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ORIGINAL

Application of an intracellular stability test of a novel missense menin mutant to the diagnosis of multiple endocrine neoplasia type 1

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Abstract. Germline *MEN1* mutation analysis is a powerful tool for an early diagnosis of multiple endocrine neoplasia type 1 (MEN1), an autosomal dominant familial cancer syndrome characterized by the parathyroid, pituitary and gastroenteropancreatic endocrine tumors. However, the clinical significance of *MEN1* gene variants, especially missense and in-frame mutations as well as some splicing mutations, is not always obvious. We have previously shown that mutant menin proteins associated with MEN1 are rapidly degraded by the ubiquitin-proteasome pathway. We also demonstrated by a fluorescent immunocytochemical stability test that the stability of missense and in-frame deletion mutants varies widely but that unstable mutants were found only in MEN1 and related disorders and not in normal polymorphisms. In the present study, we evaluated by this stability test the pathogenicity of a novel *MEN1* missense mutation, c.1118C>T, encoding a P373L mutant menin, identified in a suspected MEN1 patient. The results demonstrated that the mutant menin is highly unstable, indicating that this mutation is causative for MEN1. These findings encouraged us to proceed with presymptomatic genetic screening for this mutation among the family members, which resulted in the identification of asymptomatic mutation carriers. Thus, the information from the menin stability test was useful for genetic diagnosis and counseling of MEN1 in the case with a previously unreported *MEN1* missense mutation.

Key words: MEN1, Menin, Stability, Missense mutation

MULTIPLE ENDOCRINE NEOPLASIA TYPE 1 (MEN1) is a relatively rare autosomal dominantly inherited condition characterized by hyperplastic and neoplastic disorder of endocrine organs such as the parathyroid, anterior pituitary and gastroenteropancreatic endocrine tissues [1]. Germline mutation of the causative gene, *MEN1*, which encodes 610 amino-acid residue nuclear protein menin, can be identified in the most of the affected subjects [2, 3]. *MEN1* is a tumor suppressor gene and tumorigenesis in MEN1 by *MEN1* gene mutations can be explained by Knudson's two-hit theory [4], i.e., function of one allele is lost by a germline mutation and the inactivation of the remaining wild type allele by somatic mutation leads to tumor development.

The majority of mutations identified in affected subjects are nonsense and frameshift mutations. Splice mutations and large deletion of the *MEN1* gene have also been reported in several families [3]. It is obvious that these mutations cause loss of function of the gene and are pathogenic. On the other hand, when a novel missense mutation or an in-frame deletion or addition is identified, molecular diagnosis of MEN1 is not so simple since the pathogenicity of these mutations is not clear *per se*. Although 26% and 48% of germline *MEN1* mutations associated with MEN1 and familial isolated hyperparathyroidism, respectively, are missense mutations or in-frame deletions [3], evidence for the pathogenicity of these mutations was lacking in many cases [5-9]. As menin shows no significant homology to

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Table 1 Serum and plasma concentrations of biochemical parameters of P373L mutation carriers

		I-2*	II-2**	III-1	III-2	III-3	Normal range
Age (year)		82	57	31	28	24	
Sex		F	F	M	M	M	
Calcium	(mg/dL)	10.0	9.9	10.0	9.6	10.6	8.8-10.1
Phosphate	(mg/dL)	2.7	2.7	3.9	3.4	3.1	2.7-4.0
Intact PTH	(pg/mL)	57	119	85	32	64	10-65
Prolactin	(ng/mL)	9.1	12.2	6.5	14.1	12.2	< 12.7
IGF-1	(ng/mL)	ND	193	215	ND	ND	37-266 (II-2) 85-369 (III-1)
Glucose	(mg/dL)	188	99	82	86	105	70-109
Insulin	(μU/mL)	24.0	7.0	9.7	8.5	6.1	1.84-12.2
Gastrin	(pg/mL)	77	39	52	102	84	< 200

* I-2 has diabetes and is receiving medication. Insulin and glucose of I-2 were measured 2 hours after meal.
** Proband. ND, not determined. IGF-1, insulin-like growth factor I

other known proteins and its physiological function is not fully understood, there are no established parameters that can adequately represent impaired function of mutant menin [10-14].

