ORIGINAL

Attenuated expression of menin and p27^{Kip1} in an aggressive case of multiple endocrine neoplasia type 1 (MEN1) associated with an atypical prolactinoma and a malignant pancreatic endocrine tumor

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Abstract. Tumors in multiple endocrine neoplasia type 1 (MEN1) are generally benign. Since information on the pathogenesis of MEN1 in malignant cases is limited, we conducted genetic analysis and compared the expression of menin, p27^{Kip1}(p27)/CDKN1B and p18^{Ink4C}(p18)/CDKN2C with levels in benign cases. We describe the case of a 56 year-old male with an atypical prolactinoma and malignant pancreatic neuroendocrine tumor. At age 50, he had undergone transsphenoidal surgery to remove a prolactinoma. However, the tumor relapsed twice. Histological analysis of the recurrent prolactinoma revealed the presence of prolactin, a high MIB-1 index (32.1 %), p53-positive cells (0.2%), and an unusual association with FSH-positive cells. A few years later, he was also found to have a non-functioning pancreatic tumor with probable metastasis to the extradural region. The metastatic region tested positive for chromogranin and CD56, and negative for prolactin, with 1.2 % of cells p53-positive. Although genetic analyses of the *MEN1*, *p27*, and *p18* genes demonstrated no mutation, numbers of menin, p27 and p18 immuno-positive cells were significantly down-regulated in the recurrent prolactinoma, but that of p18 was intact in the metastatic region. Furthermore, *MEN1* and *p27* mRNA levels of the recurrent prolactinoma were down-regulated, particularly the *MEN1* mRNA level, similar to levels in 10 cases of benign prolactinoma, while the *p18* mRNA level was similar to that of normal pituitary. The tumor in this case may be a subtype of MEN1 showing more aggressive and malignant features probably induced by low levels of menin and p27.

Key words: Atypical prolactinoma, MEN1, p27, p18, Menin

MULTIPLE endocrine neoplasia type 1 (MEN1) is a rare autosomal dominantly inherited syndrome characterized by parathyroid, gastro-entero-pancreatic and anterior pituitary tumors [1-3]. Primary hyperparathyroidism is the most common clinical phenotype (>90 %), while entero-pancreatic tumors are present in 30-75 %, and pituitary adenomas in 10-60 % of cases [1, 2]. Among entero-pancreatic tumors, gastrinomas are

found in 40 % of cases, non-functioning islet adenomas in 20 %, and insulinomas in 10 % [1]. With respect to pituitary adenomas, prolactinomas or nonfunctioning adenomas are common, GH or ACTH-secreting adenomas are rare, and TSH or LH/FSH-secreting adenomas are very rare [4, 5]. Although in cases of MEN1 gastrinomas and foregut carcinoids can exhibit malignant behavior, other tumors are generally benign.

The *MEN1* gene is located on chromosome 11q13 and consists of 10 exons encoding a protein of 610 amino acids, referred to as menin [6]. A mutation of the *MEN1* gene has been identified in approximately 90 % of familial cases and 27 % of sporadic cases of MEN1 [7]. Furthermore, a mutation of the p27^{Kip1}

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(*p27*)/*CDKN1B* gene was recently implicated in a family with what appeared to be MEN1-related tumors, which has now been proposed to describe as MEN type 4 (MEN4). Pellegata *et al.* reported a family with a W76X mutation of the *p27* gene and that the proband had an invasive GH-secreting adenoma (acromegaly) with high mitotic activity and cell atypia, and hyperparathyroidism [8]. The proband's father had acromegaly. Furthermore, two other family members suffered from testicular carcinoma at the age of 28 years and renal angiomyolipoma, an MEN1-associated neuroendocrine tumor, respectively. Therefore, the MEN4 caused by the *p27* gene mutation may have more malignant features than tumors induced by the *MEN1* mutation. Furthermore, phenotypes similar to those of MEN1 have been reported in rats with a mutation of the *p27* gene [8]. In addition, Agarwal *et al.* reported a mutation of the *p18*^{Ink4C} (*p18*)/*CDKN2C* gene in a family with hyperparathyroidism [9].

