# Overexpression of the Angiogenic Tetrapeptide AcSDKP in Human Malignant Tumors

JIAN-MIAO LIU[1], MICHAL KUSINSKI[2], VESNA ILIC[1], JÉRÔME BIGNON[1], NEILA HAJEM[1], JAN KOMOROWSKI[3], KRZYSZTOF KUZDAK[2], HENRYK STEPIEN[4] and JOANNA WDZIECZAK-BAKALA[1]

*[1]Institut de Chimie des Substances Naturelles, Centre National de la Recherche Scientifique, Avenue de la Terrasse, 91198 Gif-sur-Yvette, France ; Departments of [2]Endocrinological and General Surgery, [3]Clinical Endocrinology and [4]Immunoendocrinology, Medical University of Lodz, 91-425 Lodz, Poland*

**Abstract.** *Background: The natural tetrapeptide acetyl-Ser-Asp-Lys-Pro (AcSDKP), generated from thymosin β4 following its cleavage by prolyl oligopeptidase (POP), is a physiological stimulator of angiogenesis. Because of the critical role of neovascularisation in tumor development, the expression of AcSDKP and the activity of POP were examined in different human solid malignancies. Materials and Methods: The expression of AcSDKP and the activity of POP were evaluated in human blood samples and tissue specimens of thyroid goiter and thyroid papillary carcinoma as well as in commercial cancer tissue microarray. Results: A significantly increased concentration of AcSDKP in intratumoral blood and enhanced tissular activity of POP were detected in cancer patients. The expression of AcSDKP in human breast, colon, head and neck, kidney, lung, skin, ovary and prostate cancer tissues was shown to be greater than that in normal tissues. Conclusion: AcSDKP and POP contribute to the malignant phenotype and these molecules are potentiel markers of cancer.*

Physiologically, angiogenesis ensures the proper development of mature organisms, prepares the womb for egg implantation and plays a key role in wound healing and repair of ischemic damage. On the other hand, the process of neovascularisation supports the pathological conditions associated with a number of disease states such as cancer, inflammation and ocular disorders (1, 2). Tumor angiogenesis is a prerequisite process for continuous growth and metastasis of solid malignancies. The ability to induce and sustain angiogenesis seems to be acquired during tumor development, *via* an angiogenic switch from vascular quiescence. Solid tumors appear to activate the angiogenic switch by changing the balance between angiogenesis inducers and countervailling inhibitors (3, 4) in the tumor cells and their microenvironment. The modulation of angiogenesis thus appears to be a promising stategy in the treatment of solid tumors particularly in combination with chemotherapy (5) and understanding the differences between normal and tumoral angiogenesis might potentially allow accurate distinction of benign from malignant tumors.

The natural tetrapeptide acetyl-*N*-Ser-Asp-Lys-Pro (AcSDKP), described initially as a physiological inhibitor of hematopoietic stem cell proliferation (6), has recently been recognized to be part of a complex system that contributes to the regulation of angiogenesis. Indeed, AcSDKP promoted *in vivo* the growth of new blood vessels (7, 8) and its administration reduced the extent of ischemic damage. In animal models, AcSDKP induced neovascularization following experimentally induced myocardial infarction, hindlimb ischemia and cutaneous injury (8-11).

Our recent studies have revealed that the level of endogenous AcSDKP was markedly increased in malignant neoplastic diseases including hematological malignancies and solid neoplasms. In fact, Liu *et al*. (12) have reported a dramatic increase in the concentration of endogenous AcSDKP in leukemia-inoculated mice. Moreover, a strong correlation between the levels of AcSDKP and the progression of acute myelogenous leukemia (AML) has been demonstrated. Increased levels of AcSDKP have also been detected in a pilot study on thyroid malignant tumors (13).

To verify whether AcSDKP overexpression is a general event in the process of carcinogenesis, the level of this peptide was analyzed in different human solid malignancies. Moreover, the enzymatic activity of prolyl oligopeptidase (POP), a cytosolic enzyme involved in the metabolism of many peptide hormones and neuropeptides (14) and responsible for the release of AcSDKP from its metabolic precursor thymosin β4 (15), was evaluated in the tissues of thyroid carcinomas.

