Jpn. J. Cancer Res. 84, 419–424, April 1993

A Case of Squamous Cell Lung Carcinoma with High Concentration of Parathyroid Hormone-related Peptide in Serum and Pleural Effusion Presenting Hypercalcemia

Haruo Iguchi,[1] Hideki Katakami,[2] Yukito Ichinose,[3] Yoshihiro Nishi,[4] Seiichi Tanaka,[4] Nobuyuki Hara,[3] Mitsuo Ohta,[3] Masafumi Haji[4] and Hajime Nawata[4]

[1]Department of Biochemistry and [3]Department of Respiratory Tract, National Kyushu Cancer Center, Notame 3-1-1, Minami-ku, Fukuoka 815, [2]Third Department of Internal Medicine, Miyazaki Medical College, Kiyotake-cho, Miyazaki 889-16 and [4]Third Department of Internal Medicine, Faculty of Medicine, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812

A 57-year-old man with lung squamous cell carcinoma revealed hypercalcemia, hypophosphoremia, elevation of nephrogenous cAMP and metabolic alkalosis. Serum parathyroid hormone (PTH) and $1,25(OH)_2D_3$ concentrations were not elevated. These findings were consistent with those in humoral hypercalcemia of malignancy (HHM). PTH-related peptide (PTHrP) concentrations were determined using N- and C-terminal specific radioimmunoassays (PTHrP-N, PTHrP-C), and elevation of both PTHrP-N and PTHrP-C concentrations in the serum was noted (PTHrP-N, 27 pmol/liter (normal < 5); PTHrP-C, 1408 pmol/liter (normal < 50)). High concentration of PTHrP (946 pmol/liter for PTHrP-N and 5983 pmol/liter for PTHrP-C) was also found in the pleural fluid obtained at autopsy. Immunohistochemical study, using paraffin-embedded sections of the tumor tissue obtained at autopsy, revealed numerous PTHrP-positive cells and expression of PTHrP gene was confirmed by Northern blot analysis. These findings indicate that PTHrP, produced in the tumor tissue, was secreted into the blood stream, which caused HHM in the patient. Gel permeation chromatography of the serum and pleural fluid revealed several peaks of both PTHrP-N and PTHrP-C. Molecular forms of PTHrP-N were larger than those of PTHrP-C in the serum as well as pleural fluid. These findings indicate that multiple forms of PTHrP molecules are present in the serum and pleural fluid. Granulocytosis was also noted in the patient. However, granulocyte- and granulocyte macrophage-colony stimulating factor were not detected in the serum, and the mechanism of the granulocytosis in the patient was unclear.

Key words: Lung carcinoma — Humoral hypercalcemia of malignancy — PTHrP

Parathyroid hormone-related peptide (PTHrP) was isolated from a human lung cancer-derived cell line (BEN) as a substance associated with humoral hypercalcemia of malignancy (HHM) and its primary structure was established in 1987.[1)] Subsequent studies revealed a close relationship between PTHrP and HHM *in vitro* as well as *in vivo*.[2–7)] In the human lung carcinoma, HHM is found mostly in squamous cell type, and PTHrP gene expression was demonstrated in this type of tumor.[8)] However, little information on serum PTHrP levels in patients with HHM is yet available. In the present study, we examined the serum PTHrP concentration in a patient with squamous cell lung carcinoma presenting with HHM, and changes of serum PTHrP concentration were observed during treatment of the hypercalcemia with pamidronate. Molecular size heterogeneity was also studied by using two different kinds of radioimmunoassay (RIA) for PTHrP (N- and C-terminal specific) coupled with gel permeation chromatography.

[1] To whom correspondence should be addressed.

CASE REPORT

A 57-year-old man was followed up at an outpatient clinic of our hospital after left pneumonectomy because of squamous cell lung carcinoma in February, 1990. He was admitted to our hospital in November, 1990, because of persistent thirst, appetite loss and drowsy state.

