Endocrine, *vol. 29, no. 3, 485–490, June 2006* 0969–711X/06/29:485–490/$30.00
ENDO (Online) ISSN 1559-0100

Expression and Functional Analysis of Menin in a Multiple Endocrine Neoplasia Type 1 (MEN1) Patient with Somatic Loss of Heterozygosity in Chromosome 11q13 and Unidentified Germline Mutation of the *MEN1* Gene

Junko Naito,[1] Hiroshi Kaji,[1] Hideaki Sowa,[1] Riko Kitazawa,[2] Sohei Kitazawa,[2] Toshihiko Tsukada,[3] Geoffrey N. Hendy,[4] Toshitsugu Sugimoto,[5] and Kazuo Chihara[1]

[1]Division of Endocrinology/Metabolism, Neurology and Hematology/Oncology, Department of Clinical Molecular Medicine); [2]Division of Molecular Pathology, Department of Biomedical Informatics; [3]Kobe University Graduate School of Medicine, Japan; and Tumor Endocrinology Project), National Cancer Center Research Institute, Tokyo, Japan; [4]Departments of Medicine, Physiology, and Human Genetics, McGill University and Calcium Research Laboratory and Hormones and Cancer Research Unit, Royal Victoria Hospital, Montreal, Quebec, Canada; and [5]Division of Endocrinology, Metabolism and Hematological Oncology, Shimane University School of Medicine, Japan

In some patients with multiple endocrine neoplasia type 1 (MEN1) it is not possible to identify a germline mutation in the *MEN1* gene. We sought to document the loss of expression and function of the *MEN1* gene product, menin, in the tumors of such a patient. The proband is an elderly female patient with primary hyperparathyroidism, pancreatic islet tumor, and breast cancer. Her son has primary hyperparathyroidism. No germline *MEN1* mutation was identified in the proband or her son. However, loss of heterozygosity at the *MEN1* locus and complete lack of menin expression were demonstrated in the proband's tumor tissue. The proband's cultured parathyroid cells lacked the normal reduction in proliferation and parathyroid hormone secretion in response to transforming growth factor-β. This assessment provided insight into the molecular pathogenesis of the patient and provides evidence for a critical requirement for menin in the antiproliferative action of transforming growth factor-β.

Key Words: Hyperparathyroidism; multiple endocrine neoplasia; menin; transforming growth factor-β; breast cancer.

Introduction

Multiple endocrine neoplasia type 1 (MEN1: MIM# 131100) is an autosomal-dominant disorder characterized by multiple endocrine tumors of the parathyroids, pancreatic islets, and anterior pituitary *(1)*. The *MEN1* gene on chromosome 11q13 *(2)*, encodes a 610-amino-acid protein called menin *(3,4)*, and many germline mutations scattered throughout the protein-coding region have been identified *(5–9)*. Somatic mutations have been found in the corresponding sporadic endocrine tumors *(10–12)*. Many of the mutations are clearly inactivating, giving rise to a truncated product. Consequently, lack of menin caused by loss of both alleles leads to tumor development according to Knudson's two-hit hypothesis, consistent with menin acting as a tumor suppressor *(13,14)*.

Allelic loss involving chromosome 11q13 has been reported in the majority of the MEN1-associated parathyroid tumors examined *(15,16)*. Germline mutations (either within the protein-coding exons or at the exon–intron boundaries) have been identified in approx 70% of the MEN1 kindreds examined *(5–9)*. However, for the other approx 30%, mutations within the *MEN1* gene flanking regions or introns may be present but are not detected by the present analytical methods. Germline mutations are detected much less frequently in sporadic MEN1 cases, some of which may be so-called MEN1 phenocopies *(9,17–20)*. In such patients, one way to assess whether complete loss of functional menin has taken place would be to examine the expression and function of menin in the endocrine tumors. However, this has not yet been done.

Transforming growth factor (TGF)-β causes growth inhibition of many different cell types, and genes encoding proteins that are essential for the TGF-β signaling pathway are inactivated in several cancers *(21,22)*. We previously demonstrated that menin is implicated in TGF-β signaling and interacts with Smad3, a crucial mediator of the TGF-β signaling pathway, in pituitary cells *(23)*. Moreover, we showed that menin inactivation leads to loss of TGF-β inhibition of

Received February 10, 2006; Revised March 2, 2006; Accepted March 17, 2006.