*Correspondence to:* Joanna Wdzieczak-Bakala, ICSN, CNRS, 91198 Gif-sur-Yvette, France. Tel: +33 169823098, Fax: +33 169077247, e-mail: Johanna.Bakala@icsn.cnrs-gif.fr

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## Materials and Methods

*Patients and tissue samples*. Blood samples and thyroid tissue specimens were obtained from patients who underwent curative surgery for thyroid nodular goiters and thyroid papillary carcinomas. Blood was collected from 35 (24 women and 11 men aged 14-79 years) patients. Specimens of thyroid papillary carcinoma tissue were collected from a second group of 15 patients (14 women and 1 man, aged 21-68) who had been enrolled in our former study (13). All the patients were diagnosed before surgery with an ultrasound-guided biopsy and the diagnosis was confirmed after surgery by routine histopathological examination. None of the patients had other diseases and none of them received drugs before surgery. The healthy control group included age- and sex-matched volunteers from our department. This study was approved by the Ethics Committee of the hospital and informed consent was obtained from all the participating patients. Human breast, colon, head and neck, kidney, lung, skin, ovary and prostate cancer tissues and their normal couterparts (Select Tissue Microarray: TMA2010-4) were obtained from Chemicon Int. (Temecula, CA, USA) as formalin-fixed and paraffin-embedded 4 to 8 μm-thick sections.

*Immunohistochemical staining*. The slides were deparaffinized with xylene and rehydrated in graded alcohols. The tissue sections were permeabilized with 0.1% Triton-X-100 in phosphate-buffered saline (PBS) and preincubated for 1 h in PBS containing 3% bovine serum albumin (BSA) and 10% goat serum to block non-specific binding. Subsequently, the sections were incubated overnight at 4˚C with the primary anti-AcSDKP diluted at 1:100 (rabbit anti-thymosin β4 (1-4), Biodesign International, Saco, ME, USA) and then for 2 h at room temperature with IRDye 800 CW-conjugated goat anti-rabbit secondary antibody diluted at 1:400 (Tebu-Bio, Le Perray-en-Yvelines, France). Thereafter, the slides were washed twice with PBS containing 0.1% Tween and mounted in fluorescent mounting medium (Dako, Trappes, France). Tissue sections incubated either with preimmune rabbit serum containing irrelevant antibodies or with rabbit immunoglobulins (rabbit IgG, Beckman Coulter, Villepinte, France) instead of the primary antibody were used as negative control. The slides were scanned by the Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, USA) and the data were acquired using the scanner software, exported to Excel (Microsoft, Redmond, USA) and analysed. The values of the integrated fluorescence intensity (U) are the sums of the intensity values for all pixels enclosed by a feature, multiplied by the area ($mm^2$) of the feature. Background pixels were calculated and subtracted.

*AcSDKP anay*. The intratumoral and peripheral blood were collected on lithium heparin and immediately supplemented with lisinopril (Sigma, Saint-Quentin Fallavier, France) at $10^{-6}$ M final concentration to prevent AcSDKP degradation. The intratumoral blood was collected from the vein nearest the tumor in patients with carcinoma of the thyroid gland, or from the central vein of benign goiter which is commonly the biggest vessel on the gland's surface. The blood samples were then centrifuged at 1,000 ×g for 10 min at 4˚C, the plasma was removed and stored at –20˚C until use. The AcSDKP concentration in the plasma was measured using a highly specific competitive enzyme immunoassay (EIA) with acetylcholinesterase conjugate as a tracer (SPIbio, Massy, France) as previously described (7). Each assay was performed in triplicate.

*POP enzymatic activity assay*. The human thyroid tissues obtained by open surgery were washed with cold saline and immediately frozen in liquid nitrogen. The thyroid tissue (100 mg) was homogenized in 1 ml of 10 mM Tris-HCL containing 1% of Nonidet P40 and then centrifuged at 100,000 ×g for 1 h at 4˚C. The POP activity was measured in the supernatants using the fluorogenic substrate *N*-benzyloxycarbonyl-glycyl-prolyl-7-amino-4-methylcoumarin (Z-Gly-Pro-NHMec; Bachem, Weil-am-Rhein, Germany) previously dissolved at a concentration of 5.6 mM in dimethylsulfoxide. The assay was initiated by incubating 10 μl of sample in 100 μl of assay buffer (100 mM $K_2HPO_4$, $KH_2PO_4$, pH 7.5 containing 1 mM dithiothreitol, 1 mM $NaN_3$ and 1 mM EDTA) at 37˚C. After equilibration for 3 min, 5 μl of the substrate were added. The reaction was stopped after 20 min by the addition of 500 μl of 1.5 mM acetic acid. The fluorescence was recorded at $\lambda_{ex}$=370 nm and $\lambda_{em}$=440 nm using a Victor microtiter plate fluorimeter (Perkin-Elmer, Waltham, USA). The relative fluorescence was converted to pmols of 7-amino-4-methylcoumarin released by POP from the Z-Gly-Pro-NHMec. The protein concentration in the tissue samples was measured by the method of Bradford according to the manufacturer's recommendations (Biorad, Marnes-la-Coquette, France). The POP activity was expressed in arbitrary units (AU) corresponding to the pmols of the released product per milligram of protein detected in the examined sample.

