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Defective Expression of Prohormone Convertase 4 and Enhanced Expression of Insulin-like Growth Factor II by Pleural Solitary Fibrous Tumor Causing Hypoglycemia

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Abstract. A 75-year-old man was admitted to our hospital because of unconsciousness. His plasma glucose was very low, but his serum levels of insulin and IGF-I were also low. He was found to have a giant solitary pleural tumor, which was completely resected, after which his hypoglycemia ameliorated postoperatively. Histologically, the tumor was consistent with the pathological diagnosis of a solitary fibrous tumor derived from the pleura. Immunohistochemical study revealed positive immunostaining for IGF-II in tumor cells. The presence of high molecular weight (HMW) form of IGF-II in the tumor tissue and patient's serum was confirmed by Western blot analysis. Steady-state mRNA levels of IGF-II and prohormone convertases (PC) 4, a potential protease responsible for IGF-II processing, as determined by RT-PCR were about 14-fold greater and 5-fold less in the tumor tissue than those in normal placental tissue, respectively. Therefore, it is suggested that biologically active, unprocessed HMW form of IGF-II generated from the impaired processing of IGF-II precursor by the defective PC4 expression in the tumor was responsible for the non-islet cell tumor hypoglycemia (NICTH) in the present case.

Key words: Prohormone convertase 4, Insulin-like growth factor II, Non-islet cell tumor hypoglycemia

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NON-ISLET cell tumor hypoglycemia (NICTH) is a rare cause of hypoglycemia frequently associated with a large tumor of mesenchymal origin, such as fibroma, mesothelioma, histiocytoma, hemangioblastoma and so on [1, 2]. It is now recognized that the unprocessed high molecular weight (HMW) form(s) of insulin-like growth factor (IGF)-II secreted from the tumor plays a pivotal role in the development of NICTH [2]. However, the exact molecular mechanism(s) responsible for such abnormal processing of IGF-II precursor in NICTH remains unknown.

To date, only limited information is available on the exact endoprotease(s) responsible for the physiological processing of IGF-II [3]. However, it has been recently shown that prohormone convertase (PC) 4 is involved in the processing of IGF-II precursor in human placental tissue where mature IGF-II is produced [4]. Thus PC4 may be a candidate enzyme responsible for the physiological processing of IGF-II precursor. To the best of our knowledge, the involvement of defective PC4 expression in NICTH has not been reported thus far.

We herein report a case of NICTH caused by a giant pleural solitary fibrous tumor predominantly secreting the HMW form of IGF-II in which defective PC4 gene expression may be responsible for the impaired processing of IGF-II precursor to generate this unprocessed HMW form.

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Methods

Real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the surgically resected tumor and normal placental tissue obtained from the delivery by cesarean section, using RNAzol (GIBCO/BRL, Carlsbad, CA), and cDNA synthesized using a First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ) according to the manufacturer's instructions. IGF-II and prohormone convertase (PC) 4 mRNA level was quantified with real-time quantitative RT-PCR using fluorescent SYBR green technology (LightCycler; Roche Molecular Biochemicals, Mannheim, Germany). PCR primers were synthesized by Greiner Bio-one (Tokyo, Japan). The human IGF-II primers for RT-PCR were: sense strand 5'-TCCTCCCTGGACAATCAGAC-3' and antisense strand 5'-AGAAGCACCAGCATCG ACTT-3'. The human PC4 primers were: sense strand 5'-GACCTGGAGATCTCGCTCAC-3' and antisense strand 5'-ACGTCCCCGTGTTGAAATAG-3'. The human GAPDH primers were: sense strand 5'-GCTG AGAACGGGAAGCTTGT-3' antisense strand 5'-TCT CCATGGTGGTGAAGACG-3'. The mRNA levels of the target sequence were normalized by the GAPDH mRNA levels that were used as an endogenous internal control.

DNA sequencing

Nucleotide sequence of HMW form of IGF-II transcripts in the tumor tissue was examined. First, we performed nested RT-PCR methods to obtain the cDNA fragment. The designed nucleotide sequence was: the first forward primer 5'-AAGAGTCACCAC CGAGCTT-3' and the first reverse primer 5'-GGG ATGGAACCTGATGGAAA-3'; the second forward primer 5'-AACTGCGAGGCAGAGAGACA-3' and the second reverse primer 5'-TGCAGACTTGCGG CAGTTTT-3v. Direct DNA sequencing was then performed using the cDNA fragment and the second primers.