Author to whom all correspondence and reprint requests should be addressed: Hiroshi Kaji, Division of Endocrinology/Metabolism, Neurology and Hematology/Oncology, Department of Clinical Molecular Medicine, Kobe University Graduate School of Medicine. 7-5-2 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan.E-mail: hiroshik@med.kobe-u.ac.jp

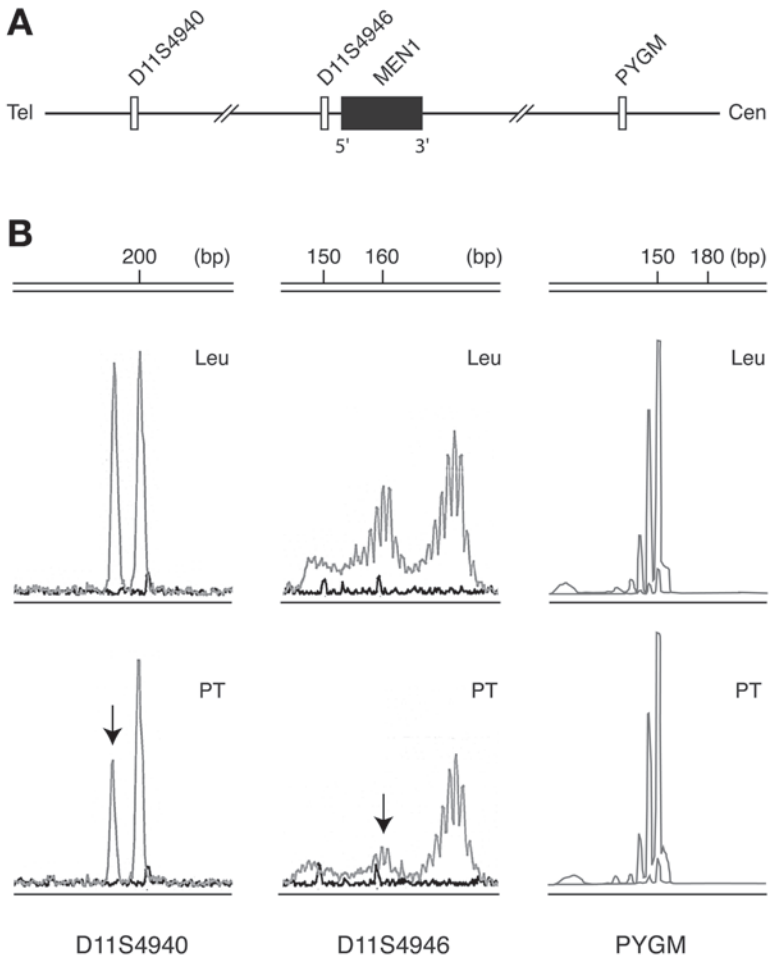

Fig. 1. Microsatellite polymorphism analysis with markers flanking the *MEN1* gene. (**A**) Schematic diagram of chromosome 11q13 showing the positions of markers D11S4940, D11S4946, and PYGM relative to the *MEN1* gene. The distance spanned by the markers is approx 300 kb. (**B**) Electrophoretograms of fluorescein-labeled PCR products generated with primers for D11S4940 (left), D11S4946 (middle), or PYGM (right) and leukocyte (Leu) or parathyroid tumor (PT) DNA. Arrows denote peaks with reduced fluorescence intensity in tumor versus leukocyte DNA.

parathyroid cell proliferation and PTH secretion by using human parathyroid cell cultures *(24)*. In the present study, we have used similar techniques to examine the expression and function of menin in neoplastic parathyroid cells of an MEN1 patient exhibiting loss of heterozygosity (LOH) of chromosome 11q13 in the tumor but in whom a germline *MEN1* mutation was not identified.

