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CASE REPORT

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Lack of allelic loss at the multiple endocrine neoplasia type 1(MEN-1) gene locus in a pancreatic ductal (non-endocrine) adenocarcinoma of a patient with the MEN-1 syndrome

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Abstract The gene responsible for multiple endocrine neoplasia type I (MEN-1) syndrome has been mapped to chromosome 11q13. It appears to function as a tumour-suppressor gene analogous to that for retinoblastoma and allelic losses involving the wild-type of the MEN-1 allele have been found in parathyroid and pancreatic endocrine tumours of MEN-1 patients. No genetic information has been provided so far on non-endocrine malignancies that may occur in MEN-1 patients. A case of exocrine pancreatic adenocarcinoma presenting as the terminal event in a woman with a long standing history of MEN-1 syndrome and multiple endocrine tumours of the pancreas was investigated for possible allelic losses at the MEN-1 gene locus using restriction fragment length polymorphisms (RFLPs) closely linked to the MEN-1 gene and polymerase chain reaction (PCR) for D11S533 locus. No allelic losses were found in tumour tissue with two informative RFLPs (D11S97, D11S146) or with PCR analysis. These findings suggest that the MEN-1 gene does not confer a predisposition to develop tumours other than those that typify the syndrome.

Key words Multiple endocrine neoplasia type 1 (MEN-1) syndrome · Pancreatic adenocarcinoma · MEN-1 gene locus · Heterozygosity

Introduction

Multiple endocrine neoplasia type 1 (MEN-1) is a proliferative multiglandular disorder, most commonly characterized by adenomas of the pituitary, adenomas or more rarely carcinomas of the pancreatic islets, and hyperplasias of the parathyroid glands [8]. In addition, gastric, duodenal, bronchial and thymic carcinoid tumours have also been found in several patients. All of these tumours are regarded to derive from cells belonging to the diffuse, peptide and/or amine producing neuroendocrine system. However, several cases of MEN-1 syndrome also presenting cancers originating in other tissues have been reported. These include endometrial adenocarcinoma [9], renal cell carcinoma [10, 23], urinary bladder carcinoma [18, 29], liposarcoma [18], papillary carcinoma of the thyroid [1], adrenal carcinoma [3] and exocrine pancreatic carcinoma [25].

Larsson et al. [21] mapped the gene for MEN-1 syndrome to chromosome 11q12-13 and suggested that it acts as a tumour-suppressor gene analogous to that for retinoblastoma. Indeed, they found allelic losses involving the wild-type of the MEN-1 allele in two malignant pancreatic insulinomas from brothers with the syndrome. Further studies showed that homozygous inactivation of the MEN-1 gene is also involved in parathyroid tumours associated with the syndrome [15, 31]. These data indirectly demonstrated that parathyroid lesions in MEN-1 syndrome, previously regarded as merely hyperplastic [8] and hence polyclonal, are often monoclonal. More recently, similar allelic deletions were observed in a gastric carcinoid of an MEN-1 patient [22].

Allelic losses of chromosome 11q13 have also been found in sporadic forms of either parathyroid adenomas [15] or pancreatic endocrine tumours [13, 28], indicating that the MEN-1 gene has a role for the development of endocrine tumours in non-MEN-1 patients. In agreement with this assumption we have recently shown that progression of parathyroid hyperplasia secondary to chronic uraemia may be associated with loss of heterozygosity at the MEN-1 gene locus [14].

To investigate whether similar allelic losses may be involved in the development of malignant non-endocrine tumours of MEN-1 patients we have analysed a case of exocrine pancreatic adenocarcinoma presenting as the terminal event in a woman with a long standing history of MEN-1 syndrome.