Western blot analysis

Serum samples (2.5 ml) from the patient and a normal subject were extracted by Sep-Pak C18 cartridge (Waters Associates, Milford, MA). Tumor and placental tissues were homogenized in an assay buffer [10 mM sodium phosphate (pH 7.4), 1% (v/v) Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.15 M NaCl, 2 mM ethylenediaminetetraacetic acid and 50 mM NaF] containing protease inhibitors (10 mM leupeptin, 1 mM pepstatin and 1 mM phenylmethanesulfonyl fluoride), centrifuged at 20,000 × g for 1 h at 4°C, and the resulting supernatants were saved [5]. For immunoblotting, serum and tissue extracts were electrophoresed on a 15% SDS-polyacrylamide gel under nonreducing conditions, and the size-fractionated proteins were then electroblotted onto a nitrocellulose sheet, which was blocked with 5% (wt/vol) skim milk, and then incubated with a mouse anti-IGF-II monoclonal antibody (Upstate Biotechnology). After extensive washing, the sheet was incubated with horseradish peroxidase-conjugated anti-mouse IgG, and the complexes were detected using an enhanced chemiluminescence (ECLplus) system (Amersham).

Immunohistochemistry

Tissue sections were exposed to 5% normal goat serum for 20 min, incubated with anti-IGF-II monoclonal antibody (Upstate Biotechnology), diluted in PBS and exposed to 3-amino-9-ethylcarbazole for 5 min as reported [6].

Case Report

A 75-year-old man was brought to the emergency room of our hospital because of unconsciousness on April 2006. He had frequent facial sweating and general fatigue during the previous two weeks. He had not been taking any hypoglycemic agents. He was 165.3 cm tall and weighed 55.6 kg (body mass index: 20.3 kg/m^2). Blood pressure was 156/90 mmHg. Physical examination showed decreased breath sounds in the left lung field. Chest X-ray revealed a large solid mass (about 20 cm in diameter) in the left lung (Fig. 1A). A small mass in the left lung had been pointed out to him by routine chest X-ray in 1994 without further examination.

Initial laboratory examination showed very low plasma glucose level (29 mg/dl), which lasted over subsequent days despite continuous daily infusion of