Results

Loss of Heterozygosity of MEN1 Gene

The proband presented clinical features of MEN1, namely, primary hyperparathyroidism and pancreatic islet cell tumor (described under *Patient* in *Subjects and Methods*). Her son, also, has been diagnosed with primary hyperparathyroidism but has not undergone parathyroidectomy. However, no mutation was identified in *MEN1* gene protein-coding exons in leukocyte DNA of the proband or her son or tumor tissue of the proband. To search for the presence of a deletion in the proband's tumor, we analyzed microsatellite polymorphisms using DNA markers, D11S4940, located approx 200 kb telomeric, D11S4946 located in the 5' flanking region, and PYGM, located approx 100 kb centromeric of the *MEN1* gene (Fig. 1A). Reduction in the signal from one of the alleles for both D11S4940 and D11S4946 markers was observed for tumor relative to leukocyte DNA (Fig. 1B). (The residual wild-type signal is likely due to some contamination of the tumor sample with normal tissue.) Also, LOH of polymorphic markers within the *MEN1* gene was observed (data not shown). The PYGM marker was uninformative (Fig. 1B). Therefore, in the patient's tumor a deletion involving the *MEN1* gene locus was demonstrated.

Microsatellite analysis using D11S4940 and D11S4946 and direct sequence analysis of leukocyte DNA indicated that the patient's son has inherited the maternal *MEN1* allele that is not lost in the mother's tumor tissue and hence represents the allele carrying the putative (unidentified) germline *MEN1* mutation (data not shown).