Laboratory data on admission are shown in Table I. The serum calcium level was 14.9 mg/dl (normal 8.4–9.8) and the value corrected for albumin was 15.8 mg/dl. The serum phosphorus level was 1.7 mg/dl (normal 2.7–4.5). Blood gas analysis revealed metabolic alkalosis. The nephrogenous cAMP was 9.0 nmol/dl GFR (normal 0.8–2.8) and serum $1,25\ (OH)_2D_3$ was 32 pg/ml (normal 20–76). Serum parathyroid hormone (PTH) concentration, determined by two different RIA kits (one specific for the middle portion and the other for the intact PTH), were below the limit of sensitivity and within the normal range, respectively. Marked granulocytosis (WBC, 51,500; granulocytes, 95%) was noted, but serum granulocyte(G)- and granulocyte macrophage-colony stimulating factor (GM-CSF) determined by using EIA kits

Table I. Laboratory Data on Admission

CBC			Blood chemistry		
RBC ($\times 10^4$)	465/mm^3	(410–530)	T.P.	7.4	g/dl	(6.7–8.3)
Hb	13.0 g/dl	(14–18)	Alb.	3.1	g/dl	(3.8–4.7)
Ht	40.1%	(40–48)	GOT	39	IU/liter	(11–37)
WBC	51500/mm^3	(4900–8400)	GPT	37	IU/liter	(7–30)
Band	14.5%		γ-GTP	53	IU/liter	(10–60)
Seg	79.5%		T. Bili.	0.5	mg/dl	(0.3–1.0)
Eosino	0.5%		ALP	118	IU/liter	(45–130)
Lymph	2.5%		LAP	55	IU/liter	(17–54)
Mono	3.0%		LDH	484	IU/liter	(200–370)
PLT ($\times 10^4$)	43/mm^3	(12–30)	BUN	32.2	mg/dl	(8.5–20)
Urinalysis			Creat.	1.2	mg/dl	(0.7–1.3)
Protein	(−)		U.A.	8.2	mg/dl	(3.7–7.8)
Glucose	(−)		Na	147	mEq/liter	(135–145)
Urobilinogen	Normal		K	2.3	mEq/liter	(3.5–4.6)
Sediment	N.P.		Cl	91	mEq/liter	(100–108)
Serological test			Ca	14.9	mg/dl	(8.4–9.8)
CRP	(3+)		P	1.7	mg/dl	(2.7–4.5)
SCC	1.7 ng/ml	(<1.5)	Blood gas analysis			
CEA	4.7 ng/ml	(<7.0)	pH	7.51		(7.38–7.42)
			Po2	88.2	mmHg	(85)
			PCO2	50.9	mmHg	(40)
			HCO^{3-}	40.3	mEq/liter	(23–28)
			BE	14.3		(−3–+3)

NcAMP	9.0	nmol/dl·GFR	(0.8–2.8)
$1,25(OH)_2D_3$	32	pg/ml	(20–76)
PTH-Int	<5	pg/ml	(23–73)
PTH-M	470	pg/ml	(230–560)
PTHrP-N	27	pmol/liter	(<5)
PTHrP-C	1408	pmol/liter	(<50)

Values in parenthesis represent the normal range.
NcAMP, nephrogenous cAMP; PTH-Int and M, PTH-immunoreactivities determined by specific RIAs for intact and middle portion of PTH; PTHrP-N and -C, PTHrP-immunoreactivities determined by specific RIAs for N- and C-terminal portion of PTHrP.

(Chugai Pharmaceutical Co. Ltd., Tokyo) were below the limit of sensitivity. The other abnormal data included serum albumin (3.1 g/dl), LDH (484 IU/liter), K (2.3 mEq/liter), Cl (91 mEq/liter), C-reactive protein (3+) and SCC antigen (1.7 ng/ml).

The patient was treated with 60 mg of pamidronate given DIV for 2 h. Serum calcium was decreased to 10.2 mg/dl (corrected for albumin) at day 4, and then gradually increased to the level of 11.3 mg/dl at day 10 (Fig. 1). However, he died of septic shock. At autopsy, metastatic tumor tissue and pleural fluid were obtained for Northern blot analysis, immunohistochemistry and chromatographic analysis. Bone metastasis was not found.

