, phospho-Akt (Ser473), phospho-Akt (Thr308), and phospho-(Ser/Thr) Akt substrates in focal follicular cell hyperplasias (FFCH), adenomas (Ad), and parenchymal carcinomas (PCa), and capsular carcinomas (CCa) at Week 10 (*open column*) and Week 15 (*black column*) of tumor promotion. Values in parentheses represent the number of lesions examined. $*P < 0.05$, $**P < 0.01$ vs. FFCH (Mann–Whitney's *U*-test)

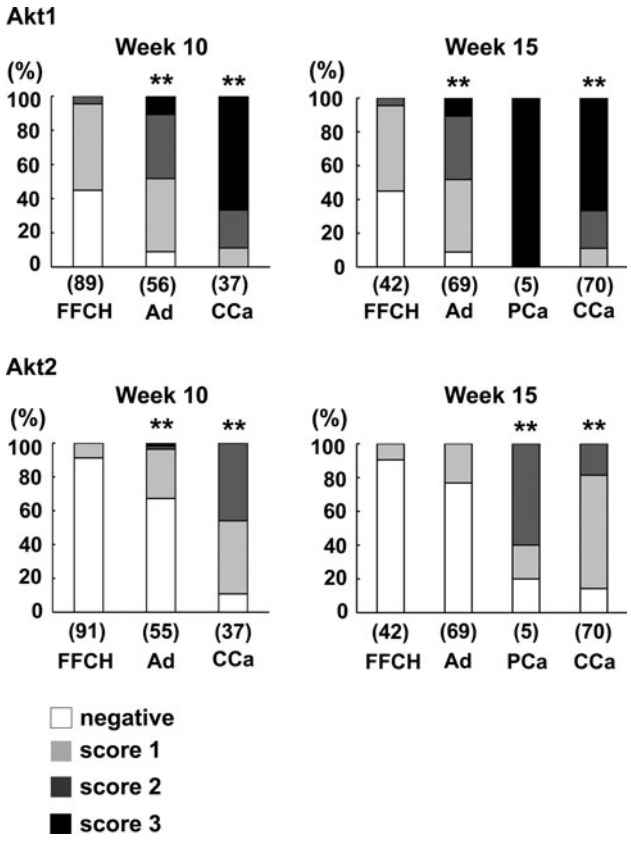


Fig. 4 Incidence of immunohistochemical staining intensity scores for Akt1 and Akt2 in focal follicular cell hyperplasias (FFCH), adenomas (Ad), parenchymal carcinomas (PCa), and capsular carcinomas (CCa) at Week 10 and Week 15 of tumor promotion. Values in parentheses represent the number of lesions examined. $**P < 0.01$ vs. FFCH in the total incidence showing ≥ score 1 (Fisher's exact probability test)

Polypeptide signal levels of PTEN/Akt signaling molecules

Although only three animals from each group were examined, the thyroid tissue from SDM-promoted rats showed increased polypeptide band intensities on Western blots for Akt1, phospho-PTEN (Ser380), p70-S6 kinase and GSK-3β compared with the untreated controls (Fig. 6). Different from phospho-PTEN, PTEN (as a total of non-phosphorylated and phosphorylated isoforms) did not show altered levels.

Discussion

Using immunohistochemistry, we found localized expression of PTEN/Akt signaling molecules in the proliferative