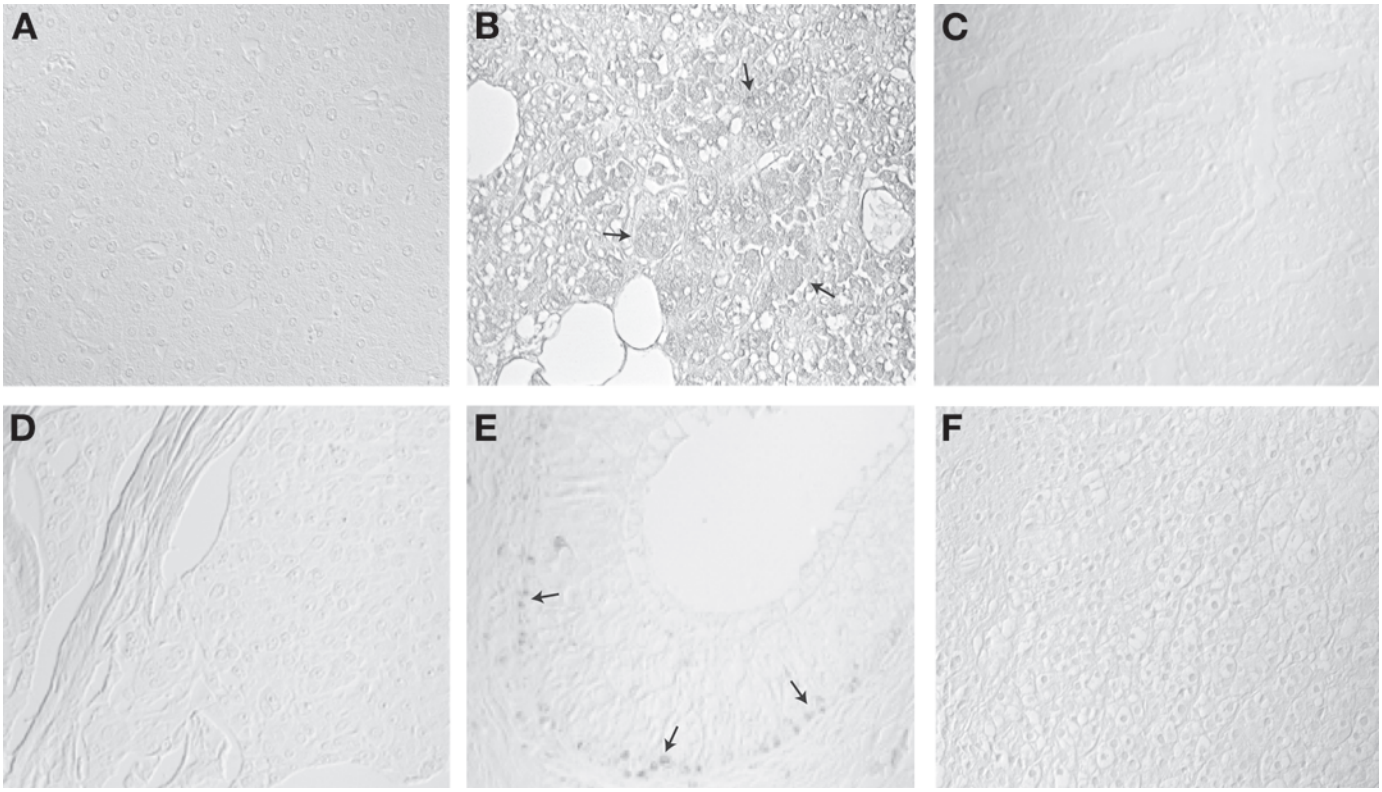

Fig. 2. Loss of menin expression in the patient's tissues. Paraffin-embedded sections were immunostained for menin as described in *Subjects and Methods*. (**A**) Parathyroid tumor from the MEN1 patient. (**B**) Positive control (parathyroid tissue from a patient with secondary hyperparathyroidism). Menin staining is observed throughout the section. Intense staining in some chief cells is indicated by the arrows. (**C**) Islet cell tumor (liver metastasis) from the MEN1 patient. (**D**) Breast cancer from the MEN1 patient. (**E**) Positive control (testis). Staining of interspersed epithelial cells is indicated by the arrows. (**F**) Nonimmune serum control (secondary hyperparathyroidism). Magnification, ×200.

Loss of Menin Expression

In the absence of definitive identification of a germline mutation, we next examined menin expression in the patient's tumor tissues. By immunohistochemistry, parathyroid tumor tissue from a patient with secondary hyperparathyroidism and testis were positive for menin expression (Figs. 2B,E). However, a similar analysis failed to identify any menin expression in the parathyroid tumor, liver metastasis of islet cell tumor and breast cancer of the patient (Figs. 2A,C,D). This would be consistent with complete inactivation of menin encoded by both of the patient's *MEN1* alleles.

Loss of Responsiveness to TGF-β

Next we assessed the functional consequences of the complete loss of menin expression in the patient's tumor tissues by examining the effects of TGF-β on the proliferation and PTH expression of cultured cells derived from the patient's parathyroid tumors. With TGF-β treatment, the number of PCNA-positive parathyroid cells from the MEN1 patient were not significantly different from those not so treated (Fig. 3A). This is in marked contrast to the effect of TGF-β in significantly reducing the number of PCNA-positive cultured parathyroid cells obtained from patients with secondary hyperparathyroidism (Fig. 3A). TGF-β treatment did not alter the number of PTH-positive cells and concentration of PTH in the medium of the cultured cells derived from the patient's parathyroid tumor tissue (Figs. 3B,C). This lack of effect of TGF-β is in contrast to the significant reductions in PTH expression in and release from cultured parathyroid cells derived from tumor tissue of patients with secondary hyperparathyroidism (Figs. 3B, C). These findings indicate that functional menin was absent in the parathyroid tumor of the MEN1 patient consistent with the lack of menin expression in the patient's tumor tissues.

Discussion

MEN1 gene sequencing detects a germline mutation in approx 70% of MEN1 kindreds. For most of the remaining approx 30%, mutations within the *MEN1* gene flanking regions or introns are likely to be causative of the disease but are not detected by the present mutation analysis methods. In MEN1 patients who present without a family history, *MEN1* mutations are found much less often. With respect to the present case in which the proband presented with MEN1-type tumors, hyperparathyroidism and pancreatic islet (although at a late age), and her son has been diagnosed with primary hyperparathyroidism, there might remain some