We previously reported that missense mutant menin proteins associated with MEN1 are unstable and rapidly degraded through ubiquitin-proteasome pathway [15]. More detailed analysis by a newly developed fluorescence immunocytochemical method revealed that the stability of missense and in-frame deletion mutants varies widely but that unstable mutants were found only in MEN1 and related disorders and not in normal polymorphisms [16]. We recently encountered a suspected MEN1 patient with a previously unreported missense mutation in the *MEN1* gene. To assess the pathogenicity of this mutation, we examined the stability of the menin protein encoded by this mutant *MEN1* gene.

Case Presentation

A 56-year-old woman was referred to Shinshu University Hospital. She had been diagnosed with primary hyperparathyroidism (PHPT) and undergone parathyroidectomy when she was 45 years old. Three enlarged glands were removed but the fourth gland was not found. She had been followed-up before being referred to us. An abdominal CT scan identified multiple contrast-enhanced nodular lesions (3-12 mm in diameter) in her pancreas, based upon which she was suspected as having MEN1. She was eucalcemic but her plasma PTH level was elevated (II-2, Table 1). Other biochemical studies including fasting plasma levels of gastrin, insulin and glucose, and glucagon revealed no abnormalities. Pancreas tumors were thus considered nonfunctioning. MRI imaging for pituitary gland revealed no abnormal findings and plasma levels of prolactin and IGF-1 (insulin-like growth factor I) were within normal range. Genetic testing of the patient, performed after obtaining written informed consent, revealed a heterozygous single nucleotide substitution (c.1118C>T) in the *MEN1* gene, which was predicted to substitute amino acid codon 373 of menin from proline (CCC) to leucine (CTC). This mutation has neither been reported [3] nor registered to mutation database (The Human Gene Mutation Database, http://www.hgmd.cf.ac.uk/ac/index.php).

Her 82-year-old mother had a history of PHPT and had undergone a single gland parathyroidectomy at the age of 69. She is currently eucalcemic (Table 1) and is receiving no medication except oral antidiabetic drugs for her type 2 diabetes. Periodic surveillance including imaging studies for pituitary and abdomen and biochemical and endocrine function tests are performed at another hospital and no MEN1-related diseases have been identified. Genetic analysis revealed that she also had the same mutation.

Tumor specimen was not available as surgeries for

the proband and her mother were undertaken at other hospitals more than 10 years ago.

Materials and Methods

The intracellular stability of missense menin variants was evaluated using a quantitative fluorescent immunohistochemical method as described previously [15, 16]. Briefly, WI38VA13 cells were transfected with a bicistronic plasmid expressing N-terminal FLAG-tagged and Myc-tagged proteins: one protein was wild type menin, which served as an internal control for transfection efficiency, and the other was the variant menin to be tested. Forty eight hours after transfection, expressed proteins were stained with FITC-labeled anti-FLAG antibody and Cy3-labeled anti-c-myc antibody, and quantified by fluorescence microscopic digital photography and an image analysis software. The ratios of the mean numerical value of fluorescence intensity for mutant menin to that for wild type menin in each nucleus was calculated, and normalized by the ratio obtained from the control plasmid expressing both FLAG- and Myc-tagged wild-type menin. As a known unstable control, L22R variant expression plasmids were used. Mutant menin was located mainly in the nucleus although the cytoplasm was also faintly stained in some cells. Only nuclear staining was analyzed.

To measure the degradation rate of menin proteins, 293T cells were transfected with plasmids expressing FLAG-tagged menin, and 28 hr after transfection, 20 μg/mL of cycloheximide (CHX) was added into the culture medium to prevent further protein synthesis. Whole-cell lysates were prepared from samples taken at 0 hr (control) and 6 hr after adding CHX, and analyzed by Western blotting with an alkaline phosphatase-conjugated anti-FLAG monoclonal antibody coupled with CDP-Star reagent. The membranes were exposed to X-ray films, and density of the target bands were scanned with a densitometer.

These studies were approved by the Institutional Review Board of both the National Cancer Center Research Institute and Shinshu University School of Medicine.

