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CASE

Parathyroid Hormone Expression in a Patient with Metastatic Nasopharyngeal Rhabdomyosarcoma and Hypercalcemia

Katy Wong, Shawn Tsuda, Ryan Mukai, Kenneth Sumida, and Richard Arakaki

Department of Medicine, John A. Burns School of Medicine, University of Hawaii-Manoa, Hawaii

Ectopic PTH secretion by tumor cells has been described as the cause of hypercalcemia associated with malignancy in the absence of osteolytic bone lesions. Although there have been case reports of elevated PTH and hypercalcemia in patients with rhabdomyosarcoma, to date ectopic PTH secretion by malignant cells has not been definitively shown. The possibility of PTH production by pleural-based metastatic nasopharyngeal rhabdomyosarcoma cells in a 62-yr-old Japanese male with hypercalcemia was investigated. The patient's serum PTH level was found to be elevated at 62.22 pmol/L, and pleural fluid PTH level was 47.28 pmol/L and PTHrP level was 3.7 pmol/L. RT-PCR of mRNA extracted from rhabdomyosarcoma cells in the pleural fluid was performed with the addition of PTH and PTHrP exonic primer sets yielded only a cDNA fragment of approx 150 bp consistent with the expected PTH fragment. Sequence analysis of a nested primer PCR fragment confirmed PTH mRNA sequence. We believe this patient to have had hypercalcemia secondary to ectopic PTH secretion, as we have identified the presence of PTH mRNA in tumor cells. We speculate that the overexpression of PTH in rhabdomyosarcoma cells results from molecular rearrangement of the PTH gene. The finding of a normal PTH DNA sequence of the PCR fragment suggests the likelihood of alterations in regulatory sequences.

Key Words: PTH; mRNA; nasopharyngeal rhabdomyosarcoma.

Introduction

In 1941 Fuller Albright first proposed that hypercalcemia associated with malignancy, in the absence of osteolytic bone lesions, is due to secretion of a parathyroid hormone (PTH)–like substance by tumor cells *(1)*. Multiple mediators, such as prostaglandin E2, cytokines, and osteoclast-activating factor, account for the humoral hypercalcemia of malignancy (HHM), yet the predominant mediator is parathyroid hormone–related peptide (PTHrP) *(2,3)*. PTHrP is expressed by many tumors including squamous, breast, and renal cell carcinomas, and has a 70% homology of the first 13 amino acids with PTH exerting its effects through the PTH receptor *(4)*.

In very rare instances, ectopic PTH secretion by tumor cells has been described as the cause of hypercalcemia associated with malignancy in the absence of osteolytic bone lesions *(5–10)*. Although there have been case reports of elevated PTH and hypercalcemia in patients with rhabdomyosarcoma, to date ectopic PTH secretion by rhabdomyosarcoma cells has not been definitively shown *(11–13)*. In this paper, we describe ectopic PTH secretion to account for hypercalcemia in an individual with nasopharyngeal rhabdomycosarcoma and confirm the presence of PTH mRNA in tumor cells.

Case Presentation

A 62-yr-old Japanese male was diagnosed with metastatic nasopharyngeal rhabdomyosarcoma in September of 1995, and initially treated with neoadjuvant chemotherapy with alternating cycles of vincristine, actinomycin-D, and cytoxan; and vincristine, adriamycin, and cytoxan. He also received radiation treatment and resection of minimal residual disease. He was continued on the same course of chemotherapy for 2 yr. Two months following his last course of chemotherapy, he developed malignant pleural effusion that required several thoracenteses for symptomatic relief. He was subsequently given salvage chemotherapy with decadron and taxotere.