Here, we present a case of MEN1 associated with an atypical prolactinoma of high malignant potential and an unusual association with FSH-positive cells, and a malignant pancreatic neuroendocrine tumor with metastasis to the extradural region. To explore the pathogenesis, we performed a genetic analysis of the *MEN1*, *p27* and *p18* genes and further examined the expression of menin, *p27* and *p18* at the protein and mRNA levels in the recurrent prolactinoma and metastatic region of the pancreatic endocrine tumor. In addition, we compared the levels of expressions with those in 10 cases of benign prolactinomas.

Materials and Methods

Genetic analysis

Written informed consent was obtained from the patient, and the study was approved by the ethics committee on human research of Gunma University. Genomic DNA was extracted from leukocytes, and direct sequencing of entire exons and flanking intron sequences of the *MEN1* (exons 2-10), *p27*, and *p18* genes was performed. In addition, somatic mutations of these genes were also analyzed in the recurrent prolactinoma, but the amount of the sample was insufficient for analysis of LOH.

RNA extraction and real-time PCR.

Some specimens of the pituitary adenomas were immersed in RNA later (Qiagen, Tokyo, Japan) at the

time of the operation; otherwise, specimens were frozen in liquid nitrogen immediately after removal during the operation. Three normal pituitaries sampled at autopsy were also used as a control. Total RNA was prepared from each adenoma using ISOGEN (Nippongene, Toyama, Japan) according to the manufacturer's instructions. Then cDNA was reverse-transcribed from 500 ng of total RNA (TaqMan Reverse Transcription Reagents, Applied Biosystems, Tokyo, Japan), and 1 µl was subjected to real-time PCR. All reactions were performed in triplicate using TaqMan probes and an Applied Biosystems 7500 sequence detection system. TaqMan probes for *MEN1* (Hs 00365720), *p27* (Hs 00153277), *p18* (Hs 00176227) and *GAPDH* (Hs 99999905) for pituitary adenomas were purchased from Applied Biosystems. The level of each mRNA relative to that of *GAPDH* mRNA was calculated using a standard curve, and relative quantification was performed as described in ABI User Bulletin #2. All experiments were repeated at least twice. The level of mRNA expression for each patient was evaluated with the median value for the normal control set as 1.0.

Immunohistochemistry

Pituitary hormone staining was performed to establish the hormone phenotype of the tumor samples. A prolactin antibody was purchased from DAKO Corp. (Kyoto, Japan) and an antibody for the common α subunit was from Abcam plc. (Cambridge, UK). Antibodies against *p27* and *p53* were purchased from Leica Microsystems (Neucastle Upon Tyne, UK), against menin (A300-105A) from Bethyl (Montgomery, TX, USA) and *p18*(sc-9965) from Santa Cruz Biotechnology Inc., (Santa Cruz, CA, USA). Samples were fixed in 10% (wt/vol) phosphate-buffered formalin at room temperature overnight and embedded in paraffin wax. The pituitaries or extradural tumor were cut at a thickness of 4 µm. The sections were pretreated with 0.3% hydrogen peroxide for 30 min for the quenching of endogenous peroxidase activity. The sections were then autoclaved with citrate buffer, pH 6.0 at 121°C for 10min (for *p27*), for 20min (for *p18*), treated with 0.01% protease (Nihirei Corp., Tokyo, Japan) at room temperature for 5min (for menin), or microwaved at 90°C for 10min (for *p53*). The sections were rinsed in PBS and incubated with 10% normal rabbit serum for 30 min at room temperature to block non-specific antibody binding, and treated with the above antibodies at 4°C overnight. After three

Fig. 1 MRI and histological analysis of the initial and recurrent prolactinomas.