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Case report

A female patient born in 1923 presented intractable ulcer disease from the age of 35. For this reason she underwent several operations comprising Billroth II gastric resection, gastro-jejunal resection, vagotomy and, eventually, total gastrectomy in 1965. At this time distal pancreatectomy was also performed for multiple endocrine tumours measuring up to 1.5 cm in diameter. Cytological analysis of 17 of these, histologically benign tumours described in detail elsewhere [4, 5, 6, 30], revealed that most of them were predominantly composed of glucagon-producing cells often together with a well represented pancreatic polypeptide (PP) cell population. In contrast, insulin and somatostatin cells were found in a minority of tumours, usually as discrete, scattered cells whereas gastrin producing cells were consistently absent.

In 1977 the patient presented with mild diabetes, requiring 30 U of insulin per day. Serum fasting gastrin, first determined in 1974, was consistently elevated on annual examinations ranging between 750 and 1300 ng/l (normal <100 ng/l). A secretin test performed in 1982 was positive for persisting gastrinoma (Δ: 770 ng/l; positive >200 ng/l). At the same time asymptomatic hyperparathyroidism was demonstrated by elevated fasting blood levels of parathyroid hormone (612 ng/l; normal <360 ng/l), and calcium (2.99 mmol/l; normal <2.74 mmol/l) and low levels of phosphorus (0.65 mmol/l; normal >0.81 mmol/l). Elevated plasma fasting levels of PP (500 ng/l; normal <100 ng/l) and somatostatin (84 ng/l; normal <20 ng/l), but not of glucagon, were also found. In the same year cholecystectomy was required for suppurative cholecystitis. Careful intraoperatory search for tumours in the pancreatic head remnant and in the duodenum was negative. The size of the sella turcica was found to be at the upper limit of normal (16 mm in length) by X-ray examination.

In 1991 she rapidly developed severe jaundice. A CT scan revealed a mass in the pancreatic head measuring 3.5 cm in diameter and multiple liver metastases. As a result of tumour compression, secondary occlusion of the bile duct was apparent. At surgery the large tumour in the pancreatic head was found to infiltrate the duodenal wall and the left lobe of the liver. Multiple metastases measuring up to 2 cm in diameter were found in both lobes of the liver. Intrasurgical inspection of the duodenum and small and large bowel was negative for primary tumours. A biliary-jejunal anastomosis was performed to divert bile flow. Serum gastrin concentration was 675 ng/l. The patient died 3 months later. Autopsy was not performed.

The family history of the patient, based on 33 family members including her 3 adult children, was negative for symptoms of MEN-1 syndrome for the 33-year-long period of clinical observation. However, 3 years after her death a sister developed Zollinger-Ellison syndrome (ZES) due to multiple duodenal gastrinomas, a finding fully consistent with the familial form of MEN-1 syndrome [27].

Material and methods

The study was performed on tumour specimens collected at surgery from the patient's liver metastases. In view of the patient's poor condition no biopsies were performed on the apparent primary tumour in the head of the pancreas.

For histology and immunohistochemistry, samples of tumour tissue were fixed in Bouin's fluid and routinely processed to paraffin. Serial, 5 μm thick sections were stained with haematoxylin and eosin, periodic acid-Schiff (PAS) method with and without previous diastase treatment, PAS-Alcian blue and the Grimelius silver method. For immunohistochemical analysis the following antisera were used: mouse antibodies (diluted according to the manifacturer's instructions) to chromogranin A (CgA; code PHE 5, Ortho Diagnostic System, Milan, Italy, diluted 1:800); human neurone specific enolase (NSE; code A008, Dakopatts, Copenhagen, Denmark); HISL-19 [7]; carcinoembryonic antigen (CEA; code SP-651, BioGenex Laboratories, San Ramon, Calif., USA); α-fetoprotein (code M873, Dakopatts); CA-19-9 (code HIS-19-9-AB 1, CIS Bio International, Gif sur Yvette, France); DU-PAN-2 (Code MAOO4-5C, BioGenex Laboratories); and rabbit antisera to gastrin, insulin, glucagon, somatostatin, serotonin, PP and α-subunit of human chorionic gonadotropin as previously described [2]. The immunoreactions were visualized with the avidin-biotin complex procedure, using diaminobenzidine-tetrahydrochloride as peroxidase substrate. For electron microscopy, specimens were fixed in 4% paraformaldehyde and 5% glutaraldehyde Karnovsky's solution in 0.1 M phosphate buffer, pH 7.3, for 3 h at room temperature, post-fixed in 1% osmium tetroxide, dehydrated and embedded in araldite. Thin sections were stained with uranyl acetate and lead citrate and examined with a Zeiss EM 109 electron microscope.