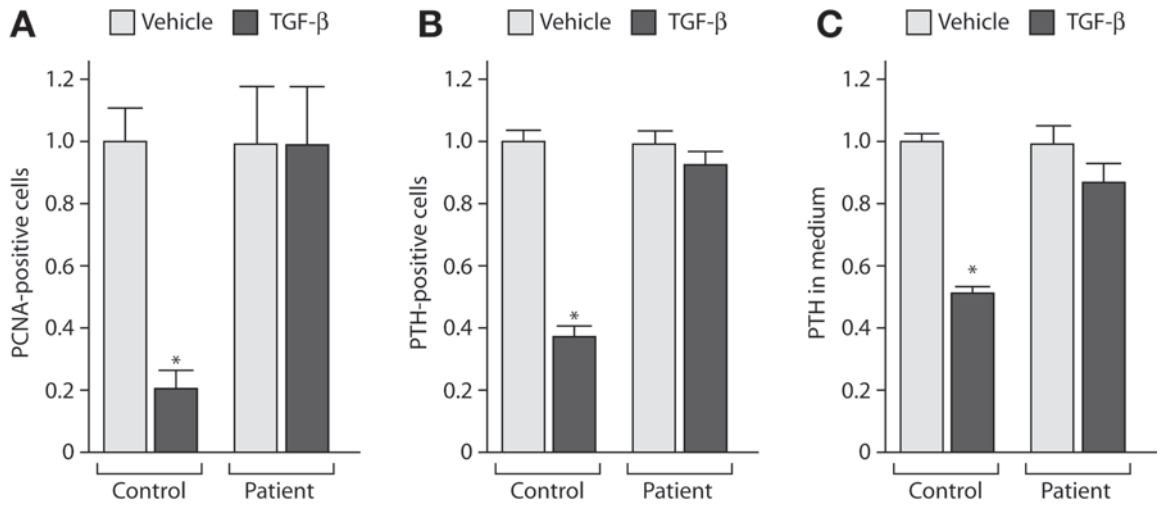

Fig. 3. Effects of TGF-β on proliferation and PTH production and secretion of parathyroid tumor cells of the MEN1 patient. Parathyroid cells [from either a patient with secondary hyperparathyroidism (Control) or the proband (Patient)] were cultured in chamber slides for 4 d, after which they were cultured in fresh medium in the absence (vehicle) or presence of 2.5 ng/mL TGF-β for 24 h. Then, PCNA immunocytochemistry and counterstaining with methyl green or PTH immunocytochemistry and counterstaining with hematoxylin were performed as described in *Subjects and Methods*. (**A**) PCNA-positive cells for each group. (**B**) PTH-positive cells for each group. (**C**) PTH levels in the culture medium were measured as described in *Subjects and Methods*. In all cases each value is the mean ± SE of triplicate determinations relative to the vehicle-treated control group. *$p < 0.01$ compared with the corresponding TGF-β-untreated group.

question as to whether this represents sporadic or familial MEN1 or an MEN1 phenocopy. In neither the proband nor her son was a germline *MEN1* mutation identified.

Loss of heterozygosity of chromosome 11q13 commonly occurs in tumors of familial MEN1 patients as well as many sporadic tumors with an *MEN1* mutation in the other allele. In the present study, LOH at chromosome 11q13 was documented in the patient's tumor tissue.

Menin is expressed ubiquitously from an early developmental stage and it has been demonstrated in mouse models that homozygous deletion of menin ($Men1^{-/-}$) is embryonic lethal. Mice that are heterozygous for menin deletion ($Men1^{+/-}$), like humans carrying the *MEN1* gene, develop tumors in select tissues throughout their lifetime *(25,26)*. By immunohistochemistry, it was demonstrated that the tumors lacked menin expression *(27)*. So far few studies of menin expression in human MEN1 tumors have been reported. By RT-PCR and immunoblotting, menin expression was reported to be down-regulated in MEN1 tumors including cases carrying biallelic ablative mutations of the *MEN1* gene *(28)*. In another case, menin expression was shown by *in situ* immunofluorescence to be absent in a gastrinoma of an MEN1 patient in whom a germline *MEN1* mutation and somatic loss of the wild-type allele was documented, whereas sporadic endocrine tumors showed variable levels of menin expression *(29,30)*. It was suggested that this type of analysis might provide an alternative method for testing for *MEN1* mutation (inactivation) in sporadic cases of hyperparathyroidism and Zollinger–Ellison syndrome *(31)*. In a previous study, we showed a lack of menin immunostaining in the parathyroid tumor of an MEN1 patient in whom a germline mutation and somatic LOH in the *MEN1* gene were found *(24)*. In the present study, we confirmed by immunohistochemistry our previous observation of menin expression in parathyroid tissue of patients with secondary hyperparathyroidism *(24)* and additionally showed menin expression in normal testis. However, no menin expression was observed in the parathyroid and pancreatic islet (liver metastasis) tumors or in the breast carcinoma of the patient. Therefore, despite not being able to identify an *MEN1* germline mutation we were able to show complete loss of menin in this way.