*Statistical analysis*. The results are expressed as mean±SEM. Statistical analysis was performed using the Mann-Whitney *U*-test. $P<0.05$ between groups was considered statistically significant.

## Results

*AcSDKP concentration in intratumoral blood from thyroid papillary carcinomas*. The level of AcSDKP in the peripheral blood taken from the patients with benign goiter (n=9) and malignant (n=26) thyroid tumors did not show any significant differences between the studied populations (Figure 1). Indeed, similar levels of AcSDKP were detected in the patients with nodular goiter and thyroid papillary carcinomas before (1.35±0.16 nM *versus* 1.66±0.27 nM), during (1.82±0.30 nM *versus* 1.62±0.14 nM) and 3 months after surgery (1.73±0.16 nM *versus* 1.29±0.22 nM). The obtained values were also not different from the control values (1.65±0.31 nM) measured in the blood of healthy subjects (n=10). However, a significantly higher ($p<0.05$) concentration of AcSDKP was found in the intratumoral blood taken intraoperatively and collected from the malignant tumors (2.86±0.33 nM) compared to the blood drawn from nodular goiters (1.52±0.27 nM).

*AcSDKP expression in different human solid neoplasms*. The immunohistochemical staining of AcSDKP in the sections of human malignant tumors provided evidence of overexpression of AcSDKP in cancer tissues (Figure 2A). The values of fluorescence intensity corresponding to the detected immunoreactivity were shown to be significantly greater in all the malignant tumors than in the corresponding normal tissues

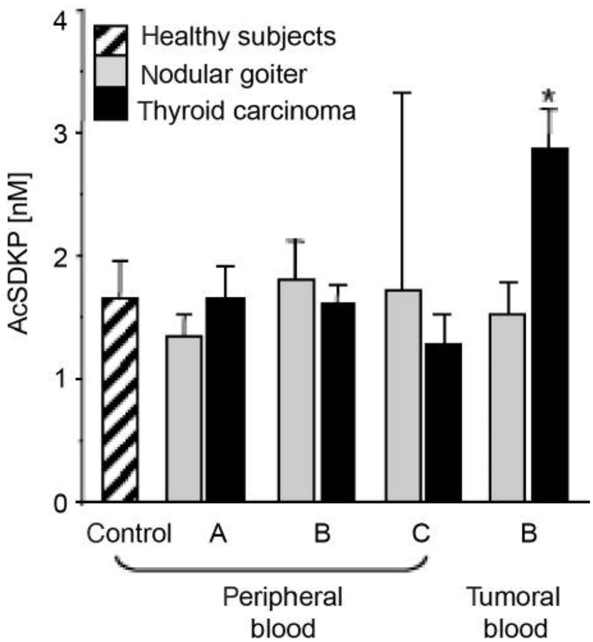


Figure 1. *Concentration of AcSDKP in peripheral and intratumoral blood of patients with thyroid goiters (n=9) and papillary thyroid carcinomas (n=26), (A) before surgery, (B) during surgery and (C) after surgery. The results are the mean±SEM; *p<0.05.*

(4.3-, 2.5-, 3.5-, 1.5-, 2,1-, 4.0-, 1.8- and 1.5-fold increase respectively in breast, colon, head and neck, kidney, lung, skin, ovary and prostate tumor). As shown in Figure 2B, the investigated sections of two different specimens of thyroid papillary carcinomas also displayed stronger immunoreactivity (2-fold increase) than that detected in the control tissue of nodular goiter. The values of fluorescence intensity were 6.06 U and 5.65 U for the carcinomas *versus* 3.01 U for the control. This overexpression of AcSDKP in surgically removed human thyroid papillary carcinomas confirms our previous results published by Kusinski *et al*. (13). The primary antibodies, as indicated in the notification issued by the supplier, do not cross-react with thymosin β4 which contains a tetrapeptide sequence at its *N*-terminus. The visualized immunostaining was specific since no signal was observed when the tissue sections were incubated either with preimmune rabbit serum or with rabbit IgG instead of the first antibody.