For restriction fragment length polymorphism (RFLP), an adequate DNA sample was obtained from cryopreserved specimens carefully cleansed of surrounding liver tissues. High molecular weight DNA was prepared from frozen tissue by pulverization with liquid nitrogen, digestion with proteinase K-sodium dodecyl sulfate (Boehringer Mannheim Italia, Milan, Italy, and IBI, New Haven, Conn., USA) at 37° C, phenol-chloroform extraction, and ethanol precipitation [16]. Leucocyte DNA from frozen whole blood samples was extracted by the guanidine hydrochloric acid technique [17]. Five micrograms of DNA were digested to completion according to the manufacturer's instruction (Promega, Madison, Wis., USA) and size fractioned by electrophoresis on 0.8–1.4% agarose gel (FMC, Rockland, Me., USA). Transfer to nylon filters (Gene Screen plus, Du Pont, Boston, Mass., USA) was performed according to the manufacturer's instructions. Samples were analysed for four RFLPs that map close to the MEN-1 gene [19]: PYGM (pMCMPI, *Taq* I), D11S97 (pMS51, *Taq* I), D11S146 (pHBI59, *Taq* I), INT 2 (pSS6, *Taq* I) (all probes were purchased from American Type Culture Collection, Rockville, Md., USA). Probes were labelled to a specific activity of approximately 10^9 cpm/μg with $dCTP^{32}$ (Amersham Italia, Milan, Italy) using a random priming method. The conditions of prehybridization, hybridization and washing were performed using the manufacturer's specifications (Du Pont). Filters were autoradiographed at –70° C for 24–96 h.

Polymerase chain reaction (PCR) analysis was performed using two oligoprimers flanking a highly degenerated sequence at locus D11S533 (gift from Dr. Glen Evans, La Jolla, Calif., USA). PCR conditions were according to the original paper [12] using a DNA Thermal Cycler 480 (Perkin Elmer Italia, Monza, Italy).

Results

The histological appearance of the hepatic metastases revealed a poorly differentiated adenocarcinoma showing

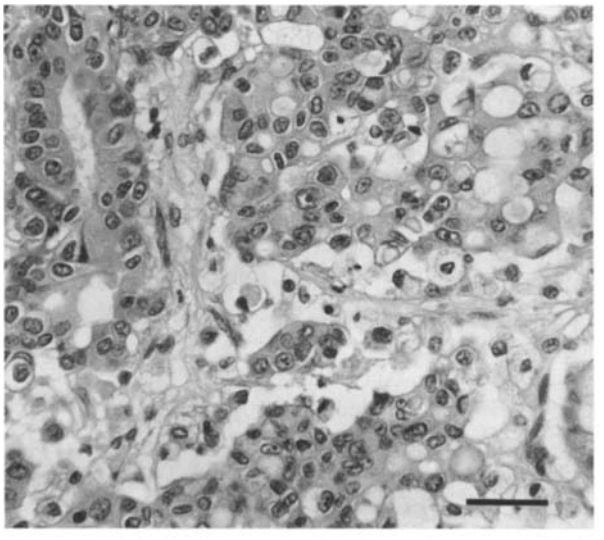

Fig. 1 Histological appearance of the tumour with few tubular structures and frequent mucin filled vacuoles. (Haematoxylin and eosin, ×220; *bar*=50 μm)