A. A pituitary mass 3.5 cm in diameter of extending into the sella turcica was observed. B. Three years later, a relapsed prolactinoma was found to have invaded the right sphenoidal sinus. C. Immunohistochemistry revealed in addition to prolactin-positive cells, many FSH and α subunit-positive cells, a high MIB-1 index (21.4%), and p53-positive cells (0.2%).

washes in PBS, the sections were incubated with secondary biotinylated anti-mouse or rabbit IgG (Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature. Finally, the slides were visualized with a solution containing 0.02% 3, 3'-diaminobenzidine tetrahydrochloride (DAB; Wako Pure Chemicals, Osaka, Japan) and 0.005% H_2O_2 , followed by light counterstaining with hematoxylin. To compare the expression of each protein, we performed the staining of a control normal organ and the patient's tissue on the same slide glass.

Statistical analysis.

Statistical analyses were performed with ANOVA and Student's *t* test or the Wilcoxon/Kruskal-Wallis test using JMP (SAS Institute Inc., Cary NC).

Results

Case report

A 56-year-old male had received surgery and subsequent radiation for an undifferentiated thymic car-

cinoma at the age of 49. He developed an aggressive pituitary prolactinoma at 50 years-old, presented with bitemporal hemianopsia, and had a prolactin concentration of 1418 ng/mL (normal value 1.9-13.2 ng/mL). MRI revealed a pituitary mass with a 3.5 cm in diameter extending into the sella turcica (Fig. 1A). Transsphenoidal surgery was performed, and the bitemporal hemianopsia improved. After the operation, his serum prolactin level was kept below 25.2 ng/mL by bromocriptine therapy. However, about three years later, his serum prolactin level rose to 282.6 ng/mL, and MRI showed a relapsed prolactinoma that had invaded the right sphenoidal sinus (Fig. 1B). He was reoperated on via the transsphenoidal route. Immunohistochemistry revealed, in addition to the prolactin-positive cells, significant number and staining of FSH, a high MIB-1 index (Ki-67), 21.4%, and 0.2 % of cells to be p53 -positive (Fig. 1C). Furthermore, the staining of the α -subunit was also observed, indicating the presence of mature FSH in the tumor. About one year later, the prolactinoma was 2.0 cm in diameter, his serum prolactin level was 1809 ng/mL, and he showed

Fig. 2 MRI and histological analysis of the metastatic extradural region.

A. MRI demonstrated an extradural tumor with 4 cm in diameter located from Th9 to Th10 as indicated by an arrow. B. Histological investigation of the tumor cells revealed a clear foamy cytoplasm with an egg-shaped nucleus containing a high level of chromatin. C. Immunohistochemistry showed chromogranin A and CD56-positive cells in the tumor, demonstrating a neuroendocrine tumor (NET). Approximately 1.9 % of cells were p53-positive.

right abducent palsy. The tumor was again removed by craniotomy, and radiation therapy administered (total dose 58 Gy). Histological analysis demonstrated the presence of prolactin, and an MIB-1 index of 32.1 %.

One year after the last surgery, he suddenly developed back pain and left lower hemiparesis. MRI demonstrated an extradural tumor 4 cm in diameter of located from Th 9 to Th 10 (Fig. 2A). Laminectomy was performed for the decompression of the spinal cord. Histological investigation of the tumor cells revealed a clear foamy cytoplasm with an egg-shaped nucleus containing a high level of chromatin (Fig. 2B). These cells were positive for chromogranin A and CD56 (neural cell adhesion molecule, NCAM), but not for prolactin, indicating they were derived from a neuroendocrine tumor [10, 11]. Furthermore, 1.9 % of cells were p53-positive (Fig. 2C).

A CT scan of the entire body demonstrated a tumor of 5 cm in a diameter at the tail of the pancreas (Fig. 3A). FDG-PET revealed the high accumulation of tumor cells (maxSUV, 5.71) at several sites in the lumbar vertebrae and abdominal paraaortic lymph nodes (Fig. 3B), suggesting a malignant pancreatic tumor that had metastasized to the extradural region and lymph nodes. Serum levels of pancreatic hormones, insulin

(7.4mU/mL), gastrin (130 pg/mL), VIP (<5.0 pg/mL), and pancreatic polypeptide (PP)(267 pg/mL), were normal except for a slightly elevated concentration of glucagon (248 pg/mL). The stimulation test with Ca^{++} in selective arteries into the pancreas revealed no increase of any hormone including insulin, gastrin, glucagon, VIP, and PP. In addition, histological examination of the extradural tumor demonstrated no staining positive of insulin, glucagon or somatostatin. These findings suggested the pancreatic tumor to be a non-functioning islet tumor with metastasis to the extradural region and lymph nodes.