We previously showed that the normal inhibitory proliferative and secretory responses to TGF-β were reduced by menin inactivation in pituitary and parathyroid cells *(23, 24)*. We demonstrated that cultured cells derived from parathyroid tissue from patients with uremic hyperparathyroidism [in which mutation of the *MEN1* gene rarely plays a role and the TGF-β signaling pathway is therefore intact *(24)*] respond to TGF-β with a decrease in cell proliferation and production and secretion of PTH *(24)*. We also showed that the parathyroid cells from an MEN1 patient with ablation of both *MEN1* alleles lacked this responsiveness. This method provided us with a means to assess the functionality of menin in the parathyroid tumor of the patient of the present case. The inhibitory responses to TGF-β with respect to cell proliferation and PTH production and secretion were clearly lost in the tumor cells of the proband. The complete loss of expression and function of menin in the parathyroid tumor is explained, in part, by the LOH of a part of chromo-

some 11q13 including the *MEN1* gene. In the absence of identifying a germline mutation within the *MEN1* gene protein-coding exons, it is not known whether the causative mutation lies within a part of the *MEN1* gene not examined or is within a (neighboring) gene that is essential for *MEN1* gene expression.

Besides parathyroid hyperplasia and pancreatic islet tumor, the patient presented with the additional complication of breast cancer. Moreover, immunohistochemistry of the breast cancer demonstrated a lack of menin expression similar to the other tumors of the patient. There are few reports of the coexistence of MEN1-type tumors and breast carcinoma, although one patient with primary hyperparathyroidism, an aldosterone-producing adrenocortical adenoma, and breast cancer has been described *(32)*. Interestingly, in that case while LOH of chromosome 11q13 markers was demonstrated in the parathyroid adenoma and breast cancer, no *MEN1* gene germline mutation was identified. In the present study, we did not have the opportunity to analyze the DNA of the breast carcinoma. However, it is possible that the pathogenesis of breast cancer in some patients might be related to the *MEN1* gene or another gene at the chromosome 11q13 locus.

In summary, we have demonstrated that menin expression and function was lost in tumor tissue of an MEN1 patient with LOH of chromosome 11q13 but in whom an *MEN1* gene germline mutation was not identified. Furthermore, this study provides further evidence for a critical requirement for menin in the anti-proliferative action of TGF-β consistent with the notion that loss of this particular signaling pathway may contribute to the generation and/or progression of MEN1-type tumors.

Subjects and Method

Patient

A 74-yr-old female was admitted to our hospital in March 2003 after she was found to have hypercalcemia. She had a history of duodenal ulcer and had taken antacid medication to prevent ulcer recurrence. She was diagnosed with primary hyperparathyroidism because of her hypercalcemia and elevated serum PTH levels. Preoperative imaging analysis—ultrasound, computed tomography (CT), magnetic resonance imaging (MRI), and technetium-99m sestamibi imaging—indicated multiple increased parathyroid masses. Serum pituitary hormone levels were normal. Serum gastrin level was high at 839 ng/mL (normal range <100 ng/mL). Bone mineral densities (determined by dual-energy X-ray absorptiometry) were decreased at lumbar spine, femoral neck, and distal radius. By contrast-enhanced abdominal CT, a round mass was detected in the pancreas. A space-occupying lesion (SOL) was detected in segment eight of the liver, the size of which increased from 19 to 22 mm in 3 mo. A left adrenal incidentaloma was also detected. Serum gastrin levels markedly increased in response to a secretin stimulation test indicative of gastrinoma. A pituitary cyst, but no apparent pituitary tumor, was detected by MRI.

The patient underwent parathyroidectomy and one gland was transplanted into the left forearm. Histology indicated parathyroid hyperplasia and postoperatively serum calcium and PTH levels normalized. By celiac artery angiography, staining was detected around the border of the pancreatic body and tail. For the localization of the gastrinoma, a selective arterial secretin injection test was performed with sampling of the hepatic vein. A significant increase in gastrin level was detected upon injection of the superior mesenteric and gastroduodenal arteries, suggesting the existence of the gastrinoma in this region. Three months later, breast carcinoma was identified, the tumor was resected, and a histological diagnosis of invasive ductal carcinoma was made. The liver tumor was resected and histological examination showed it to represent metastasis of the islet cell tumor. By immunostaining, the tumor was positive for gastrin and glucagon. The patient tumor tissues were used for further analyses as detailed below. The proband's son was diagnosed with primary hyperparathyroidism but has not yet undergone parathyroid neck exploration. The patients gave informed consent and the study was approved by the ethical committee of the Kobe University Graduate School of Medicine.

