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CASE REPORT

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In situ demonstration of parathyroid hormone-related protein mRNA in sclerosing hepatic carcinoma

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Abstract A 69-year-old man had a hepatic tumour occupying the left and half of the right lobe, with portal vein thrombus. There were hypercalcaemia and hypophosphataemia with increased nephrogenous cyclic adenosine monophosphate; bone metastases were excluded. Serum parathyroid hormone-related protein (PTHrP) was elevated, but no increase in intact parathyroid hormone (PTH) or vitamin D_3 metabolites was found. At autopsy the histological features were typical of sclerosing hepatic carcinoma. By immunohistochemistry PTHrP was detected in cancer cell nests but not in the fibrous stroma. PTHrP transcripts were demonstrated by in situ hybridization using a polymerase chain reaction (PCR)-derived single-stranded DNA probe. Tumour cells expressed AE1 and CA19-9 (markers for cholangioepithelium) and CEA (for bile canaliculi). Electron microscopy revealed microvilli on the apical surface, and secretory granules 100 nm in diameter were observed. These findings indicate that this case is one of cholangiocellular sclerosing hepatic carcinoma. The interaction between cancer and stromal cells may be the cause of PTHrP overexpression.

Key words Sclerosing hepatic carcinoma · Hypercalcaemia · Parathyroid hormone-related protein (PTHrP) · Immunohistochemistry · In situ hybridization

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Introduction

Sclerosing hepatic carcinoma was originally described as a rare variant of primary hepatic malignancy characterized as carcinoma of the liver with dense stroma [13].

Clinicopathologically, sclerosing hepatic carcinoma is frequently associated with hypercalcaemia and hypophosphataemia [3, 13]. Parathyroid hormone-related protein (PTHrP), identified in 1987 [17], is thought to be a major factor responsible for humoral hypercalcaemia of malignancy (HHM). Because of its similarity to PTH in the amino-terminal amino acids, PTHrP shares the common PTH/PTHrP receptor [7] acting on bone and kidney, and PTHrP expression in sclerosing hepatic carcinoma has been detected immunohistochemically [1]. We report an autopsy case of sclerosing hepatic carcinoma associated with hypercalcaemia and demonstrate PTHrP mRNA expression on tumour cells by in situ hybridization, using a polymerase chain reaction (PCR)-derived single-stranded antisense DNA probe. Phenotypic markers for bile epithelium and hepatocytes were investigated to elucidate the origin of the tumour cells.

Clinical history

A 69-year-old man presented with upper abdominal tumour. Ultrasonography and abdominal computed tomography (CT) revealed a hepatic mass 5 cm in diameter. Hepatocelullar carcinoma was suspected; the patient had a history of chronic type B viral hepatitis. Since angiography revealed hypovascularity of the tumour, making interventions with the catheter embolization impractical, he was kept on supportive therapy only. At the last admission, laboratory data showed GOT 99 IU/l, GPT 21 IU/l, ALP 525 IU/l, AFP 584 ng/ml, Ca 12.4 mg/dl, P 1.7 mg/dl. Abdominal CT disclosed a large tumour (8 cm in diameter) in S4–8 of the liver with daughter nodules. Serum calcium level rose further to 13.5 mg/dl with increasing nephrogenous cyclic adenosine monophosphate (cAMP; 2.97 µmol/day); serum PTHrP rose to 602 pmol/l, but intact PTH and $1,25(OH)_2$ vitamin D_3 in his serum were within the normal ranges. Bone metastasis was excluded by bone scintigraphy. These data were compatible with the syndrome of HHM caused by PTHrP. Pamidronate disodium treatment decreased his serum calcium level transiently.