Results

Stability of variant menin P373L

The intracellular stability of the putative products of the c.1118C>T mutation, P373L, was examined by comparing the relative expression levels of mutant *vs.* wild-type menin proteins expressed from a bicistronic plasmid. The L22R mutant, a disease-causing mutation associated with typical MEN1, was used as a positive control for unstable menin. Two plasmids were constructed for each mutant, one expressing FLAG-tagged wild type menin and Myc-tagged mutant menin, the other expressing FLAG-tagged mutant menin and Myc-tagged wild type menin. Using either construct, the test showed that stability of the P373L mutant was comparable to that of the L22R mutant (Fig. 1A and 1B).

To confirm that the lowered protein level of the mutant was due to rapid protein degradation, the effects of CHX on the amounts of menin proteins were analyzed. The results demonstrated the rapid reduction of P373L mutant after 6-hr treatment with CHX, while the amount of the wild type menin was almost unaffected (Fig. 1C). These findings suggest that the c.1118C>T mutation is likely a pathogenic mutation causing MEN1.

Presymptomatic genetic testing for offspring of the proband

We confirmed an association between the mutation and phenotype in the elder generation of the family (generations I and II, Fig. 2) before offering presymptomatic genetic testing for her offspring. II-1 and II-3 did not have c.1118C>T mutation, and no abnormal findings were found by biochemical and imaging studies. Genetic testing of three sons (III-1,2,3, Fig. 2) was then performed and revealed that they all had c.1118C>T mutation.

Although they were asymptomatic, biochemical screening indicated that they had early stage endocrine abnormalities, consistent with results of genetic testing. III-1 was eucalcemic but intact PTH level was above the normal range. Prolactin level of III-2 was slightly elevated, and III-3 had hypercalcemia with unsuppressed PTH (Table 1). Although observed biochemical changes were subtle and imaging studies failed to detect any abnormalities in either individual, it is likely that they had already developed the disease. Indeed, in contrast to sporadic PHPT, a significant proportion of PHPT developed in MEN1 patients show marginal biochemical abnormalities [17]. Future surveillance for three sons was thus warranted.