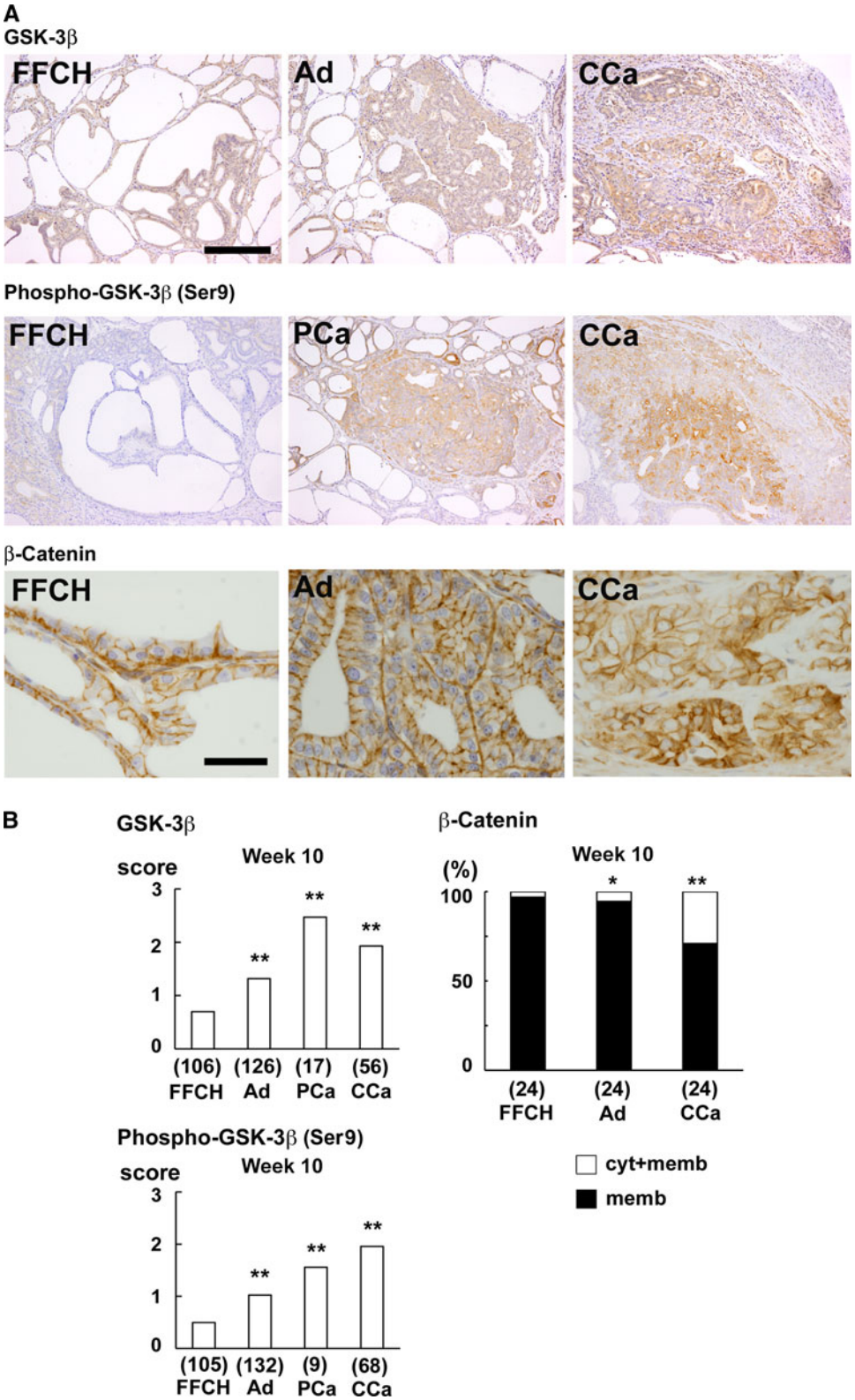


Fig. 5 Immunohistochemical distribution of glycogen synthase kinase (GSK)-3β, phospho-GSK-3β (Ser9), and β-catenin in thyroid proliferative lesions induced by promotion with sulfadimethoxine. **a** Photomicrographs of representative immunoreactivity in focal follicular cell hyperplasias (FFCH), adenomas (Ad), parenchymal carcinomas (PCa), or capsular carcinomas (CCa) induced after 10 weeks of promotion are shown ($\times$200 for GSK-3β and Phospho-GSK-3β (Ser9). *Bar* 200 μm; $\times$400 for β-catenin. *Bar* 50 μm). **b** Immunohistochemical staining intensity of GSK-3β and phospho-GSK-3β (Ser9) in FFCH, Ad, PCa, and CCa and subcellular localization of β-catenin in FFCH, Ad, and CCa after 10 weeks of tumor promotion. The ratio of lesions showing both cytoplasmic and membranous expression patterns (*open column*), or membranous expression pattern alone (*black column*), are shown. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. FFCH (Mann–Whitney's *U*-test)

lesions of follicular cells, with increased intensity in advanced neoplastic lesions. Although PTEN, a tumor suppressor (LoPiccolo et al. 2007), showed localized expression in the proliferative lesions, the phosphorylated and inactive forms of this molecule showed similar expression patterns. In contrast to the unaltered polypeptide signal levels of total PTEN (both phosphorylated and non-phosphorylated forms) in the thyroid tissues of the untreated controls and