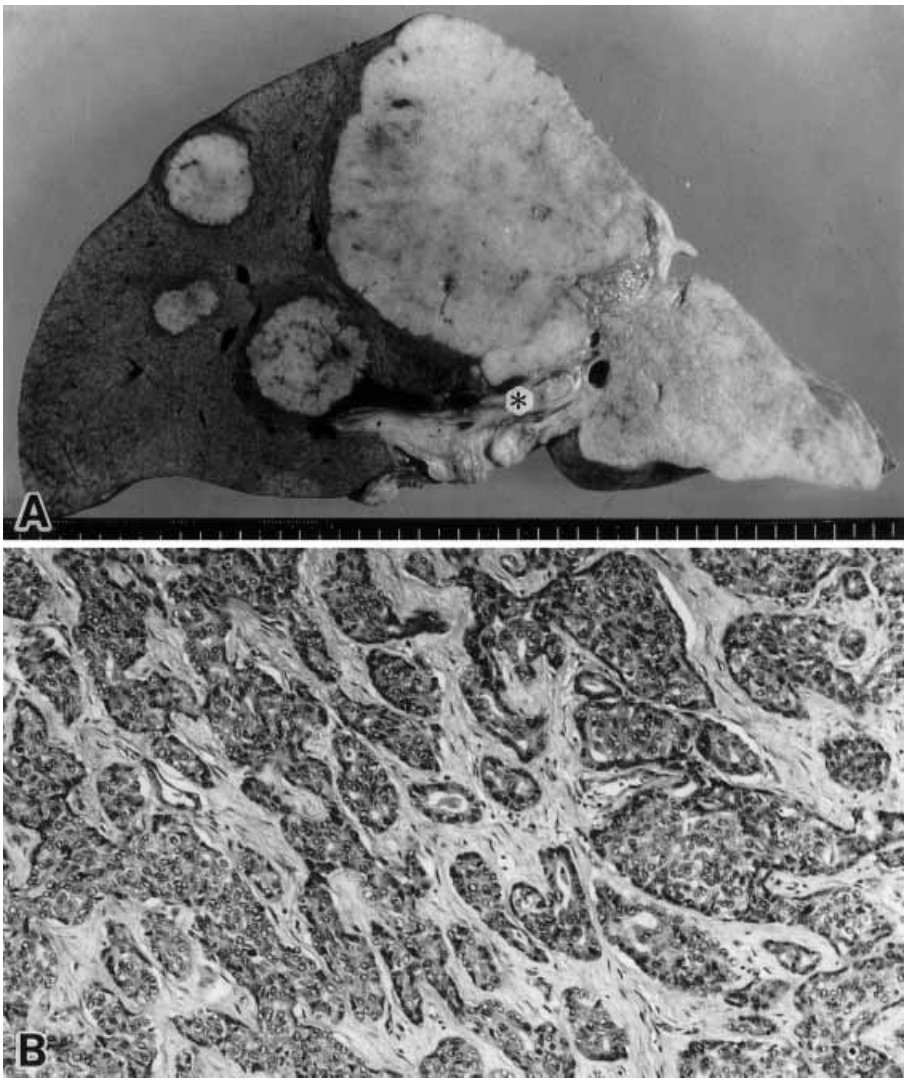

Fig. 1 **A** The cut surface of the liver. A hard, fibrous, well-circumscribed, whitish grey tumour associated with daughter nodules is present and the left lobe is retracted. *Asterisk* indicates the tumour thrombus in the portal vein. The remaining liver was not cirrhotic. **B** Photomicrographs of the tumour. Histology is typical of sclerosing hepatic carcinoma. Tubular and trabecular structures and solid nests of cancer cells are embedded within dense fibrous tissue. HE, ×100

Despite various supportive therapies, the patient died of hepatic encephalopathy and respiratory failure 4 months after the last admission.

Pathological findings

Autopsy was carried out 5 h postmortem. The enlarged liver (2000 g) with a retracted left lobe was noted. The cut surface showed a pearl grey, elastic firm tumour (7.5×14 cm) occupying the left lobe and half of the right lobe with daughter nodules; tumour embolism in the portal vein was also seen (Fig. 1A). Microscopically, tubular or trabecular structures of cancer cells were embedded in dense fibrous tissue (Fig. 1B), a picture compatible with the histology of sclerosing hepatic carcinoma. Cancer cells exhibited little bile production with focal PAS staining. The remaining liver did not manifest liver cirrhosis but revealed chronic hepatitis with mild inflammation and minimal fibrosis in the portal area (grade 1, stage 1). Metastases were observed in both lungs and the para-aortic lymph nodes (T4N1M1, stage IVB). No bone metastases were found.