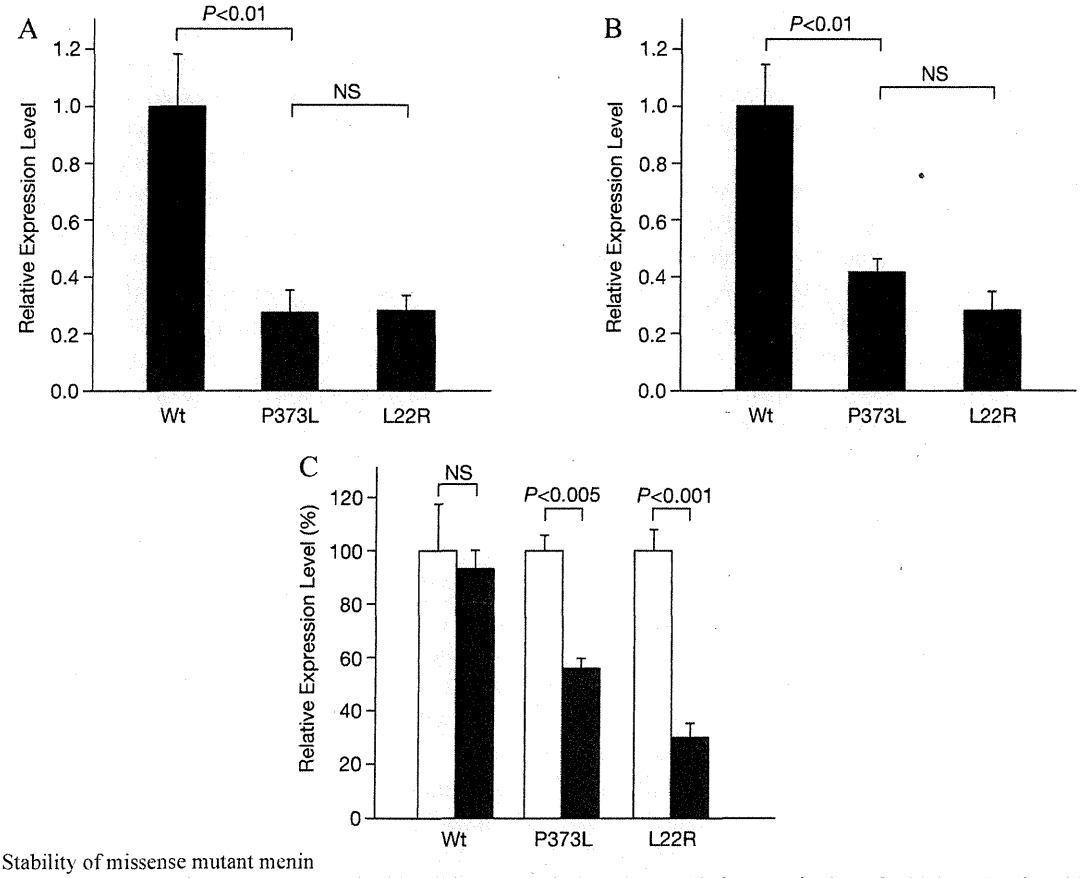

Fig. 1 Stability of missense mutant menin
Mutant menin protein was coexpressed with wild type menin in culture cells by transfection of a bicistronic plasmid vector expressing either FLAG-tagged wild type and Myc-tagged mutant menin (A) or FLAG-tagged mutant and Myc-tagged wild type menin (B). The relative expression levels of mutant to wild type menin were compared with those of control plasmid expressing FLAG-tagged and Myc-tagged wild type menin proteins (Wt). Degradation rate of menin proteins was evaluated by CHX experiments (C). The open and closed bars indicate the control and CHX-treated samples, respectively. The data are expressed as relative values, with the control levels of each menin protein being a hundred per cent. The thin bars represent standard error of the mean of three independent transfection experiments. P373L and L22R represent the missense menin mutant identified in this study and that previously reported to cause typical MEN1, respectively. NS, not statistically significant ($P>0.05$).

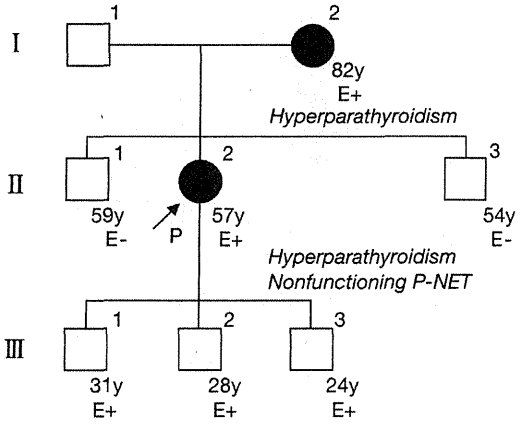

Fig. 2 Pedigree of the patient with P373L mutation
E+, have had genetic analysis and P373L mutation was identified; E-, have had genetic analysis and P373L mutation was not identified; P, proband; P-NET, pancreas neuroendocrine tumor

Discussion

In the present study, we examined stability of the mutant menin protein identified in a family with MEN1. Our case had PHPT with involvement of multiple glands and pancreas endocrine tumors. Her mother also had a history of PHPT, but she was diagnosed with PHPT at the age of 69 and only one gland was affected. Her mother has remained eucalcemic for 13 years since single gland parathyroidectomy and no other endocrine diseases had developed to date, which is an atypical clinical course of MEN1. Therefore, we were cautious to conclude that the c.1118C>T missense mutation was pathogenic based only on the segregation pattern. Although an association of a different mutation P373S at the same codon with typical MEN1 had previously been reported [18], there are a number of examples that different amino acid substitution at the same codon exerts different clinical consequences.

Our present study demonstrated that the P373L missense menin protein is highly likely pathogenic as this protein is apparently unstable compared to wild-type menin. This finding encouraged us to offer presymptomatic genetic testing for her sons, which resulted in early diagnosis of the disease. Since the menin stability test we established focuses on the stability of protein rather than its specific function, it enables a more comprehensive verification of pathogenicity of mutant menin. It might be argued that our *in vitro* method, which quantitates proteins in fibroblast-derived culture cells, may not reflect menin stability in endocrine cells. However, we have previously demonstrated an apparent correlation between the clinical phenotype and stability of missense menin tested in various non-endocrine as well as in endocrine cells [15, 16]. We are also aware that the stability of menin missense mutants is highly variable and that some mutants associated with typical MEN1 are comparatively stable [16, 19]. Therefore, the pathogenicity of a missense mutation giving rise to a stable mutant menin should be interpreted cautiously.