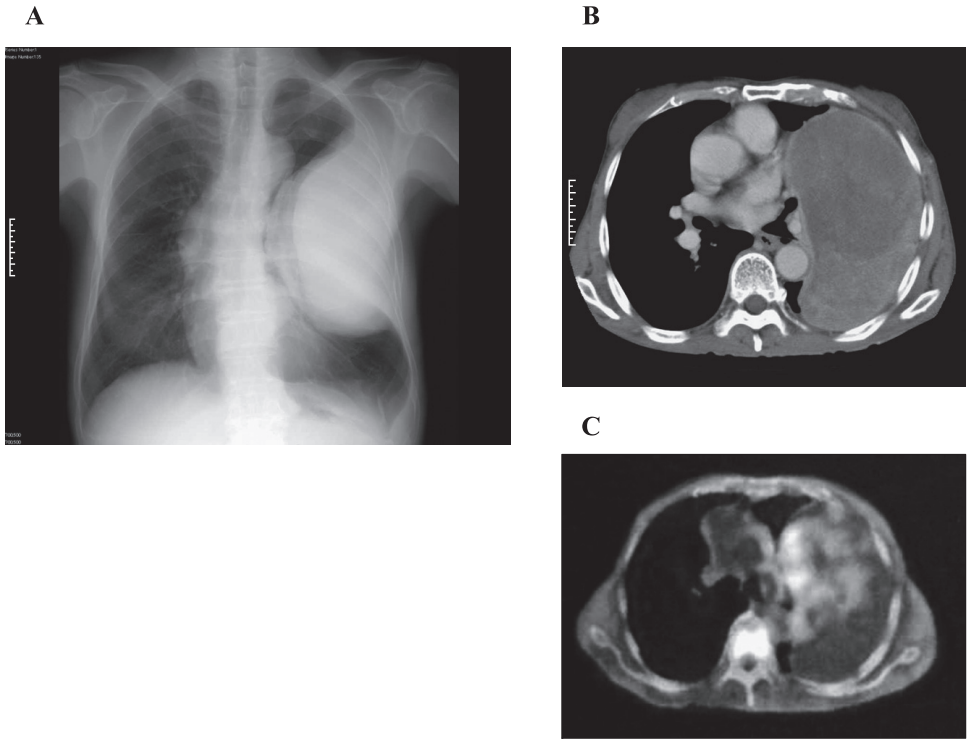


Fig. 1. Diagnostic imaging test. A) Chest X-ray: a large solid mass, approximately 20 cm in diameter, in the left intrathoracic region. B) Chest CT scan: a non-uniform large tumor with a peripheral contrast enhancement effect. C) Fluorodeoxyglucose positron-emission-tomography (FDG-PET): max SUV 3.6.

glucose. Laboratory and endocrine data are shown in Table 1. Fasting serum IRI level was undetectable (<1.0 μU/ml), and C-peptide, GH, IGF-I and IGF-binding protein (BP) 3 levels were all low. Anti-insulin antibody was negative. Oral glucose tolerance testing (oGTT) showed low IRI response. Except for the increased prolactin level, ACTH, TSH, cortisol, thyroid hormones, glucagon were all within normal ranges. Thus, insulinoma and adrenal insufficiency as a cause of his severe hypoglycemia were excluded.

A chest computed tomography (CT) scan revealed a heterogenous large mass (18 × 17 × 10 cm in size), extending from the left pleural to the thoracic cavity, with a peripheral heterogeneous contrast effect (Fig. 1B). [^{14}C]Fluorodeoxyglucose positron-emission-tomography (FDG-PET) showed a moderate uptake in the left pleural tumor (Fig. 1C).

After treatment with dexamethasone (0.5–2.0 mg/day) and continuous glucose infusion (220 g/day), plasma glucose levels were gradually elevated. He underwent operation; the left thoracic mass derived from the pleura was completely resected surgically. Postoperative course was uneventful. Plasma glucose levels increased to 141 mg/dl just after operation and remained between 71–182 mg/dl even after cessation of continuous glucose infusion (Fig. 2). He had no hypoglycemic episodes thereafter. Postoperatively, fasting plasma glucose (90 mg/dl), hemoglobin A_{1c} (4.6%), IRI (1.8 μU/ml), C-peptide (1.3 ng/ml), GH (0.99 ng/ml), IGF-I (76.8 ng/ml), IGF-BP3 (1.54 μg/ml) were all within normal limits and PRL level decreased.

Pathological, immunohistochemical, and biochemical study

The resected mass (18 × 17 × 9 cm in size, 1.36 kg in weight) was solid and lobular (Fig. 3A). The tumor cells consisted of spindle-shaped cells with atypical appearance, consistent with the pathological diagnosis of a pleural solitary fibrous tumor (Fig. 3B). IGF-II-like immunoreactivity was exclusively localized within the cytoplasm of many, but not all tumor cells by immunohistochemical analysis (Fig. 3C).

Steady-state mRNA levels of IGF-II and PC4 in the tumor tissue were about 14-fold greater than and 5-