Materials and methods

The anti-human PTHrP monoclonal antibody (MoAb), 4B3, was generated against the amino-terminus of PTHrP molecules, hPTHrP (1–34), as previously reported [9]. MoAbs such as DC 10 (against cytokeratin 8), AFP, CEA, CA19-9 and chromogranin A were purchased from DAKO (Carpinteria, Calif.), and AE1 (against cytokeratin) from Turner Japan (Osaka, Japan). Paraformaldehyde (4%) (PFA)-fixed and paraffin-embedded sections of the hapatic lesion were used for immunohistochemistry. After blocking of endogenous peroxidase activity with 0.3% H_2O_2 in methanol, the sections were incubated with the primary antibodies listed above in 50 mM phosphate-buffered saline (PBS) at a final concentration of 5 µg/ml. The avidin-biotin-peroxidase complex (ABC) method was employed with a Vectastain ABC kit (Vector Laboratory, Calif.). Final development of the sections was carried out with 3,3′-diaminobenzidine (DAB) containing 0.03% H_2O_2.

In situ hybridization was done on the 4% PFA-fixed paraffin-embedded sections of the liver tissue using digoxigenin (DIG)-labelled single-stranded antisense DNA probe as previously re-

ported [11]. Total RNA extracted from the HTLV I-infected cell line with the RNA extraction system, RNAzol (TEL-TEST, Friendswood, Tex.), was used as a template for RT-PCR. The sixth exon of human PTHrP cDNA was amplified by RT-PCR with rTth Reverse Transcriptase (Perkin-Elmer Cetus, Norwalk, Conn.) using the following pair of oligonucleotide primers:

Sense primer; 5′-CGATTCTTCCTTCACCATCT-3′

Antisense primer; 5′-TTTCTTTTCCTGCTCCTTGC-3′

An antisense primer-primed reverse transcription was done at 60°C for 60 min, followed by both sense and antisense-primer-primed PCR amplification. PCR parameters were denaturation at 94°C for 15 s, annealing at 67°C for 15 s and elongation at 72°C for 30 s, for a total of 35 cycles. The amplified PCR product was loaded onto 3% agarose gel, and DNA band of the predicted size (249 bp) was cut and purified with glass powder (Takara, Tokyo, Japan). To prepare the DIG-labelled single-stranded antisense DNA probe, purified DNA was subjected to uni-directional PCR with the antisense primer alone in the presence of DIG-dUTP (Boehringer Mannheim, Mannheim, Germany) with the same PCR parameters for a total of 40 cycles as previously described [11]. The DIG-labelled antisense DNA was purified with Quickspin Column Sephadex-G50, Fine (Boehringer Mannheim, Mannheim, Germany) at a speed of 500 *g*. For negative control, the DIG-labelled sense probe was generated with sense primer-primed unidirectional PCR.

After dewaxing and rehydrating, tissue sections were treated with 2 µg/ml proteinase K (Sigma, St. Louis, Mo.) for 10 min, refixed with 4% PFA, and acethylated in 0.1 M triethanolamine containing 0.25% acetic acid. Samples were then incubated in a hybridization medium [10 mM Tris-HCl (pH 7.3), 1 mM EDTA, 600 mM NaCl, 0.25% sodium dodecyl sulfate, 1× Denhardt′s medium, 50% (v/v) deionized formamide/ 1 ng/ml of probe DNA, 10% dextran sulfate] at 50°C in a moist chamber for 16 h. Negative controls were prepared with either a DIG-labelled sense DNA probe or RNase predigestion. After hybridization, the slides were washed with 50% deionized formamide/2× SSC, 2× SSC and 0.2× SSC at 50°C for 30 min each to remove the superfluous probe. To visualize the hybridized probe, the slides were incubated with alkaline phosphatase-conjugated anti-DIG antibody (Boehringer Mannheim, Mannheim, Germany) for 60 min after blocking with non-fat dry milk in PBS for 30 min. The colorimetric reaction was done with nitro-blue tetrazolium salt and bromo-4-chloro-3-indolyl phosphate solution (Boehringer Mannheim, Mannheim, Germany) in the dark for 4 h, then stopped with 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA. Slides were mounted with CRYSTAL/MOUNT (Biomedica, Foster City, Calif.) and analysed under a light microscope equipped with phase contrast without counterstaining.