In conclusion, we examined the pathogenicity of novel nucleotide substitution in the *MEN1* gene using a menin stability test. Our results strongly suggest that c.1118C>T mutation is pathogenic. The future collection of data on the stability of missense menin protein will be of value in understanding the molecular pathogenicity of menin variants.

Disclosure Summary

All authors have nothing to disclose.

Acknowledgments

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References

1. Marx SJ, Stratakis CA (2005) Multiple endocrine neoplasia--introduction. *J Intern Med* 257:2-5.
2. Chandrasekharappa SC, Guru SC, Manickam P, Olufemi SE, Collins FS, et al. (1997) Positional cloning of the gene for multiple endocrine neoplasia-type 1. *Science* 276:404-407.
3. Lemos MC, Thakker RV (2008) Multiple endocrine neoplasia type 1 (MEN1): analysis of 1336 mutations reported in the first decade following identification of the gene. *Hum Mutat* 29:22-32.
4. Marx S, Spiegel AM, Skarulis MC, Doppman JL, Collins FS, et al. (1998) Multiple endocrine neoplasia type 1: clinical and genetic topics. *Ann Intern Med* 129:484-494.
5. Pinna G, Orgiana G, Carcassi C, Alba F, Cetani F, et al. (2004) A novel germline mutation of MEN 1 gene in a patient with acromegaly and multiple endocrine tumors. *J Endocrinol Invest* 27:577-582.
6. Ozturk M, Chiu CY, Akdeniz N, Jenq SF, Chang SC, et al. (2006) Two novel mutations in the MEN1 gene in subjects with multiple endocrine neoplasia-1. *J Endocrinol Invest* 29:523-527.
7. Balogh K, Hunyady L, Patocs A, Gergics P, Valkusz Z, et al. (2007) MEN1 gene mutations in Hungarian patients with multiple endocrine neoplasia type 1. *Clin Endocrinol (Oxf)* 67:727-734.

8. Choi H, Kim S, Moon JH, Lee YH, Rhee Y, et al. (2008) Multiple endocrine neoplasia type 1 with multiple leiomyomas linked to a novel mutation in the MEN1 gene. *Yonsei Med J* 49:655-661.
9. Hou R, Manwaring LP, Moley JF, Whelan A (2011) A novel missense mutation in the MEN1 gene in a patient with multiple endocrine neoplasia type 1. *Endocr Pract* 17:e63-67.
10. Agarwal SK, Lee Burns A, Sukhodolets KE, Kennedy PA, Obungu VH, et al. (2004) Molecular pathology of the MEN1 gene. *Ann N Y Acad Sci* 1014:189-198.
11. Gracanin A, Dreijerink KM, van der Luijt RB, Lips CJ, Höppener JW (2009) Tissue selectivity in multiple endocrine neoplasia type 1-associated tumorigenesis. *Cancer Res* 69:6371-6374.
12. Hendy GN, Kaji H, Canaff L (2009) Cellular functions of menin. *Adv Exp Med Biol* 668:37-50.
13. Tsukada T, Nagamura Y, Ohkura N (2009) MEN1 gene and its mutations: basic and clinical implications. *Cancer Sci* 100:209-215.
14. Balogh K, Patocs A, Hunyady L, Racz K (2010) Menin dynamics and functional insight: take your partners. *Mol Cell Endocrinol* 326:80-84.
15. Yaguchi H, Ohkura N, Takahashi M, Nagamura Y, Kitabayashi I, et al. (2004) Menin missense mutants associated with multiple endocrine neoplasia type 1 are rapidly degraded via the ubiquitin-proteasome pathway. *Mol Cell Biol* 24:6569-6580.
16. Shimazu S, Nagamura Y, Yaguchi H, Ohkura N, Tsukada T (2011) Correlation of mutant menin stability with clinical expression of multiple endocrine neoplasia type 1 and its incomplete forms. *Cancer Sci* 102:2097-2102.
17. Eller-Vainicher C, Chiodini I, Battista C, Viti R, Mascia ML, et al. (2009) Sporadic and MEN1-related primary hyperparathyroidism: differences in clinical expression and severity. *J Bone Miner Res* 24:1404-1410.
18. Bergman L TB, Cardinal J, Palmer J, Walters M, Shepherd J, Cameron D, Hayward N (2000) Identification of MEN1 gene mutations in families with MEN 1 and related disorders. *Brit J Cancer* 83:1009-1014.
19. Murai MJ CM, Reddy G, Grembecka J, Cierpicki T (2011) Crystal structure of menin reveals binding site for mixed lineage leukemia (MLL) protein. *J Biol Chem* 286:31742-31748.