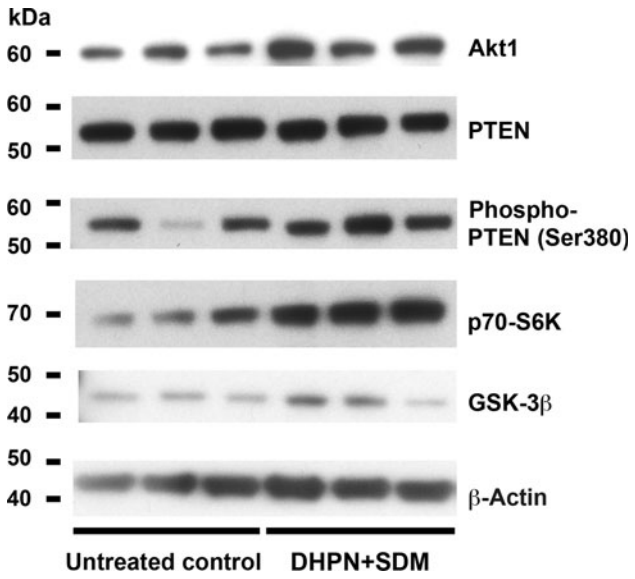


Fig. 6 Western blot analysis of Akt1, phosphatase and tensin homolog (PTEN), phospho-PTEN (Ser380), p70-S6 kinase, glycogen synthase kinase (GSK)-3β, and β-actin in the thyroid of rats after 15 weeks of promotion with sulfadimethoxine (SDM). Thyroid tissues from three untreated controls and three SDM-promoted cases were analyzed

the SDM-promoted rats, the increased signal levels for phospho-PTEN seen in SDM-promoted rats suggest a relative increase in inactive PTEN localized to the proliferative lesions. As well as the co-localization of phosphorylated Akt in these lesions, the higher incidence of positive immunoreactivity as with higher staining intensity seen for Akt1 than for Akt2 suggests that Akt1 is the major molecule driving signaling. Also, multiple downstream molecules of Akt signaling (at least GSK-3β and p70-S6K) are co-localized in these proliferative lesions or up-regulated in the thyroid tissues in a similar manner to the upstream molecules. There are no studies reporting activation of PTEN/Akt signaling by antimicrobial sulfonamide, such as SDM. Obtained results thus suggest the involvement of PTEN/Akt signaling specific to the process of progression of thyroid carcinogenesis, including the generation of capsular carcinomas in the SDM-promoted rat model.

PTEN activity is lost by mutations, deletions, or promoter methylation silencing at high frequency in many primary and metastatic human cancers (Blanco-Aparicio et al. 2007). Mice harboring a dominant-negative mutant thyroid hormone receptor-β (TR$\beta^{PV/PV}$ mice) spontaneously develop follicular thyroid carcinomas and distant metastases, and haploinsufficiency of PTEN accelerates progression and invasiveness of these carcinomas (Guigon et al. 2009). Interestingly, in the present study, localized expression of PTEN was observed in thyroid proliferative lesions with increased immunoreactivity in carcinomas using antibodies that recognize both the phosphorylated and non-phosphorylated forms of PTEN. Expression of phosphorylated PTEN was also increased in these proliferative lesions, as was that of activated downstream Akt molecules, suggesting that the accumulation of inactive PTEN may be responsible for the activation of the PI3K/Akt signaling pathway. In human cancers, there is no such signal activation mechanism related to the acquisition of malignant phenotypes, while there is one study reporting that increased phosphorylation of PTEN and Akt in human uterine leiomyomas possibly contributes to cellular survival mechanism (Kovács et al. 2007). Therefore, PTEN-inactivation, through increased expression of its phosphorylated isoform, may be a unique mechanism linking the activation of downstream signaling molecules to the progression of the SDM-promoted thyroid capsular carcinomas.

In this thyroid capsular carcinoma model, severe capsular inflammation associated with fibrous reaction is considered to be prerequisite for the frequent induction of invasive carcinomas (Imai et al. 2004). Promotion with propylthiouracil, another anti-thyroid agent that is less potent for inducing capsular inflammation than SDM, produces less frequent capsular invasive tumors, in parallel with weak capsular inflammation and fibrosis (Imai et al. 2005). Imai et al. (2007) suggested that development of invasive carcinomas in the present model is enhanced by capsular inflammation mediated by T cells, and inducible nitric oxide synthase induction in the inflamed lesions may play a role in tumor progression. Thus, although neoplastic cell invasion itself can trigger fibrous reactions, fibrotic tissue formed during the capsular inflammation may provide the place of neoplastic cell invasion in response to inflammatory reactions. Patients with Hashimoto thyroiditis are three times more likely to have thyroid cancer compared with patients without this autoimmune inflammatory disease, suggesting a strong link between chronic inflammation and cancer development (Larson et al. 2007). Also, activation of PI3K/Akt observed in both Hashimoto thyroiditis and cancer suggests a possible molecular mechanism for thyroid carcinogenesis (Larson et al. 2007).