After removal of the extradural tumor and subsequent radiation with a total dose of 30 Gy, the paralysis of the left lower limb and back pain improved, and he was discharged. A somatostatin analogue (octreotide Acetate, 20 mg/month) had no significant effect on the pancreatic tumor, which has showed slow growth for 5 years.

The patient's younger brother has a bone tumor (no details available), but no other family history regarding endocrine and non-endocrine tumors exists.

Genetic analysis

After genetic counseling, written informed consent

Fig. 3 CT of the pancreatic tumor and FDG-PET of the whole body
 A. A CT scan demonstrated a tumor 5 cm in diameter at the tail of the pancreas as indicated by an arrow. B. FDG-PET revealed the accumulation on the tumor (maxSUV, 5.71) as indicated by an arrow and several affected sites in the lumbar vertebrae and abdominal paraaortic lymph nodes as indicated by arrow heads.

was obtained for a genetic analysis. Sequencing of the *MEN1*, *p27*, and *p18* genes of genomic DNA from peripheral lymphocytes including the entire coding region and donor-acceptor site for the introns revealed no mutation. In addition, analysis of the cDNA of the recurrent prolactinoma showed no mutation of these three genes.

Expression of menin, p27 and p18 in the tumors.

Since no mutation was found in the *MEN1*, *p27* and *p18* genes, we next examined the expression of menin, p27 and p18 at the protein and mRNA level by immunohistochemistry and real-time PCR. As shown in the upper panel of Fig. 4, an immunohistochemical analysis of the normal pituitary revealed strong staining of menin, p27 and p18 in the nucleus and weak staining in the cytoplasm. In the recurrent prolactinoma, however, approximately 8.5 % of cells tested negative for menin and 33 % and 39 % negative for p27 and p18 staining, respectively.

In contrast to the normal pituitary, normal islets showed weak expression of menin in the nucleus and cytoplasm. Furthermore, in most of the metastatic region of the pancreatic endocrine tumor, the expression of menin was lost in the nucleus, though weak staining was still observed in the cytoplasm (Fig. 5). In addition, significant staining of p27 and p18 was evident in nucleus of the normal pancreatic islets, while approxi-

mately 25 % of the cells tested negative for p27 but there was no change of p18 in the metastatic region.

Expression of the p27, p18 and MEN1 mRNA levels in the recurrent prolactinoma.

Having recently reported low levels of *MEN1*, *p27* and *p18* mRNA in prolactinomas as compared with normal pituitary [12], we examined both parameters in the present case, ten cases of prolactinoma, and three samples of normal pituitary. The patients with prolactinoma had a mean age of 37.4 ± 14.0 years, and included 6 females. All the prolactinomas showed low levels of *MEN1* mRNA in comparison with normal pituitary (the median for normal pituitary was set at 1.0, and the median for prolactinomas was 0.041). Among the prolactinomas, the tumor of the present case indicated by the square in Fig. 6A had a significantly low level of *MEN1* mRNA. Similarly, the level of *p27* mRNA was significantly lower in the prolactinomas than normal pituitary with a median of 0.14. However, the *p27* mRNA level was relatively high in our case, but still low as compared to the level in normal pituitary. In addition, the *p18* mRNA level in the patient was normal although many prolactinomas showed low levels. Furthermore, as shown in Fig. 6B, the expression level of *MEN1* mRNA was well-correlated with the level of *p27* mRNA ($r=0.66$, $p=0.027$), but not with *p18* (data not shown) in prolactinomas. The level of *p27* mRNA

Fig. 4 Immunohistochemistry of menin, p27 and p18 in a normal pituitary and the recurrent prolactinoma in the patient. Most cells tested positive for menin in the normal pituitary particularly in the nucleus, but 8.5 % of cells in the recurrent prolactinoma tested negative (upper panel). Similarly, as shown in the lower panel, most cells were positive for p27 and p18, but about one third (33 % and 39 %) were negative for p27 and p18.