After PFA fixing, cancer tissue was refixed with 2% osmic acid, dehydrated through a series of graded ethanols and embedded in Epon. Ultrathin sections stained with lead citrate were prepared and observed by electron microscopy.

Results

As shown in Fig. 2A, immunoreactivity for human PTHrP (1–34) was detected in tumour cells. Dense fibrous tissue surrounding the cancer cell nests was negative for PTHrP. Negative control prepared with nonspecific mouse myeloma IgM showed no staining (data not shown). By in situ hybridization with a DIG-labelled single-stranded antisense DNA probe, the transcripts of PTHrP were clearly demonstrated in the cytoplasm of cancer cells (Fig. 2B), which is consistent with the localization of PTHrP protein. Signals were detected neither in the specimen hybridized with a DIG-labelled sense DNA probe (Fig. 2C) nor in that predigested with RNase (data not shown).

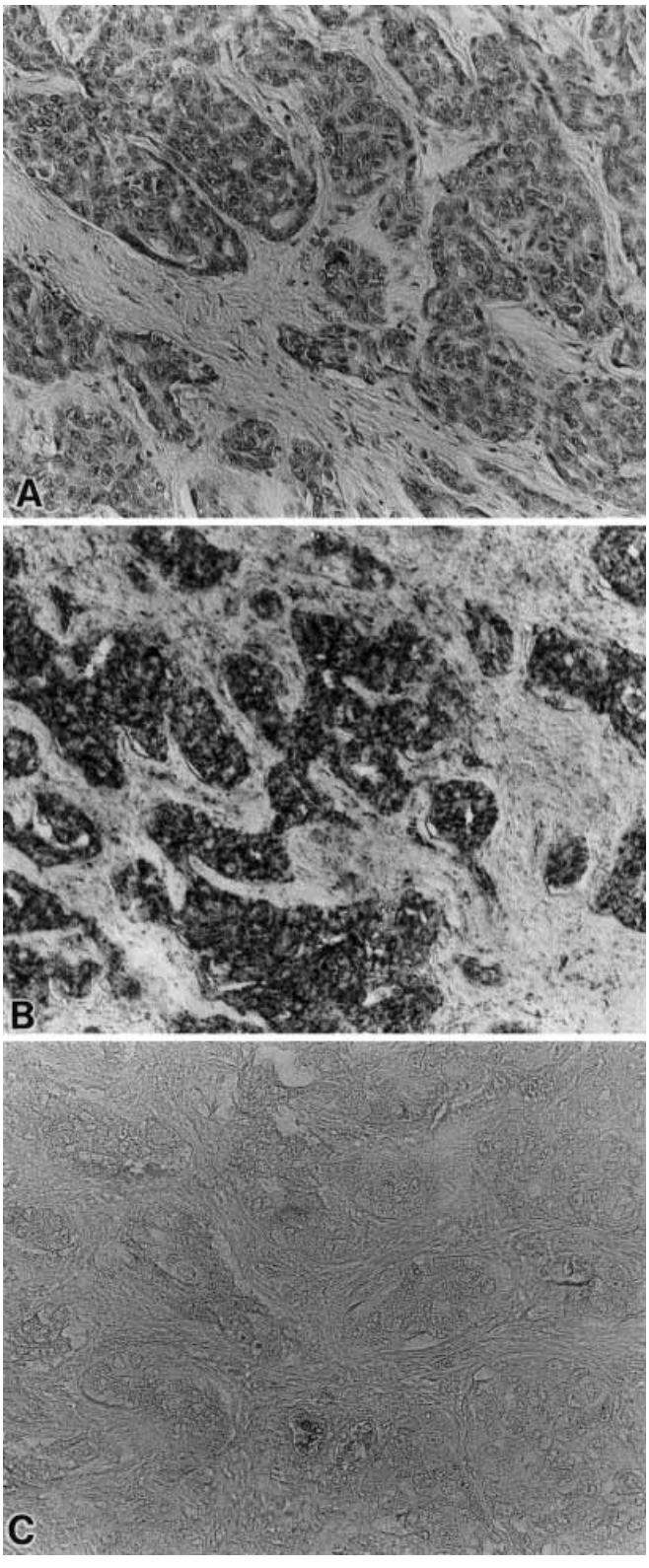

Fig. 2A–C In situ demonstration of PTHrP production in cancer tissue. ×200. **A** Immunohistochemical demonstration of PTHrP in sclerosing hepatic carcinoma cells. ABC, counterstaining with haematoxylin. **B** Expression of PTHrP mRNA in the cytoplasm of carcinoma cells detected by in situ hybridization with a DIG-labelled single-stranded antisense DNA probe. **C** The section hybridized with a DIG-labelled single-stranded sense DNA probe