MATERIALS AND METHODS

RIA Two RIAs specific for N- and C-terminal portions of PTHrP were employed. In the N-terminal-specific RIA (PTHrP-N RIA), the antiserum (#23) used was raised against human(h)PTHrP(1-34) in a rabbit, and did not crossreact with hPTH(1-34) or hPTH(1-84) (<500 nmol/liter). hPTHrP(1-34) and ^{125}I-Tyr-hPTHrP-(1-34), iodinated by the chloramine T method,[9] were used as the standard and the tracer, respectively. The assay procedure was described previously.[10] Intra- and interassay coefficients of variation were less than 8.3% and 12.5% (n=5), respectively and the sensitivity of the assay was 5 pmol/liter. The C-terminal specific RIA (PTHrP-C RIA) was carried out according to the procedure described by Kasahara *et al.*[11] The antiserum used was raised against hPTHrP(109-141), and did not crossreact with hPTHrP(1-34), hPTH(1-84), hPTH(39-84) or calcitonin. Intra- and interassay coefficients of variation were less than 10%, and the sensitivity of the assay was 2 pmol/liter. A parallel displacement of the dilution curve of serum samples with RIA standards was obtained in both RIAs.

PTH was measured by using two different RIA kits, one specific for the middle portion (PTH-M) (Yamasa Shoyu Co. Ltd., Tokyo) and the other for the intact PTH (Japan Mediphysics Co., Tokyo).

Gel permeation chromatography Gel permeation chromatography of the serum sample and pleural fluid was performed on a Sephadex G-75 column (95×1.4 cm). Two ml samples were layered onto the column, and eluted with 1 *M* acetic acid containing 0.1% BSA at a rate of 7 ml/h at 4°C. Fractions (1.3 ml) were collected, dried and reconstituted with buffer before assay. The column was calibrated with protein markers (Vo, catalase; 43 kDa, ovalbumin; 25 kDa, chymotrypsinogen A; 13.7 kDa, ribonuclease).

Immunohistochemistry Paraffin-embedded sections of the tumor tissue obtained at autopsy were immunostained using the PTHrP antiserum (#23) specific for the N-terminal portion according to the ABC method.[12]

Northern blot analysis Total RNA was extracted from the tumor tissue obtained at autopsy by the guanidium thiocyanate technique. Aliquots of total RNA were electrophoresed on a 1% agarose-formaldehyde gel, transferred to a nylon membrane and immobilized with UV light. A segment (522 bp; −36 to +138 amino acids of preproPTHrP) of full-length PTHrP cDNA (gift from Dr. T. Watanabe, Cancer Institute, Tokyo) was amplified by polymerase chain reaction (PCR), and was labeled with ^{32}P-dCTP to a specific activity of 1.5×10^9 dpm/μg DNA by the random priming technique. Blots were hybridized with 1×10^6 cpm/ml ^{32}P-labeled cDNA probe for 12 h at 48°C according to the method described previously.[13] Autoradiography was performed using Fuji X-ray film (AIF RX, Fuji Photo Film Co., Kanagawa) for 60 h at −70°C in the presence of an intensifying screen.

RESULTS

PTHrP concentration in the serum and pleural effusion and its change during treatment of hypercalcemia with pamidronate Serum PTHrP concentrations in normal subjects were below the limit of sensitivity (<5 pmol/liter) for PTHrP-N-like immunoreactivity (LI) (n=60) and 21–51 pmol/liter (mean±SD, 31±7.4 pmol/liter; n=76) for PTHrP-C-LI. Therefore, the normal range was defined as less than 5 pmol/liter for PTHrP-N-LI and less than 50 pmol/liter (the mean+2SD of the normal subjects) for PTHrP-C-LI, respectively. Serum PTHrP-N- and PTHrP-C-LI concentrations of the patient were 27 and 1408 pmol/liter, respectively. As shown in Fig. 1, serum calcium levels (corrected for albumin) dropped from 15.8 to 10.2 mg/dl at day 4 after treatment with pamidronate, and were maintained around this level until day 10. Increase of serum PTH concentration determined by an RIA kit for PTH-M in response to the decline of serum calcium levels was observed, while serum PTHrP-N-LI concentration tended to be de-