Materials

Human recombinant TGF-β was from Sigma (St. Louis, MO), anti-proliferating cell nuclear antigen (PCNA) antibody was from R&D Systems, Inc. (Minneapolis, MN). A polyclonal antibody against the NH_2-terminus of human PTH was from Chemicon (Temeala, CA). The goat polyclonal antibody against the COOH-terminus of human menin was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Mutation and Microsatellite Analyses

MEN1 gene mutation and chromosome 11q13 microsatellite analyses were performed as described previously *(33,34)*.

Cell Culture

Cells were prepared from hyperplastic parathyroid glands removed surgically from the patient, as well as from uremic hemodialysis patients, as described *(24)*. Tissues were minced into small fragments and digested at 37°C with collagenase type II (Wako, Osaka, Japan) in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) with L-glutamine and without sodium bicarbonate. The parathyroid tissue fragments were mechanically dispersed by aspiration every 15 min with a serological pipet. After 90 min, the suspension was centrifuged at 800*g*. The resulting pellet was washed with serum-free RPMI 1640 and resuspended in the same medium supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin (Life Technologies, Inc,). After trypan blue exclusion test for cell viability, the dispersed cells were distributed into tissue culture dishes or chamber

slides (Lab Tek, Naperville, IL) and incubated in a humidified 95% air/5% CO_2 atmosphere at 37°C for 4 d.

Immunohistochemistry and Immunocytochemistry

For immunohistochemistry, formalin-fixed and paraffin-embedded sections of surgically removed specimens were deparaffinized with xylene and rehydrated through a series of graded alcohols, whereas for immunocytochemistry, the primary cultured parathyroid cells in chamber slides were fixed in 4% formalin for 10 min. After blocking endogenous peroxidase activity with 0.3% H_2O_2 in methanol, the sections were incubated with the specific first antibody in a humidified chamber at room temperature for 1 h. The avidin–biotin–peroxidase complex method was used with the Dako Lab kit (Dako, Carpinteria, CA). Final development of the sections was carried out with 3,3'-diaminobenzidine containing 0.03% H_2O_2.

Measurement of PTH Secretion

Cells were cultured in six-well plates in supplemented medium until subconfluent, and then treated with TGF-β. After a 24-h incubation, PTH in the medium was measured by radioimmunoassay (PTH Kit; Yamasa Corp., Choshi, Japan) as described *(35)*.

Statistics

Data are expressed as mean ± SE. Statistical analysis was performed using an unpaired *t* test.

Acknowledgments

We thank Yoshiko Higashimaki and Kana Takeuchi for expert technical assistance and Akira Kobayashi for help in culturing parathyroid cells. This work was partly supported by Grants from the Uehara Memorial Foundation (to H. K.), Grant-in-Aid 16590313 from the Ministry of Science, Education, and Culture of Japan (to R. K.) and Canadian Institutes of Health Research Grant MOP-9315 (to G. N. H.).