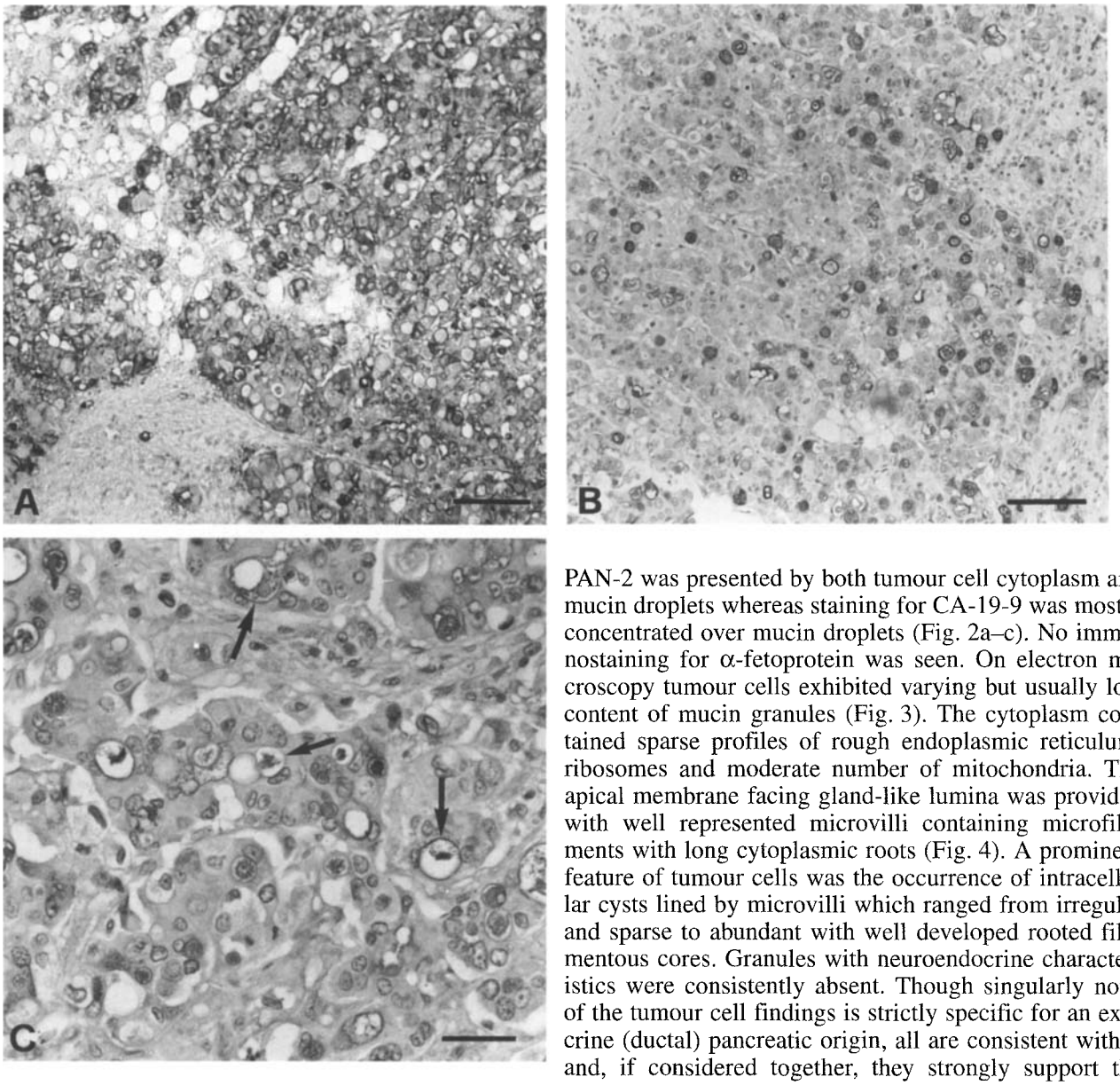

Fig. 2A–C Immunohistochemical characterization of the tumour. Intense immunoreactivity of both tumour cell cytoplasm and mucin droplets for carcinoembryonic antigen (**A**) and DU-PAN-2 (**B**). Staining for CA-19-9 (**C**) is mostly concentrated over mucin droplets (*arrows*). (Immunoperoxidase staining with haematoxylin counterstaining; **A** and **B**, ×105, *bar*=100 μm; **C**, ×220, *bar*=50 μm)