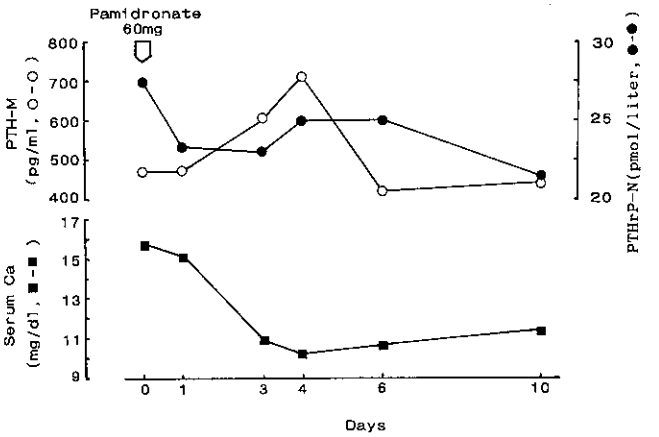

Fig. 1. Changes of serum calcium (lower panel), PTH-M (upper panel) and PTHrP-N (upper panel) concentrations after DIV administration of pamidronate (60 mg).

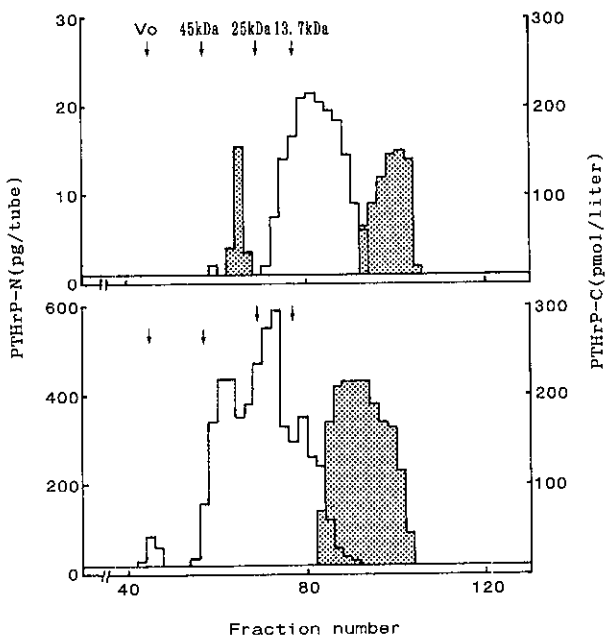

Fig. 2. Gel permeation chromatography of PTHrP-N (□) and PTHrP-C (▨) in serum (upper panel) and pleural fluid (lower panel) obtained from the patient on a Sephadex G-75 column (95×1.4 cm). The column was calibrated with protein markers (Vo, catalase; 45 kDa, ovalbumin; 25 kDa, chymotrypsinogen A; 13.7 kDa, ribonuclease). The concentrations of PTHrP-N and PTHrP-C are expressed as pg/tube and pmol/liter, respectively.

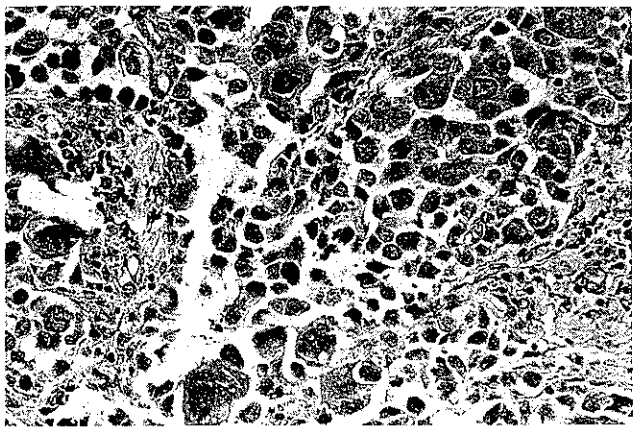

Fig. 3. Immunostaining of PTHrP in a section of the tumor tissue obtained at autopsy (×200).