By the fall of 1997, he had completed his third cycle of salvage chemotherapy, when he presented with weakness, fatigue, and confusion. Admission serum calcium level was 3.89 mmol/L (normal range: 2.09–2.54 mmol/L) with an albumin level of 30 g/L (normal range: 34–50 g/L). Serum calcium level 2 mo prior was only 2.15 mmol/L. The patient's hypercalcemia was treated with aggressive fluid hydration and 90 mg of pamidronate given intravenously over a 24-h period. His serum calcium levels gradually decreased over 5 d to 2.44 mmol/L. A bone scan revealed no focal abnor-

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Author to whom all correspondence and reprint requests should be addressed: Richard Arakaki, MD, Department of Medicine, 1356 Lusitana Street, 7th Floor, Honolulu, Hawaii 96813. E-mail: rfarakak@hawaii.edu

malities to suggest metastatic disease to bone. A computed tomography scan of the chest revealed diffuse pleural-based metastases with a left hilar mass extending into the mediastinum, and subcarinal adenopathy. Admission laboratory results revealed a BUN and creatinine of 12.50 mmol/L (normal range: 2.14–6.78 mmol/L) and 132.6 μmol/L (normal range: 35.36–97.24 μmol/L), respectively, phosphorus level was 0.872 mmol/L (normal range: 0.839–1.453 mmol/L), and a serum PTH level was found to be elevated at 62.22 pmol/L (normal range: 0.74–5.62 pmol/L). Serum PTHrP level was not performed on admission. Nine days into his hospitalization, which was a day prior to his discharge, the patient underwent thoracentesis with cytological evaluation of pleural cells confirming the presence of rhabdomycosarcoma cells. The patient's serum calcium level was 1.65 mmol/L (normal when corrected for albumin level), BUN and creatinine decreased to 4.64 mmol/L and 114.9 μmol/L, respectively, and the blood PTH level was decreased from admission levels to 13.46 pmol/L.

Two months later, after completion of 4 wk of radiation therapy to the chest, the patient was readmitted to the hospital with a calcium level of 4.34 mmol/L. Serum phosphorus level was 1.48 mmol/L, BUN and creatinine levels were 22.5 mmol/L and 335.9 μmol/L, respectively, and the serum PTH level was 59.78 pmol/L. The patient had a protracted hospital course and died from respiratory failure due to pneumonia. A postmortem examination was not performed.

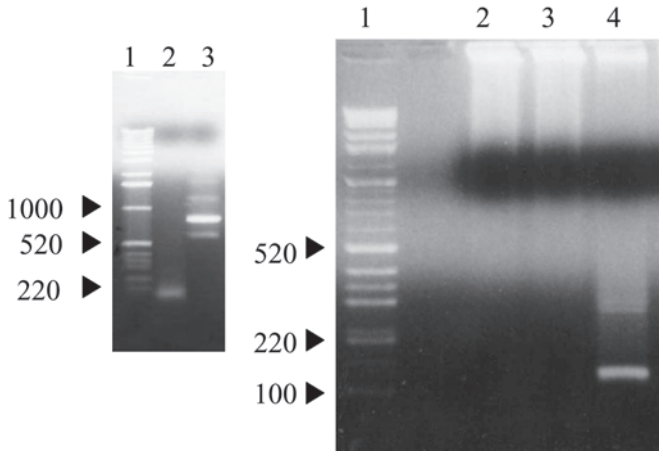

Fig. 1. A three-primer set combined RT-PCR and nested PCR reaction with primer-set for PTH of mRNA from rhabdomyosarcoma cells. mRNA from malignant cells was extracted as described in Methods. All three primer sets for PTH, PTHrP, and actin along with reverse transcriptase, Taq polymerase, nucleotides, and buffer with 1 m*M* MgCl2 were included in a single reaction mixture. The results of the reaction were examined with a 2 % agarose gel electrophoresis. Left panel: lane 1: 1 kb DNA ladder; lane 2: combined reaction mixture; lane 3: human actin control reaction. Nested primer set for PTH was added to separate PCR using the isolated 150 bp fragment from the three-primer set combined RT-PCR. The results of the reaction were examined with a 2% agarose gel electrophoresis. Right panel: lane 1: 1 kb DNA ladder; lane 2: no primers; lane 3: PTHrP primer set; lane 4: PTH primer set.

Results

The PTH level in the pleural fluid was 47.28 pmol/L, and the PTHrP level in the pleural fluid was 3.7 pmol/L. Although measured ranges for these hormones in pleural fluid or other body cavity fluids have not been established, the high levels of PTH indicate secretion from pleural-based malignant cells, because the blood level of PTH was 13.46 pmol/L.