*POP activity in human thyroid papillary carcinomas*. POP activity was measured in extracts of the operatively removed thyroid papillary carcinomas (n=8) and thyroid nodular goiters (n=6). As shown in Figure 3, the enzymatic activity of POP was higher in all the specimens of malignant tumor tissues studied (12.3-92.5 AU) compared to the activity detected in the control tissues (0.6 - 11.5 AU).

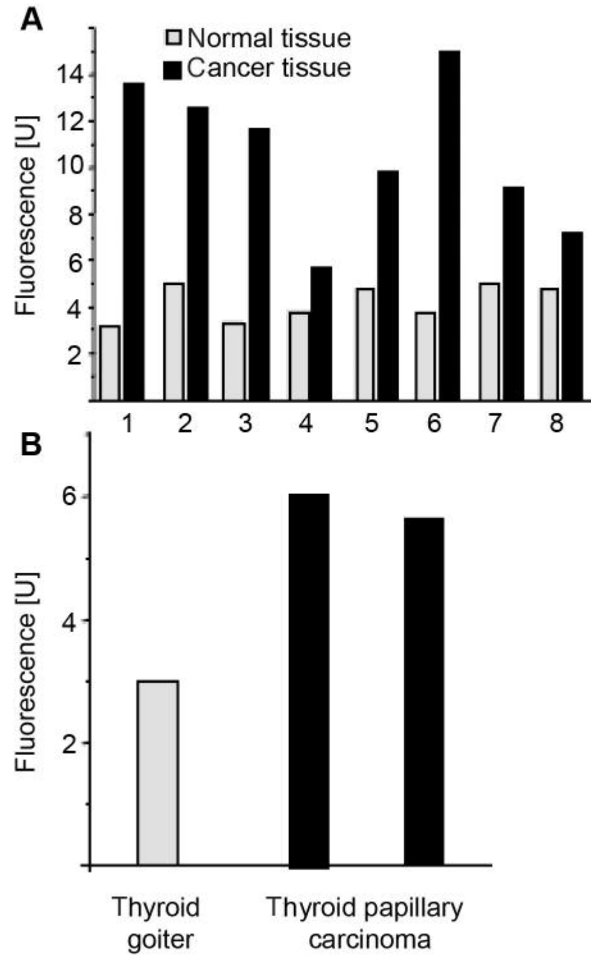


Figure 2. *Immunohistochemical expression of AcSDKP in human cancer tissues and their normal counterparts. A. Fluorescence intensity corresponding to AcSDKP immunostained sections of (1) breast: invasive ductal carcinoma; (2) colon: poorly differentiated adenocarcinoma; (3) muscle: Hodgkin's lymphoma neck mass; (4) kidney: renal cell carcinoma; (5) lung: well differentiated squamous cell carcinoma; (6) skin: melanoma; (7) ovary: ovarian carcinoma; (8) prostate: moderately differentiated adenocarcinoma. B. Fluorescence intensity, corresponding to AcSDKP, in immunostained sections of thyroid goiter and papillary thyroid carcinomas. No immunostaining was detected when the tissue sections were incubated either with preimmune rabbit serum or with rabbit IgG instead of primary antibody.*

## Discussion

Evaluation of the AcSDKP levels in the blood of 26 patients bearing papillary carcinoma provided evidence of a two-fold increase in AcSDKP concentration exclusively in the intratumoral blood in comparison with the controls. The elevated levels of AcSDKP detected locally in the intratumoral blood were compatible with the significantly greater immunostaining of AcSDKP observed in the surgical specimens of human thyroid carcinomas than in