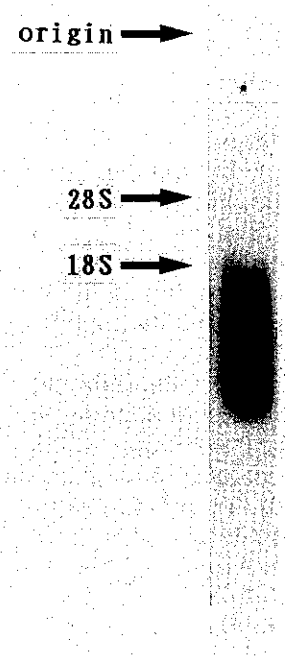

Fig. 4. Northern blot analysis of the PTHrP gene. Total RNA was extracted from the tumor tissue obtained at autopsy. Ten micrograms of total RNA was electrophoresed and subjected to Northern blot analysis as described in "Materials and Methods."

creased (Fig. 1). Serum PTHrP-C-LI concentration, on the other hand, did not seem to be altered during the treatment (data not shown). PTHrP-N- and PTHrP-C-LI concentrations in the pleural fluid were 946 and 5983 pmol/liter, respectively.

Gel permeation chromatography Figure 2 shows the elution profiles of PTHrP-LIs in the serum and pleural effusion on Sephadex G-75. One major peak of PTHrP-N-LI close to 13.7 kDa and two peaks of PTHrP-C-LI, one between 45 kDa and 25 kDa and the other far smaller than 13.7 kDa, were found in the serum (Fig. 2, upper panel), while several peaks of PTHrP-N-LI between Vo and 13.7 kDa and one peak of PTHrP-C-LI smaller than 13.7 kDa were found in the pleural effusion (Fig. 2, lower panel).

Immunohistochemistry As shown in Fig. 3, numerous cells in the tumor tissue were positively stained for PTHrP.

Northern blot analysis A hybridization band around 18S was detected in total RNA extracted from the tumor tissue (Fig. 4).

DISCUSSION

The patient revealed hypercalcemia, hypophosphoremia and increased nephrogenous cAMP. However, serum PTH and $1,25(OH)_2D_3$ levels remained in the normal range. These biochemical features are similar to those of HHM. In fact, the serum PTHrP concentration was elevated in the patient and a high concentration of PTHrP was also present in the pleural effusion. Finally, the Northern blot analysis and immunohistochemical study confirmed production of PTHrP in the tumor tissue of the patient. Therefore, PTHrP could have been produced in the squamous cell lung carcinoma and secreted into the blood stream, causing HHM in the patient.

Regulation of PTHrP secretion is not well understood. Available data about the effect of extracellular calcium on PTHrP secretion are inconsistent among the cell lines used.[14-19] In the present study, hypercalcemia was treated with pamidronate and a decrease of serum calcium was observed. A transient increase of serum PTH was noted during the course of the treatment, while serum PTHrP determined by PTHrP-N RIA was decreased in response to the decrease of serum calcium, although serum PTHrP-C-LI was unchanged. Rizzoli and Bonjour[17] found a stimulatory effect of extracellular calcium on PTHrP production in rat Leydig tumor cells associated with HHM *in vitro*. Henderson *et al.*[19] reported that reduction of extracellular calcium reduced PTHrP secretion from keratinocytes transfected with HPV16 DNA or pSV2ras DNA. The reduction of serum PTHrP in response to decrease of serum calcium seen in the present patient is consistent with the findings described by Rizzoli and Bonjour[17] and Henderson *et al.*[19] However,

further studies with many patients will be needed in order to elucidate the regulation of PTHrP secretion in humans.

Molecular heterogeneity of PTHrP was found in a variety of tumors or cell lines.[20,21] A PTHrP molecule contains numerous basic amino acid residues among residues 84–108, suggesting that proteolytic cleavage into small fragments may occur.[1] The presence of PTHrP(1–74) in the human plasma was demonstrated by using immunoradiometric assay.[7] In the present gel permeation chromatography, PTHrP-N-LI with an apparent molecular weight of 13.7 kDa was found. On the other hand, two PTHrP-C-LIs, one with an apparent molecular weight of 30 kDa and the other with a molecular weight of less than 13.7 kDa, were found. The smaller one seems to be a C-terminal fragment of PTHrP, but the nature of the larger one is unclear. In the pleural effusion, the molecular form of PTHrP-N-LI was larger than that in the serum. Similarly, the molecular form of PTHrP-C-LI in the pleural effusion was larger than that of the smaller form in the serum, although the larger molecular form (around 30 kDa) of PTHrP-C-LI was not found in the pleural effusion. Numerous tumor cells were found in the pleural effusion, so destruction of the tumor cells together with low protease activity as compared to that in serum may result in the presence of such larger molecular forms of PTHrP-LIs.