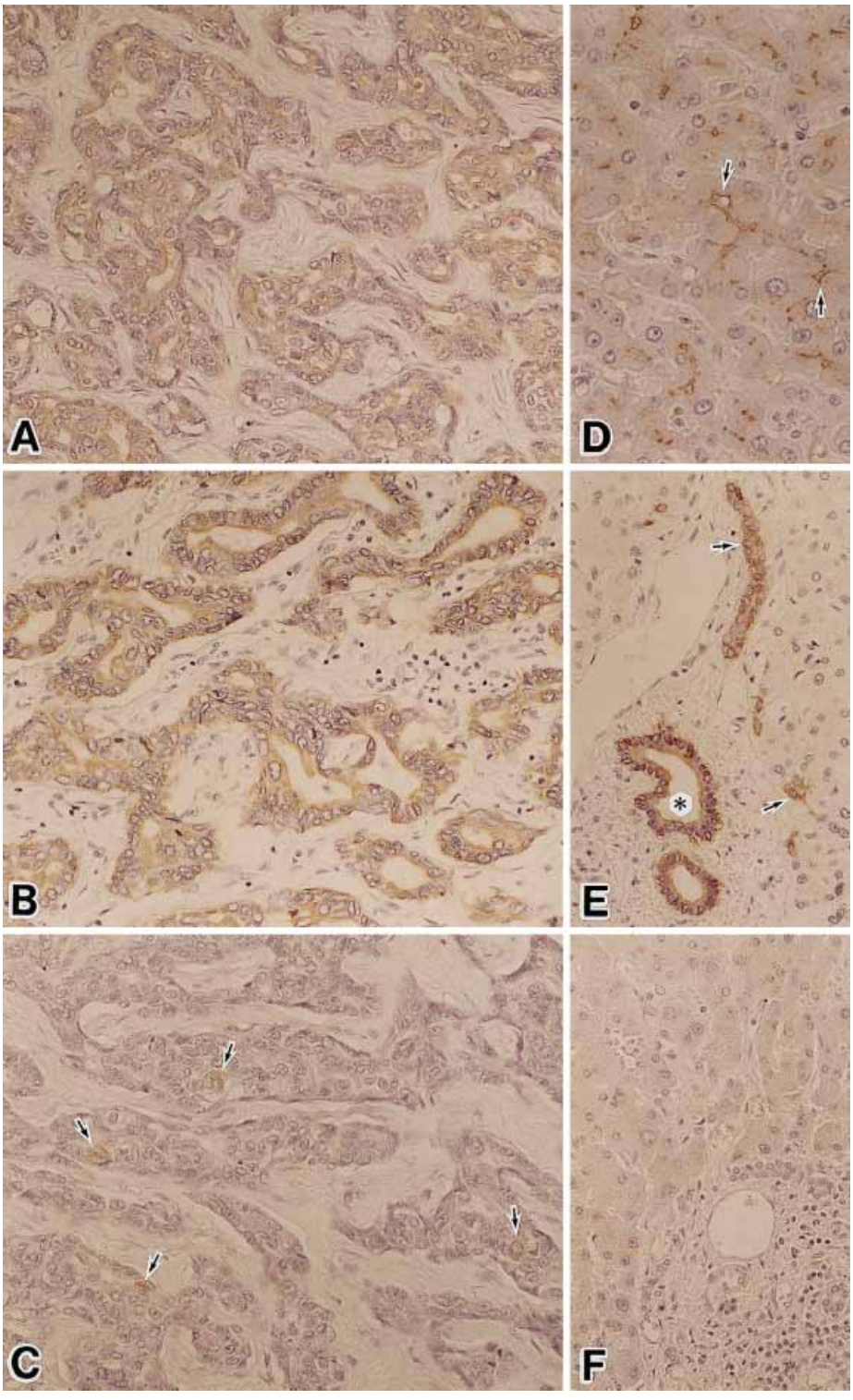

Fig. 3 Immunohistochemical analysis of **A**, **B**, **C** cancer tissue compared with **D**, **E**, **F** noncancerous liver tissue. **A**, **D** CEA, **B**, **E** AE1 and **C**, **F** AFP immmunostaining, ×200. Immunoreactivity for CEA is seen diffusely in **A** cancer cells and **D** bile canaliculi (*arrows*). Immunoreactivity for AE1 is seen mainly in the tubular structure of the cancer foci (**B**). In the portal tract, AE-1 is seen in the interlobular bile ducts (*asterisk*) and the canals of Hering (**E** *arrows*). Immunoreactivity for AFP detected focally in tumour cells (**C** *arrows*)

To investigate the origin of tumour cells of sclerosing hepatic carcinoma, we further analysed the markers for hepato- and cholangiocellular phenotypes in tumour cells by