Fig. 5 Immunohistochemistry of menin, p27 and p18 in normal pancreatic islets and the extradural metastatic region. The upper panel shows that normal islets with weak expression of menin in the nucleus and cytoplasm. In most of the metastatic region of the pancreatic endocrine tumor, however, menin expression was lost in the nucleus, though weak staining was still observed in the cytoplasm. Significant stainings of p27 and p18 were observed in the nucleus in the normal islets, while approximately 25 % of cells were negative for p27 but similar staining for p18 was observed in the metastatic region (lower panel).

of the present case indicated was slightly higher than that expected from the *MEN1* mRNA level.

Discussion

We presented here a rare case of relapsed prolactinoma showing malignant features and a non-function-

ing pancreatic neuroendocrine tumor with a metastasis into the extradural region. Furthermore, the patient had a tumor in the thymus. Approximately 2 % of cases of *MEN1* involve a thymus carcinoid [1], but there has been no report of thymus carcinoma. Although in the present case the primary diagnosis for the thymus disorder was undifferentiated carcinoma, we could not

Fig. 6 Comparison of levels of *MEN1*, *p27* and *p18* mRNA in the recurrent prolactinoma with those in 10 cases of benign prolactinomas.

A) Levels of *MEN1*, *p27* and *p18* mRNA were assessed by real-time PCR. The median of the level in the normal pituitary was set as 1.0. As reported, all *MEN1* and *p27* mRNA levels were low, and about 70 % of prolactinoma showed low expression of *p18* mRNA as compared to that in the normal pituitary. Squares indicate levels in the present case. The level of *MEN1* mRNA in the present case was extremely low compared to levels in the other prolactinomas, and the expression of *p27* mRNA was relatively high, but still low compared to levels in the normal pituitaries. The level of *p18* mRNA of the present case was similar to that of the normal pituitary. B) Correlation between *MEN1* and *p27* mRNA levels in the prolactinomas. The *MEN1* mRNA levels converted as a log scale exhibited high correlation ($r=0.66$) with the level of *p27* mRNA level, but not with *p18* mRNA level (data not shown). The levels of the present case are indicated by the square.

exclude the possibility of a misdiagnosis, since staining with chromograninA and CD56 was not performed at the time about 14 years ago.

The association of a pituitary adenoma with a pancreatic neuroendocrine tumor led us to a diagnosis of MEN type 1 (MEN1). However, this patient showed several unusual features. First, although most of the pituitary adenomas observed in cases of MEN1 are benign and pancreatic endocrine tumors are relatively more benign than sporadic tumors [13], the present case showed malignant features including relapse, invasiveness, a high MIB-1 index (>30 %), p53-positivity and metastasis. Second, while approximately 90 % of patients with MEN1 have parathyroid tumors [1-3], however, our patient did not. Third, although no details were available, his brother had a history of bone tumor. We therefore analyzed the *MEN1* gene, responsible for most of MEN1, and the *p27* and *p18* genes, candidates for causative genes in patients showing MEN1-like symptoms (MEN4) and familial hyperparathyroidism. However, we did not detect any mutations, suggesting the present case to be caused by a mutation in the promoter region of these genes or an

unknown gene.