RT-PCR of mRNA from rhabdomyosarcoma cells with the addition of all three sets of primers yielded an approx 150 bp fragment consistent with the expected PTH fragment (Fig. 1, left panel). The approximate and predicted 300 bp PTHrP and 800 bp actin fragments were not observed. Additionally, only the 150 bp fragment of PTH was observed in separate RT-PCR reactions with individual primer sets for PTH and PTHrP (Fig. 1, right panel). Nested primer PCR amplification of the purified PTH fragment produced the approximate and expected 100 bp fragment, and sequence analysis confirmed the PTH mRNA sequence. When compared to the published PTH sequence, sequence abnormalities within the 102 bp fragment were not observed *(14)*.

Because the primer sets used in the RT-PCR reactions were exonic oligomers used to differentiate between PTH and PTHrP mRNA, the potential for amplification of PTH genomic DNA of the oligo-dT mRNA extraction remained possible. Thus, RT-PCR and subsequent PCR were also performed with PTH intron 2 sense primer (5'-GTACTGTTTT GCCTTGGA-3') and initial and nested, antisense exon 2 primers (nucleotide sequence 201–533; ref. *15*) as described above. Results of these amplifications failed to demonstrate any cDNA fragments of approx 330 bp (predicted size 332 bp, data not shown). Therefore, genomic DNA in oligo-dT extraction did not contribute to the finding of an overabundance of PTH cDNA fragments.

Discussion

Humoral hypercalcemia of malignancy in the absence of osteolytic bone lesions is mediated predominantly by PTHrP and is associated with a suppressed PTH level *(16)*. Rarely is ectopic secretion of PTH responsible for HHM; however, in this patient, the serum PTH level was markedly elevated. We believe this patient to have had hypercalcemia secondary to ectopic PTH secretion, as we have identified the presence of PTH mRNA in tumor cells. Because a postmortem examination was not performed, the parathyroid glands were not studied, and the possibility of concomitant primary hyperparathyroidism with malignancy could have existed. However, we feel that this situation is unlikely. First, the patient's clinical presentation is not typical of primary hyperparathyroidism, where patients often present with a chronic course of asymptomatic hypercalcemia. The patient's calcium level was not elevated 2 mo prior to the admission,

which suggested a newly activated process such as malignancy. Second, high PTH levels as compared with PTHrP levels were detected in the pleural fluid where rhabdomyosarcoma cells were clearly found. Third, using the RT-PCR method, we have confirmed PTH mRNA in these rhabdomyosarcoma cells. Strikingly, only the PTH fragment was observed in RT-PCR reactions of mRNA from these cells when examined in separate or combined reactions with primers for PTHrP and Actin. This finding suggests high PTH mRNA expression in these rhabdomyosarcoma cells. The sequence analysis did not identify any mutations in the 100 bp fragment, which suggests that changes within the untranslated region of the PTH mRNA may account for the high expression.

In patients with rhabdomyosarcoma, the findings of elevated PTH levels and hypercalcemia have been reported by other investigators *(11–13)*. However, confirmation of PTH secretion by malignant rhabdomyosarcoma cells has not been demonstrated. Ectopic PTH secretion has been documented in patients with other malignancies, such as small cell carcinoma of the lung, papillary adenocarcinoma of the thyroid, and thymoma *(5,7,8)*. It is unclear why a rhabdomyosarcoma, a nonparathyroid tumor, and in particular arising from undifferentiated mesenchymal tissue, produced an endocrinopathy.

In a study of ectopic PTH production in a patient with ovarian carcinoma and hypercalcemia, genomic analysis of tumor DNA identified an increase in copy number and rearrangement in the 5' upstream regulatory region of the PTH gene *(9)*. These findings suggested the possibility of uncovering an enhancer sequence or deleting a suppressor sequence to account for high PTH mRNA expression. We speculate that the overexpression of PTH in rhabdomyosarcoma cells results from a similar molecular rearrangement of the PTH gene. The finding of a normal PTH DNA sequence of the PCR fragment suggests the likelihood of alterations in regulatory sequences. Sequence determination of the regulatory region of the PTH gene will provide a greater understanding of PTH overexpression in these rhabdomyosarcoma cells. The development of hypercalcemia with the progression of this patient's malignancy, suggests *de novo* changes of the PTH gene or activation of suppressor/enhancer genes in these rhabdomyosarcoma cells. Although unlikely, but certainly possible, is that these changes could have resulted from exposure to the various DNA-targeted chemotherapeutic agents such as taxotere, cytoxan, actinomycin-D, and adriamycin. There is also the possibility that oncogenic activation such as *ras* mutations could result in PTH gene amplification with protein production that result in hypercalcemia.