few tubular structures and more frequent solid collections of cells often presenting a large, mucin filled vacuole (Fig. 1). The stroma was generally scanty. The mucin content was often alcianophilic. The cytoplasm of the tumour cells was usually abundant whereas the nuclei were polymorphic with frequently pronounced nucleoli. Multinucleate giant cells were sometimes encountered. Mitoses were frequent. Necrosis was only rarely seen. Immunohistochemical investigation for general neuroendocrine markers (CgA, NSE, HISL-19) as well as for all hormonal substances tested proved to be consistently negative. Intense immunostaining for CEA and DU-PAN-2 was presented by both tumour cell cytoplasm and mucin droplets whereas staining for CA-19-9 was mostly concentrated over mucin droplets (Fig. 2a–c). No immunostaining for α-fetoprotein was seen. On electron microscopy tumour cells exhibited varying but usually low content of mucin granules (Fig. 3). The cytoplasm contained sparse profiles of rough endoplasmic reticulum, ribosomes and moderate number of mitochondria. The apical membrane facing gland-like lumina was provided with well represented microvilli containing microfilaments with long cytoplasmic roots (Fig. 4). A prominent feature of tumour cells was the occurrence of intracellular cysts lined by microvilli which ranged from irregular and sparse to abundant with well developed rooted filamentous cores. Granules with neuroendocrine characteristics were consistently absent. Though singularly none of the tumour cell findings is strictly specific for an exocrine (ductal) pancreatic origin, all are consistent with it and, if considered together, they strongly support the clinical evidence of a primary pancreatic tumour.

RFLP analysis showed that the patient was heterozygous for at least one chromosome 11 polymorphism (D11S97, D11S146) whereas two loci (PYGM, INT 2) were not informative. We did not detect any allelic loss in the patient's tumour tissue (Table 1, Figs. 5, 6). By PCR analysis the patient exhibited a heterozygous state at locus D11S533 with no allelic losses identified in tumour tissue (Table 1).

Discussion

In the present case MEN-1 syndrome was characterized by the association of multiple endocrine tumours of the pancreas and hyperparathyroidism whereas pituitary involvement was only suspected. An additional typical finding of MEN-1 syndrome was represented by the association of hypergastrinaemia and ZES with the absence of gastrinomas among the multiple pancreatic tumours [6,