Table 1. Laboratory and endocrine data on admission

CBC						
White blood cells	7900/mm^3					
Red blood cells	429/mm^3					
Platelets	22.2/mm^3					
Biochemistry						
Total protein	7.2 g/dl					
Blood urea nitrogen	12 mg/dl					
Creatinine	0.64 mg/dl					
Sodium	140 mEq/l					
Potassium	4.1 mEq/l					
Calcium	9.6 mEq/l					
Asparate aminotransferase	51 U/l					
Alanine aminotransferase	67 U/l					
Alkaline phosphatase	335 U/l					
Glucose	29 mg/dl					
Hemoglobin A_{1c}	5.0%					
C-reactive protein	2.5 mg/dl					
CA19-9	12.9 U/ml					
CEA	7.2 ng/ml					
NSE	5.6 ng/ml					
ProGRP	40.6 pg/ml					
Hormones						
Blood						
GH	0.38 ng/ml					
Prolactin	60.6 ng/ml					
LH	24.4 mIU/ml					
FSH	49.7 mIU/ml					
ACTH	40 pg/ml					
TSH	1.04 μU/ml					
Cortisol	11.4 μg/dl					
Free triiodothyroxine	1.79 pg/ml					
Free thyroxine	1.22 ng/dl					
Glucagon	89 pg/ml					
IRI	<1.0 μU/ml					
C-peptide	0.5 ng/ml					
IGF-I	59.7 ng/ml					
IGF-BP3	1.61 μg/ml					
Urine						
C-peptide	17.3 μg/day					
Cortisol	30.6 μg/day					
OGTT						
Time (min)	0	30	60	90	120	180
Glucose (mg/dl)	82	150	205	185	127	43
IRI (μU/ml)	<1.0	6.5	9.3	8.7	4.2	<1.0

CA19-9; carbohydrate antigen, CEA; carcinoembryonic antigen, NSE; neuron specific enolase, ProGRP; progastrin releasing peptide, IGF-I; Insulin-like growth factor-I, IGF-BP3; insulin-like growth factor binding protein 3.

fold less than those in the placental tissue, respectively (Fig. 4). Direct nucleotide sequencing of HMW form of IGF-II transcript in the tumor tissue showed no mutations at the PC4 cleavage sites (data not shown).

Western blotting of IGF-II in patient's serum extract revealed the presence of two distinct bands: a major band with a HMW form (approximately 14.3 kDa) and a minor band with a small molecular weight (7.5 kDa) (Fig 5A, lane 1), while control serum displayed only a 7.5 kDa band corresponding to the size of IGF-II (Fig. 5A, lane 2). In contrast to the immunoblotting pattern of tumor extract (Fig. 5B, lane 1) similar to that of patient's serum, the placental tissue extract contained only a 7.5 kDa band without a distinct HMW form band (Fig. 5B, lane 2).

Discussion

Recently, 'big' IGF-II has been shown to play a causative role in the development of hypoglycemia in NICTH [2]. Since the majority of IGF-II presents in tumor tissue and serum in patients with NICTH is the HMW form of IGF-II, the demonstration of the elevated HMW form of IGF-II in patient's serum is highly diagnostic for NICTH. In the present case, the diagnosis of NICTH was established by the following evidence: 1) hypoinsulinemic hypoglycemia with low IGF-I level; 2) the presence of a large pleural tumor and the disappearance of hypoglycemia after tumor resection; 3) the presence of HMW form of IGF-II in patient's serum and tumor tissue by Western blot analysis; and 4) overexpression of IGF-II mRNA and protein by the tumor as demonstrated by real-time RT-PCR and immunohistochemistry, respectively.

In most cases of NICTH, the serum levels of the HMW form of IGF-II, but not those of mature IGF-II, are elevated, suggesting that the unprocessed HMW form rather than the mature IGF-II plays a crucial role in the development of hypoglycemia [1, 7, 8]. The HMW form of IGF-II is considered to have greater bioavailability than the mature IGF-II [9]. In normal human serum, about 70–80% of the mature IGF-II form a ternary 150 kDa complex with IGF-BP3 and its acid-labile subunit. By contrast, the HMW form of IGF-II, which is not able to form the ternary complex, primarily forms a smaller binary 40 kDa complex with IGFBPs [10]. A binary complex can pass more readily through the capillary membrane due to its small molecular size to exert its insulin-like effects on target tissues via insulin receptors [9]. The increased levels of free IGF-II in the plasma of some NICTH patients have also been suggested to be involved in the devel-