Materials and Methods

Examination of pleural fluid and analysis of cells were performed with informed consent obtained from the patient. The pleural fluid was obtained by thoracentesis prior to discharge during the patient's fall admission in 1997.

PTH and PTH-rp Determination

Serum PTH level and pleural fluid PTH and PTHrP levels were measured in single assays by Quest Diagnostics at Nichols Institute (San Juan Capistrano, CA) using an immunochemiluminometric assay (ICMA) that detects intact PTH, and an immunoradiometric assay (IRMA) for PTHrP.

RNA Extraction, RT-PCR, and PCR Sequencing

The rhabdomyosarcoma cells were isolated from pleural fluid by centrifugation, and stored at −70°C. RNA was extracted from these cells using the Oligotex Direct mRNA Micro Kit (Qaigen, Inc).

Oligonucleotide primers that targeted specific sequences for PTH and PTHrP were obtained from Integrated DNA Technology (Coralville, IA). The oligomers targeted PTH amino acid sequence of 25–29 (5'-CGTAAGAAGCTGC AG, sense) and 71–75 (5'-5 CACATCAGCTTTGTC, antisense) that predicted a PTH fragment of 152 bp *(14)*. A set of nested sense and antisense primers (5'-AATTTTGTTG CCCTT, sense; 5'-AAGACTTTTTTC, antisense) that predicted a 104 bp PTH fragment were obtained from Life Technologies (Grand Island, NY). Primers for PTHrP mRNA were obtained targeting the amino acid sequences 25–29 (5'-CACCATCTGATCGCA, sense) and 137–141 (5'-ATG CCTCCGTGAATC, antisense) that predicted a fragment size of 306 bp *(4)*. Nested primers for PTHrP were also obtained to examine the specificity of mRNA expression. Additionally, oligomers were obtained from Clontech, Inc (Palo Alto, CA) for human actin cDNA that predicted an 838 bp fragment and served as a control template.

The Titan One Tube RT-PCR System (Boehringer Mannheim, Indianapolis, IN) was utilized to generate DNA fragments from the patient's extracted mRNA. In final concentration, the reaction mixture contained 1.0 μg of oligo-dT RNA, 0.4 μ*M* primers, 0.2 m*M* nucleotides, 5.0 m*M* DTT, 10 IU RNAse Inhibitor, 0.5 units AMV reverse transcriptase, and Expand High Fidelity enzyme blend (Taq and Pwo DNA polymerases), RT-PCR buffer with 1.5 m*M* $MgCl_2$ buffer. All three sets of primers were initially added to a single reaction mixture, and RT-PCR was performed according to manufacturer's instructions using a Perkin Elmer thermocycler (Foster City, CA). After a single, 2-min denaturation period, a 10-cycle amplification with 30 s denaturation and annealing times, and 45 s elongation time per cycle was followed by a 25-cycle amplification, which included progressively variable elongation period (5 s per cycle) after the tenth cycle, and finished with a single, 7-min elongation time. Following the initial RT-PCR, additional 25-cycle PCR amplifications were performed with separate sets of original and nested primers using the Titan One Tube PCR system. DNA fragments used as templates for additional PCR were isolated with the Geneclean III kit from Bio 101 (La

Jolla, CA) from the three-primer set combined reactions. The DNA fragments from RT-PCR and PCR were analyzed with 2.0% agarose gel electrophoresis.

The DNA fragment from nested PTH-primer PCR was sequenced using fluorescent dye-termination method with an ABI Prism 377 DNA Sequencer (University of Hawaii Biotechnology Instrumentation and Training Facility).

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