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ORIGINAL ARTICLE

An analysis of genotype–phenotype correlations and survival outcomes in patients with primary hyperparathyroidism caused by multiple endocrine neoplasia type 1: the experience at a single institution

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Abstract
Purpose To examine the clinical characteristics and survival outcomes of patients with primary hyperparathyroidism (PHPT) in multiple endocrine neoplasia type 1 (MEN1) in relation to the *MEN1* gene mutation.
Methods The study population included the patients, positive for the *MEN1* gene mutation, who underwent parathyroidectomy between 1983 and 2009 at a single tertiary referral center. Manifestations of the syndrome, other tumors and causes of death were retrospectively correlated with the specific types and locations of *MEN1* gene mutations.
Results Thirty-two patients from 19 families were diagnosed as having MEN1 on genetic examinations. Mutations were most common in exons 2, 7 and 10. A phenotypic analysis of the main MEN1 tumor types among the 32 patients revealed that PHPT was the most common (100 %), followed in order by pancreatic neuroendocrine tumors (PNETs) (53 %) and pituitary tumors (38 %). Death due to MEN1-related disease occurred in five patients (16 %), including malignant PNET in three cases (exons 2, 3), pituitary crisis in one case (exon 2) and thymic cancer in one case (large deletion).
Conclusions Premature deaths related to MEN1 are due to the development of malignant PNET, pituitary crisis or thymic tumors associated with mutations in exons 2, 3 and a large deletion.

Keywords Multiple endocrine neoplasia type 1 · Primary hyperparathyroidism · Genotype–phenotype analysis

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Introduction

Multiple endocrine neoplasia type 1 (MEN1) is an autosomal-dominant disease characterized by tumors of the parathyroid, pancreas and pituitary. Its prevalence has been estimated to be 1–10 per 100,000 individuals [1]. The clinical manifestations of MEN1 include hyperparathyroidism (over 90 % of cases), pancreatic islet cell tumors (30–80 % of cases) and pituitary tumors (15–50 % of cases) [1, 2]. Other complications such as facial angiofibromas, lipomas, carcinoids and adrenal tumors have also been identified [1, 2].

The locus of the gene responsible for MEN1 was assigned to the long arm of chromosome 11 in 198 [3, 8] and the gene was isolated by positional cloning in 1997 [4]. *MEN1* is a tumor-suppressor gene spanning 9 kb and containing 10 exons. It encodes the 610-amino-acid protein known as menin [5].

Investigators have attempted to correlate various mutations of *MEN1* with clinical manifestations or patient survival; however, no significant correlations have emerged. We herein present an analysis of the genotype–phenotype expression and genotype–prognosis correlations based on long-term observation of MEN1 patients who underwent

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parathyroid surgery for primary hyperparathyroidism (PHPT) at our institution.

Methods

Patients

Between 1983 and 2009, 68 patients from 46 kindreds with PHPT or multiglandular disease underwent parathyroidectomy at our department. To be deemed as having MEN1, they had to meet the following criteria: (1) multiglandular disease with a histopathological diagnosis of parathyroid hyperplasia and (2) satisfying the practical criteria for MEN1 reported by Brandi et al. [2].

Clinical manifestations

All patients had PHPT with serum calcium levels exceeding 10.0 mg/dl (normal 8.5–9.9 mg/dl) and serum intact-PTH levels exceeding 66 pg/ml (normal 10–65 pg/ml). The presence or absence of MEN1-associated diseases was determined by measuring the serum levels of gastrin, insulin, glucagon, prolactin, GH and IGF-1 and by performing imaging studies (MRI, CT) of the pituitary and pancreas.

MEN1 gene

All 68 patients had undergone a *MEN1* gene analysis after providing their informed consent. From each patient, a blood sample was collected and genomic DNA was extracted using a QIA Amp blood kit (Qiagen, Hilden, Germany). All protein-coding regions of exons 2 through 10 of the *MEN1* gene were amplified using polymerase chain reaction (PCR). A PCR assay and sequence analyses were performed, as previously described elsewhere [6]. All identified mutations were confirmed by cloning the PCR products into the pCR vector (Invitrogen, Carlsbad, CA, USA) and nucleotide sequencing. For one case involving a large deletion, the mutation was detected as reported previously [7].