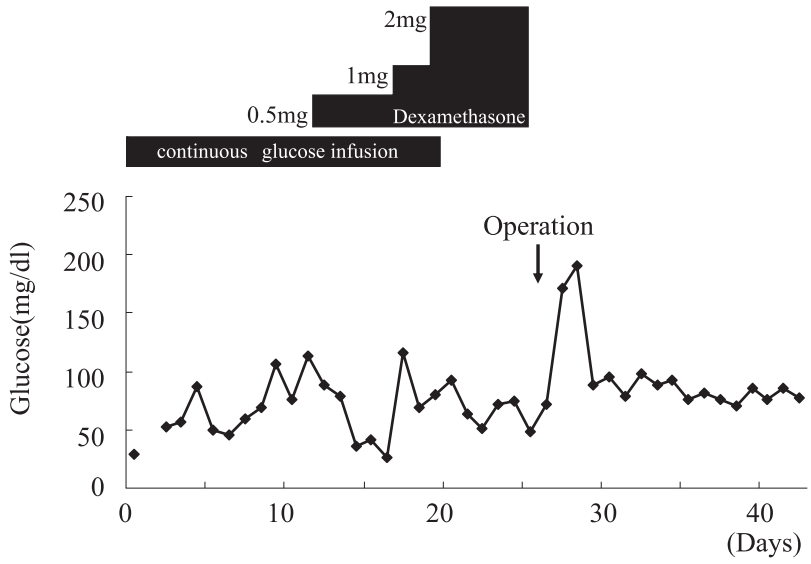


Fig. 2. Clinical course during hospitalization. Changes in blood glucose levels before and after surgery are shown. Treatment is shown in the upper panel.

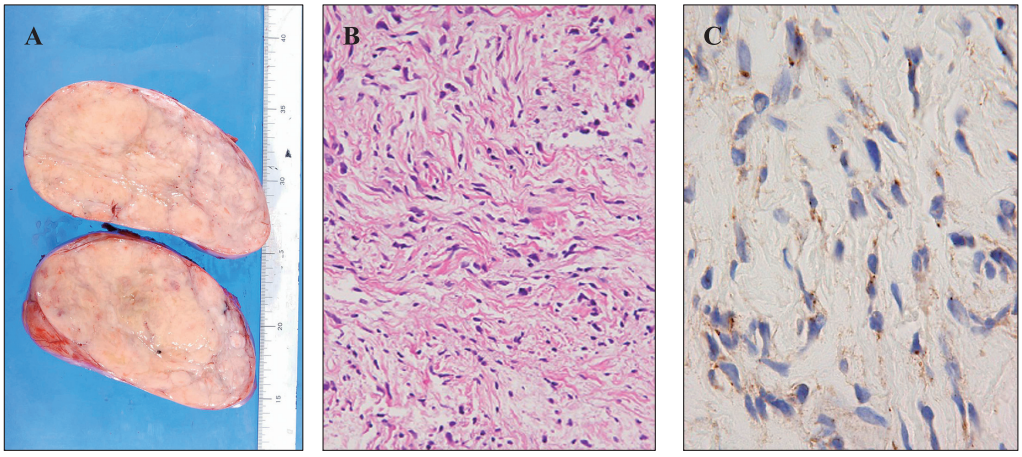


Fig. 3. Resected pleural tumor. A) Gross appearance: a solid, lobular mass (18 × 17 × 9 cm in size), B) Microscopic appearance: spindle-shaped tumor cells (HE stain, ×400), C) Immunohistochemistry: IGF-II-like immunoreactivity is localized within cytoplasm of many tumor cells (×1000).

opment of hypoglycemia [11].

The molecular mechanism(s) by which the HMW form of IGF-II is produced by tumors with NICTH remains unknown. PCs, which belong to the family of serine protease, cleave precursors of many polypeptide hormones at the C-terminal single or paired basic residues. To date, seven PC isozymes have been identified: furin, PC1/3, PC2, PC4, PACE4, PC5/PC6, and PC7. Among them, it has been recently shown that PC4 is responsible for the physiological processing of IGF-II precursor in the placenta [4]; PC4 expressed in human placenta specifically cleaves the 156-residue pro-IGF-II at Arg-104 and Arg-68 to generate a HMW form and a mature 67-residue IGF-II [4, 12].