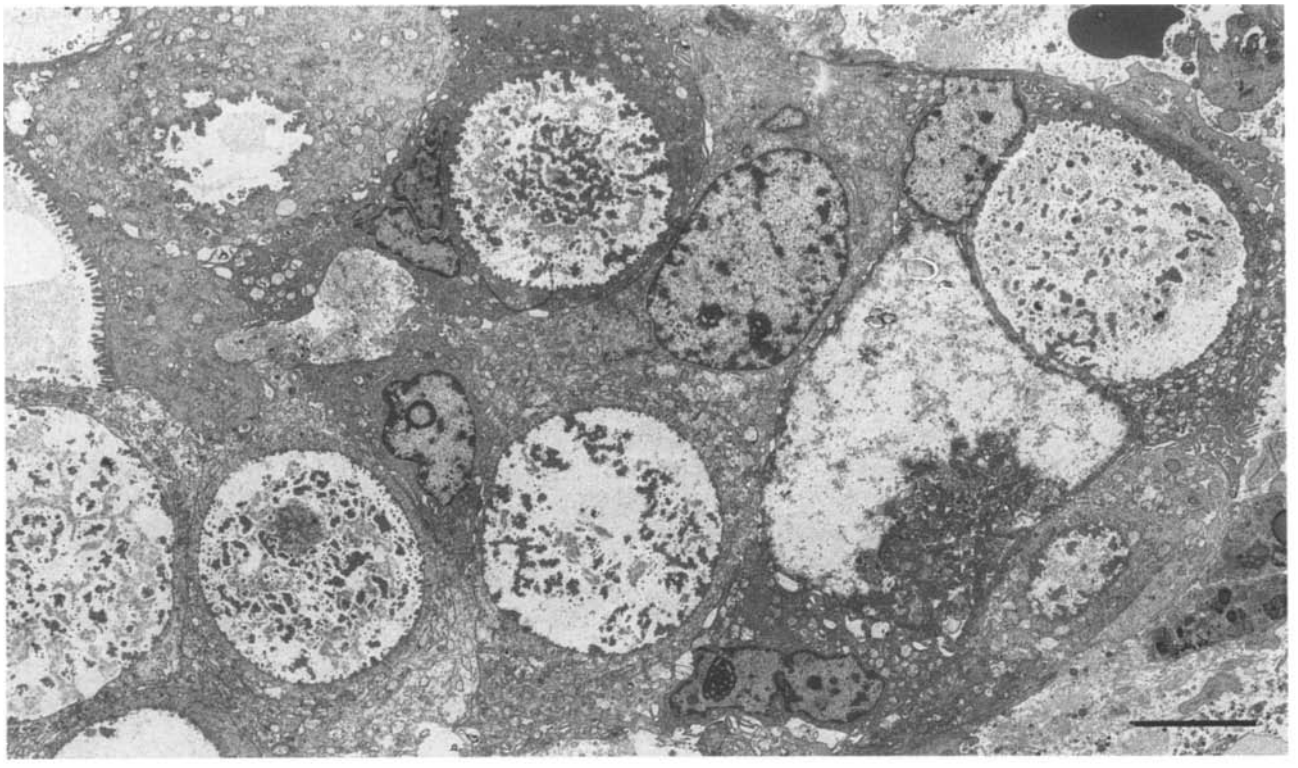

Fig. 3 Ultrastructure of tumour cells with frequent intracellular cysts (×3200, *bar* 5 μm)