immunohistochemistry for tumour markers [2] and keratin profiles [6, 19]. As summarized in Table 1, carcinoma cells exhibited immunoreactivity for DC 10 (cytokeratin 8), and AFP shared in part with the cytoplasm of normal hepatocytes. CEA, localized exclusively in bile canaliculi of hepatocytes, as demonstrated in Fig. 3D, was found diffusely in cancer cell nests (Fig. 3A). Immunoreactivities for AE1 (Fig. 3B) and CA19-9 were observed mainly in the tubular structures of the cancer foci. In the portal tract of the noncancerous area, both AE1 (Fig. 3E) and CA19-9 were detected in the canals

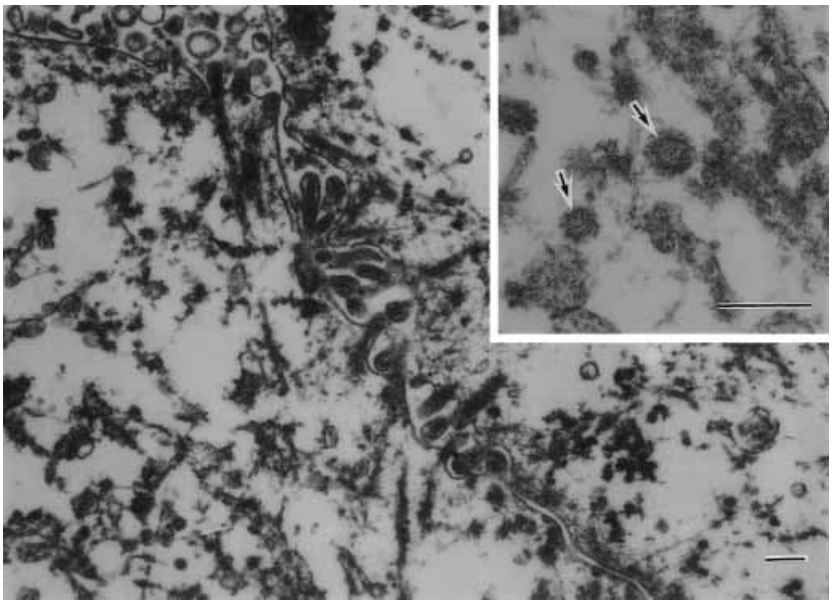

Fig. 4 Electron micrograph of cancer cells with microvilli on the apical surface. Occasional secretory granules 100 nm in diameter (*arrows*; *insert*) are observed in the cytoplasm. *Bars* 200 nm