References

1. Brandi, M. L., Gagel, R. F., Angeli, A., et al. (2001). *J. Clin. Endocrinol. Metab.* **86,** 5658–5671.
2. Larsson, C., Skogseid, B., Oberg, K., Nakamura, T., and Nordenskjold, M. (1988). *Nature* **332,** 85–87.
3. Chandrasekharappa, S. C., Guru, S. C., Manickam, P., et al. (1997). *Science* **276,** 404–407.
4. Lemmens, I., Van de Ven, W. J., Kas, K., et al. (1997). *Hum. Mol. Genet.* **6,** 1177–1183.
5. Agarwal, S. K., Kester, M. B., Debelenko, L. V., et al. (1997). *Hum. Mol. Genet.* **6,** 1169–1175.
6. Bassett, J. H., Forbes, S. A., Pannett, A. A., et al. (1998). *Am. J. Hum. Genet.* **62,** 232–244.
7. Cardinal, J. W., Bergman, L., Hayward, N., et al. (2005). *J. Med. Genet.* **42,** 69–74.
8. Ellard, S., Hattersley, A. T., Brewer, C. M., and Vaidya, B. (2005). *Clin. Endocrinol.* **62,** 169–175.
9. Klein, R. D., Salih, S., Bessoni, J., and Bale, A. E. (2005). *Genet. Med.* **7,** 131–138.
10. Heppner, C., Kester, M. B., Agarwal, S. K., et al. (1997). *Nat. Genet.* **16,** 375 378.
11. Carling, T., Correa, P., Hessman, O., et al. (1998). *J. Clin. Endocrinol. Metab.* **83,** 2960–2963.
12. Farnebo, F., Teh, B. T., Kytola, S., et al. (1998). *J. Clin. Endocrinol. Metab.* **83,** 2627–2630.
13. Knudson, A. G. (1996). *J. Cancer Res. Clin. Oncol.* **122,** 135–140.
14. Pannett, A. A. J. and Thakker, R. V. (2001). *J. Clin. Endocrinol. Metab.* **86,** 4371–4374.
15. Nakamura, Y., Larsson, C., Julier, C., et al. (1989). *Am. J. Hum. Genet.* **44,** 751–755.
16. Lubensky, I. A., Debelenko, L. V., Zhuang, Z., et al. (1996). *Cancer Res.* **56,** 5272–5278.
17. Hai, N., Aoki, N., Shimatsu, A., Mori, T., and Kosugi, S. (2000). *Clin. Endocrinol.* **52,** 509–518.
18. Burgess, J. R., Nord, B., David, R., et al. (2000). *Clin. Endocrinol.* **53,** 205–211.
19. Nishimura, Y., Yamashita, K., Yumita, W., et al. (2004). *Endocrine J.* **51,** 75–81.
20. Sakurai, A., Katai, M., Yumita, W., Minemura, K., and Hashizume, K. (2004). *Endocrine* **23,** 45–49.
21. De Caestecker, M. P., Piek, E., and Roberts, A. B. (2000). *J. Natl. Cancer Inst.* **92,** 1388–1402.
22. Hata, A. (2001). *Exp. Cell Res.* **264,** 111–116.
23. Kaji, H., Canaff, L., Lebrun, J.-J., Goltzman, D., and Hendy, G. N. (2001). *Proc. Natl. Acad. Sci. USA* **98,** 3837–3842.
24. Sowa, H., Kaji, H., Kitazawa, R., et al. (2004). *Cancer Res.* **64,** 2222–2228.
25. Crabtree, J. S., Scacheri, P. C., Ward, J. M., et al. (2001). *Proc. Natl. Acad. Sci. USA* **98,** 1118–1123.
26. Bertolino, P., Tong, W. M., Galendo, D., Wang, Z. Q., and Zhang, C. X. (2003). *Mol. Endocrinol.* **17,** 1880–1892.
27. Crabtree, J. S., Scacheri, P. C., Ward, J. M., et al. (2003). *Mol. Cell Biol.* **23,** 6075–6085.
28. Bhuiyan, M. M., Sato, M., Murao, K., Imachi, H., Namihira, H., and Takahara, J. (2000). *J. Clin. Endocrinol. Metab.* **85,** 2615–2619.
29. Cavallari, I., D'Agostino, D. M., Ferro, T., et al. (2003). *J. Clin. Endocrinol. Metab.* **88,** 3893–3901.
30. Theodoropoulou, M., Cavallari, I., Barzon, L., et al. (2004). *Endocrine-Related Cancer* **11,** 333–344.
31. Cebrian, A., Ruiz-Llorente, S., Cascon, A., et al. (2003). *J. Med. Genet.* **40,** e72.
32. Honda, M., Tsukada, T., Horiuchi, T., et al. (2004). *Int. Med.* **43,** 310–314.
33. Shimizu, S., Tsukada, T., Futami, H., et al. (1997). *Jap. J. Cancer Res.* **88,** 1029–1032.
34. Kishi, M., Tsukada, T., Shimizu, S., et al. (1998). *Jap. J. Cancer Res.* **89,** 1–5.
35. Togashi, K., Takahashi, N., Ando, K., Tsukamoto, Y., and Marumo, F. (1990). *Int. J. Art Organs* **13,** 77–82.