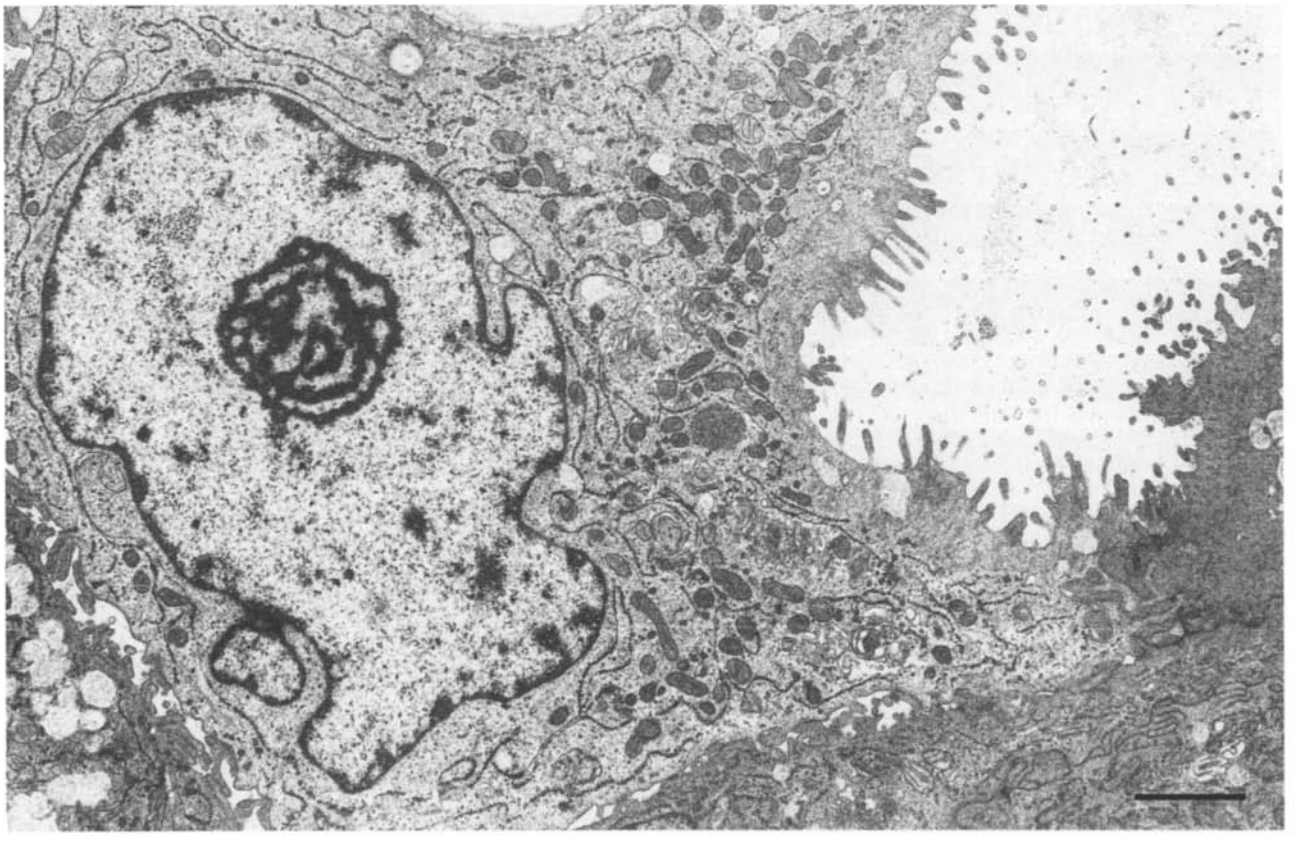

Fig. 4 Higher power of tumour cells showing sparse rough endoplasmic reticulum profiles, ribosomes and well represented microvilli in the apical membrane. Mucus granules are seen in the lower left corner. Neuroendocrine features are conspicuously absent. (×9600, *bar*=1.5 μm)

Table 1 Distribution of alleles of four restriction fragment length polymorphisms (RFLPs) mapping close to the multiple endocrine neoplasia type I gene and by polymerase chain reaction analysis of the D11S533 locus in tumour tissue and blood leucocytes of the present case. (Alleles molecular weights: *A2* 1.4 kb, *B1* 1.2 kb, *B2* 0.8 kb, *C2* 2.3 kb, *1* 2.2 kb, *2* 1.8 kb, *D1* 0.6 kb, *D2* 0.5 kb)

DNA	PYGM	D11S97	D11S146	INT 2	D11S533
Tumoural	A2/A2	1/2	B1/B2	C2/C2	D1/D2
Constitutive	A2/A2	1/2	B1/B2	C2/C2	D1/D2

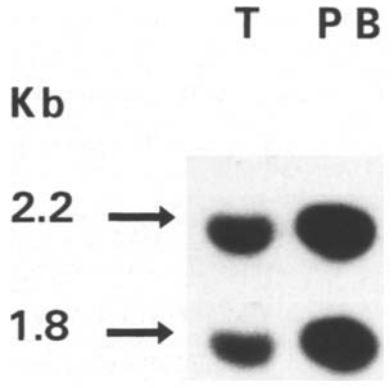

Fig. 5 Southern blot of tumour (*T*) and peripheral blood (*PB*) genomic DNA using the D11S97 (pMS51) probe. Similar results were obtained using the D11S146 (pHBI59) probe. No allelic loss in tumour tissue could be observed using these RFLPs. Allelic molecular sizes are reported on the left line