Granulocytosis was sometimes accompanied with hypercalcemia in patients with squamous cell carcinoma, and CSF, which was produced by the tumors, has been shown to induce such granulocytosis.[22] In our patient, granulocytosis was also noted, though G- and GM-CSF were not detected in the serum. The granulocytosis did not seem to be related to CSF in the patient. Cytokines and/or unknown substances may be involved in the pathogenesis.

In conclusion, we have demonstrated production and gene expression of PTHrP in squamous cell lung carcinoma of a patient who had presented with HHM. Serum PTHrP-LI, which was elevated in the patient, could have been derived from the tumor and caused HHM. Molecular heterogeneity of PTHrP in the serum and pleural effusion was apparent.

(Received July 22, 1992/Accepted January 27, 1993)

REFERENCES

1) Suva, L. J., Winslow, G. A., Wettenhall, R. E. H., Hammonds, R. G., Moseley, J. M., Diefenbach-Jagger, H., Rodda, C. P., Kemp, B. E., Rodriguez, H., Chen, E. Y., Hudson, P. J., Martin, T. J. and Wood, W. I. A parathyroid hormone-related protein implicated in malignant hypercalcemia: cloning and expression. *Science,* **237,** 893–896 (1987).
2) Kemp, B. E., Moseley, J. M., Rodda, C. P., Ebeling, P. R., Wettenhall, R. E. H., Stapleton, D., Diefenbach-Jagger, H., Ore, F., Michelangeli, V. P., Simmons, H. A., Raisz, L. G. and Martin, T. J. Parathyroid hormone-related protein of malignancy: active synthetic fragments. *Science,* **238,** 1568–1570 (1987).
3) Yates, A. J. P., Gutierrez, G. E., Smolens, P., Travis, P. S., Katz, M. S., Aufdemorte, T. B., Boyce, B. F., Hymer, T. K., Poser, J. W. and Mundy, G. R. Effects of a synthetic peptide of a parathyroid hormone-related protein on calcium homeostasis, renal tubular calcium reabsorption, and bone metabolism *in vivo* and *in vitro* in rodents. *J. Clin. Invest.,* **81,** 932–938 (1988).
4) Ikeda, K., Mangin, M., Dreyer, B. E., Webb, A. C., Posillico, J. T., Stewart, A. F., Bander, N. H., Weir, E. C., Insogna, K. L. and Broadus, A. E. Identification of transcripts encoding a parathyoid hormone-like peptide in messenger RNAs from a variety of human and animal tumors associated with humoral hypercalcemia of malignancy. *J. Clin. Invest.,* **81,** 2010–2014 (1988).
5) Stewart, A. F., Elliot, J., Burtis, W. J., Wu, T. and Insogna, K. L. Synthetic parathyroid hormone-like protein-(1-74): biochemical and physiological characterization. *Endocrinology,* **124,** 642–648 (1989).
6) Rizzoli, R., Sappino, A. P. and Bonjour, J.-P. Parathyroid hormone-related protein and hypercalcemia in pancreatic neuro-endocrine tumors. *Int. J. Cancer,* **46,** 394–398 (1990).
7) Burtis, W. J., Brady, T. G., Orloff, J. J., Ersbak, J. B., Warrell, R. P., Olson, B. R., Wu, T. L., Mitnich, M. E., Broadus, A. E. and Stewart, A. F. Immunochemical characterization of circulating parathyroid hormone-related protein in patients with humoral hypercalcemia of cancer. *N. Engl. J. Med.,* **322,** 1106–1112 (1990).
8) Martin, T. J., Allan, E. H., Caple, I. W., Care, A. D., Danks, J. A., Diefenbach-Jagger, H., Ebeling, P. R., Gillespie, M. T., Hammonds, G., Heath, J. A., Hudson, P. J., Kemp, B. E., Kubota, M., Kukreja, S. C., Moseley, J. M., Ng, K. W., Raisz, L. G., Rodda, C. P., Simmons, H. A., Suva, L. J., Wettenhall, R. E. H. and Wood, W. I. Parathyroid hormone-related protein: isolation, molecular cloning, and mechanism of action. *Recent Prog. Horm. Res.,* **45,** 467–506 (1989).
9) Hunter, W. M. and Greenwood, J. C. Preparation of iodine-131 labelled human growth hormone of high specific activity. *Nature,* **194,** 495–497 (1962).
10) Katakami, H., Hidaka, H., Matsukura, S., Tamura, K., Yokogoshi, Y., Saito, S. Plasma PTH-related protein-like immunoreactivity in patients with humoral hypercalcemia of malignancy. *Proc. Jpn. Cancer Assoc., 48th Annu. Meet.,* 331 (1989).