Table 1 Results of immunohistochemistry for cancer lesions and noncancerous structures

	Cancer cells	Hepatocytes	Bile ducts
DC 10 (cytokeratin 8)	+	++	+–
AFP[a]	+–	–	–
CEA[a]	++	+–	–
CA19-9	++	–	+
AE1 (cytokeratin 10,14,15,16,19)[a]	++	–	+
Chromogranin A	+	–	–

[a] Appears in Fig. 3

of Hering and the interlobular bile ducts. Tumour cells were positive for chromogranin A, whereas both hepatocytes and the bile ducts were negative.

Electron microscopic analyses revealed the microvillous structure on the apical surface and secretory granules 100 nm in diameter in the cytoplasm of cancer cells (Fig. 4).

Discussion

All biochemical data in this case were compatible with HHM, and no bony metastases were found. Omata et al. have reported that 70% of sclerosing hepatic carcinoma cases show hypercalcaemia with hypophosphataemia, which has been called pseudohyperparathyroidism [13]. Hypophosphataemia reflects the renal action of PTHrP through the common PTH/PTHrP receptor with an increase in nephrogenous cAMP production. In our case, circulating PTHrP was found to be elevated, which was suspected to be the cause of hypercalcaemia. The production of PTHrP in the tumour was demonstrated both by in situ hybridization and by immunohistochemistry. Various nucleic acid probes have been developed, and DIG-labelled single-stranded RNA probes transcribed in vitro are in commonly use [18], since although RNA-RNA hybridization is highly sensitive, RNA probes are susceptible to unexpected degradation by RNase. Indeed, a double-stranded cDNA probe previously used to detect PTHrP mRNA in cancer tissues [8], although stable, had the drawback of the unavailability of the sense probe control. The stability of the probe and the sensitivity of hybridization were assured by the use of a single-stranded antisense cDNA probe, and a sense-probe control was prepared. Without in vitro transcription, a reliable and stable DNA probe specific to PTHrP was generated by this system.

Recently Albar et al. have reported a PTHrP-producing sclerosing hepatic carcinoma with the phenotype of hepatocytes [1]. Differential diagnosis of sclerosing hepatic carcinoma includes conventional cholangiocellular carcinoma, metastatic adenocarcinoma and hepatocellular carcinoma with sclerosis [4]. In our case, tumour cells predominantly expressed bile epithelial markers predominantly: CEA in bile canaliculi, AE1 and CA19-9 in Hering and interlobular bile ducts. Sclerosing hepatic carcinoma cases have been divided into three subgroups based on the histological features [4, 13]: hepatocellular, cholangiocellular and cholangiolocellular [16, 20], and using these criteria we assigned our case to the cholangiocellular subgroup. Tumour cells seem to produce PTHrP irrespective of their histological subtypes and tumour origins, but in the common types of primary hepatic malignancies (hepatocellular carcinoma and cholangiocellular carcinoma) HHM caused by PTHrP overproduction is rarely found, although PTHrP distribution in normal and neoplastic liver tissues [14, 15] and the autocrine actions of PTHrP on hepatoma cells [12] have been described. Since the presence of dense fibrous stromal tissue surrounding cancer cell nests is indispensable in sclerosing hepatic carcinoma,

stromal and tumour cell interaction may affect PTHrP expression. Indeed, the cancer-stromal interaction modulating PTHrP production has been observed in vivo, as in our previous report on uterine cervical cancer [10], and in vitro [5].

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