Statistical analysis

The overall survival after initial parathyroidectomy was estimated with the Kaplan–Meier method using the JMP (version 8, SAS Institute Japan Ltd., Tokyo, Japan) statistical software package.

We analyzed genotype–phenotype correlations and the survival outcomes of the MEN1 patients.

Results

Among the 68 patients, 25 did not undergo genetic examinations, while one was negative and 42 were positive for the *MEN1* gene mutation. Ten of the latter 42 patients were excluded from the study due to incomplete clinical data. The remaining 32 patients from 19 kindreds comprised the study population. Five patients were male and 27 were female. The average age and standard deviation at initial parathyroidectomy was 45.6 ± 15 years (range 20–73 years), and the average follow-up period and standard deviation was 153 ± 97 months (range 1–396 months).

A phenotypic analysis of the main MEN1 manifestations among the 32 patients revealed that PHPT was the most common (32 patients, 100 %), followed in order by pancreatic neuroendocrine tumors (PNETs) (16 patients, 50 %) and pituitary tumors (15 patients, 47 %). In the 26 patients without *MEN1* gene confirmation, PHPT was the most common manifestation (26 patients, 100 %), followed in order by pancreatic tumors (10 patients, 38.5 %), pituitary tumors (nine patients, 24.6 %), adrenal tumors (three patients, 11.5 %), thymic tumors (three patients, 11.5 %) and malignant tumors (two patients, 7.7 %) (Table 1).

Genotype–phenotype analysis

The locations and types of *MEN1* mutations are summarized in Table 2. Mutations were most common in exons 2 (10 patients from four kindreds) and 10 (five patients from four kindreds). In exons 3 and 7, five patients (three kindreds) and four patients (three kindreds) had mutations, respectively. The genotype–phenotype associations of tumors of the pancreas and pituitary are shown in Table 3. Sixteen patients developed tumors of the pancreas. Seven

Table 1 Characteristics of the patients without *MEN1 gene* confirmation

Total	26
PHPT	26 (100 %)
Pituitary tumor	9 (34.6 %)
PRL	5
Microadenoma	4
Pancreatic tumor	10 (38.5 %)
Non-functioning	7
Gastrinoma	2
Gastrinoma	1
Adrenal tumor	3 (11.5 %)
Thymic tumor	3 (11.5 %)
Others	2 (7.7 %)
Abdominal desmoids	1
Laryngeal Cancer	1

Table 2 The site and type of the *MEN 1* gene

Location	Type	No. of kindreds	No. of patients
Exon 2			
249_252delGTCT	Frameshift	2	5
358_360delAAG	In-frame deletion	1	4
133G > T	Nonsense	1	1
Exon 3			
455T > A	Nonsense	1	2
512_520delGGGATGTCC	In-frame deletion	2	3
Intron 4			
784 − 9G > A	Splicing site mutation	1	1
824 + 1G > A	Splicing site mutation	1	1
Exon 6			
878delC	Frameshift	1	1
Exon 7			
959C > T	Missense	1	1
955_956insT	Frameshift	1	1
914G > A	Missense	1	2
Exon9			
1324C > T	Nonsense	1	2
Exon 10			
1546_1547insC	Frameshift	3	4
1387G > T	Nonsense	1	1
Large deletion		1	3

patients had PNETs, including four patients with gastrinomas, one patient with an insulinoma, one patient with a glucagonoma and one patient with a malignant tumor. Non-functional pancreatic tumors were observed in nine patients. Mutations in the *MEN1* gene were found at exons 2, 3, 6 and 10, and a large deletion was present in one case. Among 11 patients with functional pituitary adenomas, nine had prolactinomas, one had an ACTH-producing adenoma and one had an adenoma that produced both prolactin and growth hormone. Non-functional pituitary tumors were found in two patients. No particular trends were observed between the sites of mutation and the clinical manifestations in the pancreas or pituitary.