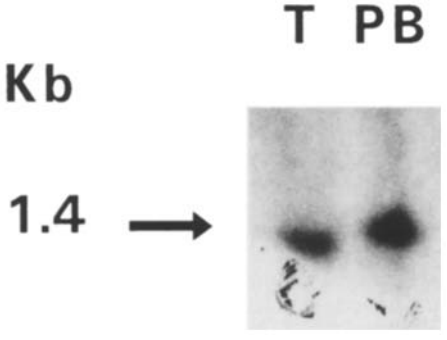

Fig. 6 Lack of informativeness of the PYGM (pMCMPI) probe in the analysis of tumoral (*T*) and peripheral blood (*PB*) genomic DNA. Similar results were obtained using the INT2 probe. Allelic molecular size is reported on the left line

20, 27]. It has been advocated that in these cases the source of the inappropriate gastrin release resides in duodenal microgastrinomas easily escaping an accurate search [27]. This explanation could also hold true for the present case. The familial background of MEN-1 syndrome in our patient was recognized only after her death by the development of a typical MEN-1 type ZES in one of her sisters.

In this patient an otherwise typical 33-year-long history of MEN-1 syndrome ended in a rapidly fatal development of a poorly differentiated non-endocrine adenocarcinoma of the pancreas with extensive liver metastases. The patient's condition did not allow for biopsies to be performed on the reputed primary tumour mass in the residual pancreatic head. However, the histological appearance of the liver metastases coupled with the results of immunohistochemical and ultrastructural studies were fully consistent with the tumour being of pancreatic exocrine origin. In this regard the present case is similar to that reported by Oliver et al. [25]. This patient was also a woman with multiple islet cell tumours and an apparently sporadic form of MEN-1 syndrome. Due to the relatively short observation period, however, an occult familial type of the syndrome cannot be ruled out, as exemplified by our case. At variance with the present patient, Oliver's case presented with Cushing's disease and hyperinsulinism but not hypergastrinaemia and developed the exocrine pancreatic carcinoma much earlier (at the age of 32 years).

RFLP and PCR analysis of the exocrine adenocarcinoma of the pancreas of our patient did not disclose the allelic losses at the MEN-1 gene region in chromosome 11q13 similar to those found in endocrine tumours of MEN-1 patients [15, 21, 22, 31] as well as in sporadic endocrine tumours [13, 15, 28]. According to the knowledge accumulated from other suppressor genes already sequenced and cloned, such as the retinoblastoma gene [11] and the familial adenomatous polyposis gene [24] in familial cases these allelic losses operate by removing the wild type gene in a patient having an inherited mutated allele in the germ line. Having a familial form of MEN-1 syndrome, our patient already carried a mutated MEN-1 gene in her germ line. Point mutations or small deletions of the wild type MEN-1 gene in her pancreatic adenocarcinoma cannot be definitely excluded unless the gene is cloned and sequenced. On a probability basis, however, their occurrence seems to be unlikely. A different oncogenic mechanism, therefore, appears more probable. For instance, exocrine pancreatic tumours were found to develop in cell lines derived from transgenic mice when SV-40 T large antigen gene is coupled to the elastase promoter [26].

This is the first case of analysis of the MEN-1 gene locus in a non-endocrine cancer of an MEN-1 patient. If confirmed by additional cases, our findings may support the suggestion that the MEN-1 gene does not confer a predisposition to develop tumours other than those that typify the syndrome.

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Note added in proof

Since the paper was accepted for publication the patient's samples were further analyzed for another RFLP that maps close to the MEN 1 gene: D11S288 (p3C7, Msp I; American Type Culture Collection, Rockville, MD, USA) and proved to be heterozygous in constitutive (blood leukocyte) DNA (A1/A2; allele M.W.: 10.0 kb and 7.0 kb, respectively). Also with this probe no allelic loss was detected in tumor tissue.