11) Kasahara, H., Tsuchiya, M., Adachi, R., Horikawa, S., Tanaka, S. and Tachibana, S. Development of a C-terminal region specific radioimmunoassay of parathyroid hormone-related protein. *Biomed. Res.*, **13**, 155–161 (1992).
12) Hsu, S. M., Raine, L. and Fanger, H. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J. Histochem. Cytochem.*, **29**, 577–580 (1981).
13) Sambrook, J., Fritsch, E. F. and Maniatis, T. "Molecular Cloning," 2nd Ed., pp. 7.37–7.52 (1989). Cold Spring Harbor Laboratory Press, New York.
14) Connor, C. S., Drees, B. M., Thruston, A., Forte, L., Hermreck, A. S. and Hamilton, J. W. Bovine parathyroid tissue: a model to compare the biosynthesis and secretion of parathyroid hormone and parathyroid hormone-related peptide. *Surgery*, **106**, 1057–1062 (1989).
15) Ikeda, K., Weir, E. C., Sakaguchi, K., Burtis, W. J., Zimering, M., Mangin, M., Dreyer, B. E., Brandi, M. L., Aurbach, G. D. and Broadus, A. E. Clonal rat parathyroid cell line expresses a parathyroid hormone-related peptide but not parathyroid hormone itself. *Biochem. Biophys. Res. Commun.*, **162**, 108–115 (1989).
16) Zajac, J. D., Callaghan, J., Eldridge, C., Hannelore, D.-J., Suva, L. J., Hudson, P., Moseley, J. M., Michelangeli, V. P. and Pasquini, G. Production of parathyroid hormone-related protein by a rat parathyroid cell line. *Mol. Cell. Endocrinol.*, **67**, 107–112 (1989).
17) Rizzoli, R. E. and Bonjour, J.-P. High extracellular calcium increases the production of parathyroid hormone-like activity by cultured Leydig tumor cells associated with humoral hypercalcemia. *J. Bone Miner. Res.*, **4**, 839–844 (1989).
18) Brandt, D. W., Pandol, S. J. and Deftos, L. J. Calcium-stimulated parathyroid hormone-like protein secretion: potentiation through a protein kinase-C pathway. *Endocrinology*, **128**, 2999–3004 (1991).
19) Henderson, J., Sebag, M., Rhim, J., Goltzman, D. and Kremer, R. Dysregulation of parathyroid hormone-like peptide expression and secretion in a keratinocyte model of tumor progression. *Cancer Res.*, **51**, 6521–6528 (1991).
20) Stewart, A. F. and Broadus, A. E. Clinical review 16. Parathyroid hormone-related proteins: coming of age in the 1990s. *J. Clin. Endocrinol. Metab.*, **71**, 1410–1414 (1990).
21) Brandt, D. W., Burton, D. W., Gazdar, A. F., Oie, H. E. and Deftos, L. J. All major lung cancer cell types produce parathyroid hormone-like protein: heterogeneity assessed by high performance liquid chromatography. *Endocrinology*, **129**, 2466–2470 (1991).
22) Kondo, Y., Sato, K., Ohkawa, H., Ueyama, Y., Okabe, T., Sato, N., Asano, S., Mori, M., Ohsawa, N. and Kosaka, K. Association of hypercalcemia with tumors producing colony-stimulating factor(s). *Cancer Res.*, **43**, 2368–2374 (1983).