Among the Akt isoforms, we found that enhanced expression of Akt1 was more prominent than that of Akt2, in accordance with lesion development. Recently, Akt1 has been shown to limit the invasive migration of breast cancer cells, suggesting that Akt1 may have a dual role in tumorigenesis; acting not only to promote oncogenesis by suppressing apoptosis, but also by inhibiting oncogenesis by suppressing invasion and metastasis (Wyszomierski and Yu 2005; Toker and Yoeli-Lerner 2006). However, there is increasing evidence regarding the role of Akt2 in promoting carcinogenesis. Overexpression of Akt2 leads to the up-regulation of integrins, increased invasion, and metastasis of human breast and ovarian cancer cells (Arboleda et al. 2003). Interestingly, opposing roles of Akt1 and Akt2 for

the induction and metastasis of mammary carcinogenesis have been reported in mice and humans (Maroulakou et al. 2007; Dillon et al. 2009).

In the present study, we also found enhanced expression of inactive phospho-GSK-3β, similar to other PTEN/Akt signaling molecules in accordance with the development of lesion stages. GSK-3 is a critical downstream element of the PI3K/Akt pathway, and its activity can be inhibited by Akt-mediated phosphorylation (Srivastava and Pandey 1998; Cross et al. 1995). GSK-3β has been shown to regulate cyclin D proteolysis and subcellular localization (Diehl et al. 1998). In a previous study, we found similar expression patterns for the inactive form of GSK-3β, phosphorylated at serine 9, and cyclin D in accordance with the development of the different stages of thyroid proliferative lesions (Ago et al. 2010). These results suggest the significance of the inactivation of GSK-3β in the tumor-promotion process, while the role of upstream Akt isoforms (either Akt1 or Akt2) is still not clear.

Imai et al. (2004) reported that cellular proliferative activity in capsular carcinomas is higher than in FFCH and adenomas, which also suggests that PTEN/Akt signaling molecules contribute to tumor progression. Although the induction is less frequent than capsular carcinomas, parenchymal carcinomas in the present study also showed activation of the PI3K/Akt pathway similarly to capsular carcinomas, suggesting this pathway as a common signaling for tumor progression, irrespective of the site of occurrence. As mentioned earlier, capsular inflammation might be responsible for frequent induction of capsular carcinomas. Interestingly, expression of phosphorylated isoforms of Akt was lower than that of PTEN and phospho-PTEN in each type of proliferative lesion. Especially, FFCH and adenomas scarcely expressed phosphorylated active Akt, in contrast to the higher expression values of phosphorylated PTEN in these lesions. Mean values in the staining intensity of total PTEN and phosphorylated PTEN were similar in each type of proliferative lesion, suggesting that PTEN is equally phosphorylated in proliferative lesions without association to lesion development. With regard to the regulation of the activation and deactivation of Akt molecules, complex interactions of lipid kinases, protein kinases, lipid phosphatases, and protein phosphatases are involved. Upon inactivation of PTEN, constitutive Akt activation occurs in a manner dependent on the cascade involving the lipid kinase PI3K (Cantley and Neel 1999). Activated PI3K generates membrane-bound phosphoinositides, which act as second messengers and serve to recruit Akt. Therefore, activation of PI3K may be prerequisite for the activation of Akt molecules, and such activation may trigger malignant transformation as reported by others (Krasilnikov 2000; Lipscomb and Mercurio 2005; Kallergi et al. 2007).

While in the present study we could only detect changes in β-catenin localization from the cell membrane to the cytoplasm in the capsular carcinomas, Imai et al. (2004) found nuclear localization of β-catenin, a molecule negatively regulated by GSK-3β, in capsular carcinomas in the SDM-promotion model. On the other hand, activated PI3K signaling leads to the activation of the downstream Akt-mammalian target of rapamycin (mTOR)-p70-S6K pathway that can potentiate enhanced cell proliferation and suppression of apoptosis (Asnaghi et al. 2004). In the present study, we also found increased expression of p70-S6K in the thyroid tissues after 15 weeks of tumor promotion with SDM, suggesting the activation of the Akt-mTOR-p70-S6K pathway. Activation of the AKT-mTOR-p70-S6K pathway and inactivation of GSK-3β may promote cellular proliferation in capsular carcinomas.

In conclusion, we have shown that activation of PI3K/PKB signaling occurs in parallel with lesion development through mechanisms involving the selective expression of the inactive isoform of PTEN. These signaling events may play an important role in the acquisition of a malignant phenotype, resulting in capsular invasion.

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Conflict of interest statement All authors disclose that there are no conflicts of interest that could inappropriately influence the outcome of the present study.

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