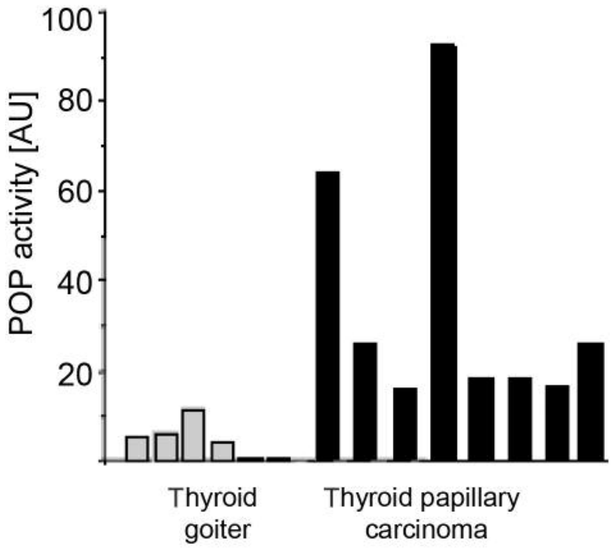


Figure 3. *Enzymatic activity of prolyl oligopeptidase in tissue of thyroid goiters and thyroid papillary carcinomas.*

the specimens of benign nodular goiter. These data reinforced our first report on the increased level of AcSDKP evidenced in the total extracts of malignant thyroid tumors (13).

It is well documented that the two prominent histological types of thyroid carcinoma originating from follicular cells, papillary carcinoma and follicular carcinoma, are hypervascular (16, 17). Moreover, a clear correlation between increased vascular endothelial growth factor (VEGF) expression and more aggressive thyroid tumor behavior and metastasis has been demonstrated (18). The present findings strongly associate thyroid cancer with the high expression of another angiogenic factor, the tetrapeptide AcSDKP. The absence of significant differences between AcSDKP levels in the peripheral blood of patients with thyroid cancer and healthy controls may be due, at least in part, to the extremely short life of this tetrapeptide in the blood (19). These observations are consistent with a previous study reporting the concentration of plasma AcSDKP in patients with various solid tumors similar to the values measured in healthy controls (20).

Further investigations using a cancer tissue array provided evidence of the tissular overexpression of AcSDKP in all the examined solid tumors. This abundant production of AcSDKP, probably by the malignant cells or/and tumoral vessels, implicated this peptide in the physiopathology of cancer. Therefore, the analysis of a large series of tumor samples associated with the clinical data is required to assess the potential of AcSDKP detection as a diagnostic and/or prognostic tool for human neoplasias.

All these findings are in agreement with the previously reported overexpression of thymosin β4 in a large variety of neoplastic tissues including thyroid, breast, uterine, lung, colon and kidney carcinomas (21-25), and with its potential role in tumorigenesis (26, 27). Indeed, *in vivo* sampling techniques using capillary ultrafiltration probes to collect secretomes directly from tumor masses and mass spectrometric proteomic approaches have allowed the identification of thymosin β4 as one of the important biomarkers and therapeutic targets for cancer therapy (28). Thymosin β4, the major actin-sequestering molecule in eukaryotic cells (29) and a potent angiogenic agent (30, 31), was proposed as the most likely precursor of AcSDKP as it possesses the necessary sequence in its *N*-terminus (32). The elevated activity of POP detected in the tissues of the thyroid tumors was consistent with the overexpression of AcSDKP in solid malignancies and consequently with the role of this enzyme in the biosynthesis of AcSDKP. The increased POP activity demonstrated in the current work was in agreement with the results of a previous study reporting the significantly elevated activity of this peptidase in prostate, lung and sigmoid tumors (33). The use of specific inhibitors of POP should thus establish whether this enzyme, as well as AcSDKP, is directly involved in the process of malignancy.

Over the last decade, assessment of angiogenesis has emerged as a potentially useful biological prognostic and predictive factor in human solid tumors. The majority of published studies have shown a positive correlation between intratumoral microvessel density and the prognosis of solid tumors. Moreover, the assessment of angiogenic factor expression is often useful for the diagnosis and prognosis of a large variety of human carcinomas. The present findings provide evidence to support follow-up studies to link clinical data with the potential consideration of AcSDKP as a new biomarker of solid malignancies.

Although high levels of AcSDKP have now been reported both in solid tumors and in hematological malignancies, it is still unknown whether this peptide at elevated concentrations has any physiological function related to cancer development. A final assessment of AcSDKP function in tumor formation will need direct *in vivo* testing of AcSDKP administration as well as suppression of this endogenous tetrapeptide in tumor-bearing animals. The role of AcSDKP in carcinogenesis could open a new therapeutic window for this peptide as a potential new target of anticancer therapy.


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