The definition of pituitary carcinoma reported by WHO in 2000 is a tumor with invasion into the dura, subarachnoid region, or spinal cord. No details regarding the relapse and prevalence of metastasis of prolactinomas in MEN1 are available at present, but only a single case of malignant prolactinoma in MEN1 has been reported among 48 reported cases of malignant prolactinomas [14, 15]. However, our case did not meet the criteria for a pituitary carcinoma. On the other hand, an atypical prolactinoma is histologically defined by 1) high mitosis, 2) an MIB-1 index > 3%, and 3) p53-immunopositive cells and clinical relapse. The prolactinoma in the present case relapsed twice and had a number of mitotic cells and an extremely high MIB-1 index. Therefore, although the proportion of p53-positive cells was only 0.2 %, the prolactinoma in this case could be considered an atypical prolactinoma. Furthermore, the pituitary adenoma showed a rare combination of prolactin and FSH-positive cells, suggesting this tumor to be immature.

Although the most frequent pancreatic tumor found in cases of MEN1 is gastrinoma, approximately 20 %

are reported to be nonfunctioning [1]. Non-functioning pancreatic endocrine tumor (NFPET) less than 2-3cm in size seem to have difficulty metastasizing and are relatively benign [16-18]. It has also been reported that 50% of cases with an MEN1-related NFPET larger than 3 cm in size frequently showed metastasis [19]. For non-MEN1-related NFPETs, 5-10 % of cases showed metastasis at diagnosis: to be lymph node, 40-45 %; liver, 25-70 %; and lung and bone, 2-3 % [17, 19], but there is no such report regarding the extradural region. To our knowledge, this is the first reported case of metastasis to the extradural region by a NFPET in MEN1.

To explore the mechanism of tumorigenesis in this case, we examined the expression of menin, and p27 and p18, and found a significant number of cells negative for menin, p27 and p18 in the recurrent prolactinoma and negative for menin and p27 in the probable metastatic region of the dulla of the NFPET. Furthermore, we found levels of *MEN1* and *p27* mRNAs to be low in the recurrent prolactinoma. Although most of previous studies reported low expression of p27 in the sporadic pituitary adenomas, expression levels of menin and *MEN1* mRNA have been inconsistent [20-27]. The discrepancy between our data and previous reports may be due to the different antibody used for immunocytochemistry or the method for real-time PCR; we used TaqMan probes and others used regular RT-PCR and semiquantitative analysis. Furthermore, although a decrease of *p18* mRNA has recently been reported in 70 % of the cases of prolactinoma, our case showed similar expression with the normal pituitary [28, 29]. Therefore, down-regulation of p18 in the present case observed in immunohistochemistry might be due to a posttranslational mechanism such as regulating the rate of degradation, and the *p18* and *p27* genes may be regulated in a different manner. Indeed, our findings demonstrated that the expression level of *MEN1* mRNA was well correlated with the level of *p27* mRNA, but

not with *p18*, suggesting that the menin/mixed lineage leukemia (MLL) complex may more predominantly regulate the expression of the *p27* gene than the *p18* gene in prolactinomas.

Down-regulation of p27 expression has been implicated in a variety of human cancers, and an inverse correlation between p27 protein levels and prognosis has been reported for several tumor types [30-32]. Recent studies suggest that menin, the product of the *MEN1* gene, interacts with MLL proteins in a histone methyltransferase complex that activated transcription of the *p27* and *p18* genes [33, 34]. Therefore, p27 may be an important readout of the menin signaling pathway in endocrine cells. In fact, p27-deficient mice showed hyperplasia of the pituitary intermediate lobe, which develops into an adenoma with age [35, 36]. When challenged with mutagenic agents, heterozygous p27 knockout mice can develop multiple tumor at several sites, suggesting that p27 acts as a dose-dependent tumor suppressor in mice. A similar dosage effect could be seen in human leukemia and lymphomas with hemizygous deletions of the *p27* locus at 12p13 and without mutation of the wild-type allele. Therefore, the reduction in p27 may be related to tumorigenesis in the present case. Furthermore, expression of the *p27* mRNA level was relatively higher than that expected from the level of *MEN1* mRNA, suggesting that expression of *p27* mRNA is also regulated by a factor other than menin in the present case.

In conclusion, we presented here a rare case of MEN1 with aggressive and malignant features. Although the patient tested negative for mutations of the *MEN1*, *p27* and *p18* genes, a down-regulation of menin and p27 expression may be related to the tumorigenesis.

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