We therefore determined and compared the steady-state mRNA levels of PC4 and IGF-II between tumor tissue and normal placental tissue by RT-PCR. The present study clearly demonstrated a markedly greater expression of IGF-II mRNA and a lesser expression of PC4 mRNA in the tumor tissue compared to those in placental tissue. Western blotting revealed more abundant expression of the HMW form compared to that of

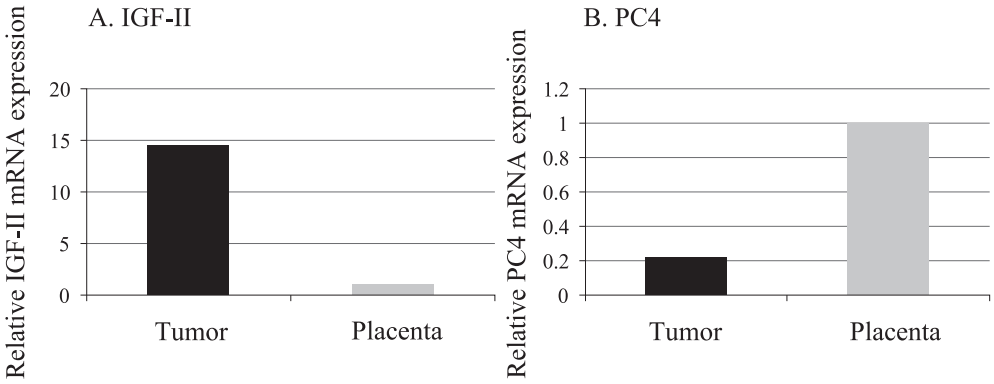


Fig. 4. Expression of IGF-II and PC4 genes in tumor and placental tissues. Expression of (A) IGF-II mRNA (B) PC4 mRNA in tumor and placenta tissues as determined by RT-PCR is shown as the ratio of each mRNA to that of GAPDH.

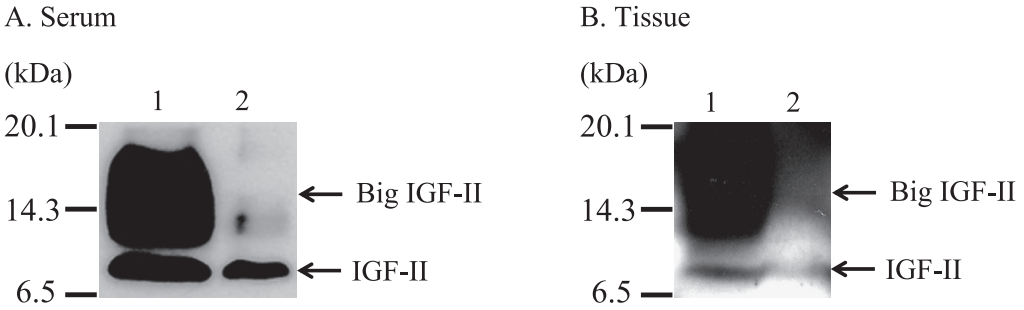


Fig. 5. Western blotting of IGF-II of serum and tissue extracts. Immunoblotting of IGF-II; (A): lanes 1) patient's serum, 2) normal serum. (B): lanes 1) tumor tissue, 2) placental tissue. Molecular size markers (kDa) are indicated by arrows in the left.

the mature IGF-II in the tumor tissue. By contrast, only the mature IGF-II, but not the big form, was detectable in placental tissue. Our data thus suggest that the defective expression of PC4, a possible processing enzyme for pro-IGF-II, may be involved in the impaired processing of pro-IGF-II, thereby leading to the predominant production of the unprocessed HMW form of IGF-II causing NICTH. To the best of our knowledge, this is the first NICTH case showing the possible involvement of defective PC 4 expression in the tumor thus far reported.

Alternatively, abnormal glycosylation of IGF-II may be responsible for the formation of the HMW form in NICTH. For example, the lack of O-linked glycosylation associated with impaired processing [13], and the differential glycosylation distinct from normal IGF-II have been reported [14]. It remains to be determined whether abnormal glycosylation of IGF-II is involved in the formation of the HMW form in our case.

Finally, it should be noted that our patient had hyperprolactinemia before surgery, which decreased after the removal of the tumors. He neither took any medications to induce PRL secretion, nor were structural hypothalamic-pituitary lesions identified by brain MRI. Ectopic production of PRL by the tumor was excluded by the negative immunostaining for PRL in the resected tumor (data not shown). Since he had a giant pleural solitary fibrous tumor, it seems large enough to mechanically stimulate afferent nerves to trigger PRL secretion, as is often observed in the irritative lesions of chest walls [15].

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