Table 3 also shows the genotype–phenotype correlations of thymic tumors and other malignant diseases. Three patients had lesions in the thymus. One patient with a large deletion mutation had a carcinoma of the thymus, and her son was also found to have a thymic carcinoid. The clinical details of another patient with an exon 3 mutation were not available. Other malignant tumors were found in three patients: one patient with a mutation in exon 2, who also had malignant PNET, died of meningioma; another patient with a mutation in exon 2 developed lung cancer; one patient who had a mutation in exon 10 developed both papillary carcinoma of the thyroid and osteomyelodysplasia.

Survival

Five (16 %) patients died of MEN1-related disease during the follow-up period. The overall survival rates of the patients at 5, 10 and 20 years were estimated to be 99.6 % (95 % CI 88.4–99.9), 95 % (95 % CI 80.8–98.9) and 74.5 % (95 % CI 51.1–89.1), respectively (Fig. 1). The mutations in the patients who died involved exons 2 and 3, and a large deletion. Two patients died of liver metastases from gastrinomas, one patient died of malignant PNET and meningioma and one patient died due to pituitary crisis. The patient with the large deletion died of thymic cancer.

Discussion

The first description of a patient with both parathyroid and pituitary tumors was published in 1903 [8]. Half a century later, in 1952, based on a case series of patients with tumors of the parathyroid, pituitary and pancreatic islets, Underdahl et al. [9] recognized the combination to be a new syndrome. Subsequently, Wermer [6] hypothesized that the syndrome was caused by a mutation in an autosomal gene with a high penetrance.

In our present series, *MEN1* gene mutations were observed in exons 2, 3, 4, 6, 7, 9 and 10, and a large deletion was also recognized. The most frequently observed mutation site was exon 2. The distribution of the mutation sites in our patients was similar to that in a previous report of 24 other Japanese cases [10], although it was different from the results reported by Turner [11]. In our series, frame-shift mutations were the most common form, in agreement with the results of previous reports [12, 13].

Although most manifestations of MEN 1 are thought to be benign, the life expectancy of affected patients has become an important issue. Dean et al. [7] reported that MEN1 patients have a lower 20-year survival rate than normal age- and sex-matched US citizens (64 vs. 81 %). Wilkinson et al. [14] reported the results of a retrospective survey on causes of death in a MEN1 family in Tasmania dating from 1861. Of the 46 family members who had a high probability of having MEN1, 20 (43 %) died due to MEN1-associated conditions, comprising 12 cases of neoplasia and eight cases of hypercalcemia [14]. Wilson et al. [15] observed a dramatic change in the causes of death in their population. Gastrointestinal (GI) hemorrhage was the most common cause of death in the young generation

Table 3 Clinical characteristics of the patients according to the *MEN 1* gene mutation

Location	Pancreatic tumor	Pituitary tumor	Thymic tumor	Other tumors	Number of deaths and cause of death
Exon 2					
249_252delGTCT	5: Gas(1), Glu(1), Malig(1) Non(2)	3: PRL(3)	0	Meningioma (1)	2: Meningioma (1), pituitary crisis (1)
358_360delAAG	2: Non(2)	2: PRL(2)	0	Lung cancer (1)	0
133G > T	1: Non(1)	0	0	–	1: Liver metastases from gastrinoma
Exon 3					
455T > A	2: Gas(1) Non(1)	2: PRL(2)	0	–	1: Liver metastases from gastrinoma
512_520del GGGATGTCC	1: Non(1)	0	Unknown (1)	–	0
Intron 4					
784-9G > A	0	0	0	–	0
824 + 1G > A	0	0	0	–	0
Exon 6					
878delC	1: Non(1)	1: Non(1)	0	–	0
Exon 7					
959C > T	0	1: PRL(1)	0	–	0
955_956insT	0	0	0	–	0
914G > A	0	0	0	–	0
Exon 9					
1324C > T	0	0	0	–	0
Exon 10					
1546_1547insC	2: Gas(1) Non(1)	3: PRL(1), PRL and GH(1), ACTH(1)	0	Papillary carcinoma of the thyroid and osteomyelodysplasia (1)	0
1387G > T	1: Ins(1)	1: Non(1)	0	–	0
Large deletion	1: Gas(1)	0	2: carcinoid and cancer	–	1: Thymic cancer

Gas gastrinoma, *Glu* glucagonoma, *Ins* insulinoma, *PRL* prolactinoma, *GH* growth hormone producing adenoma, *ACTH* ACTH producing adenoma, *Non* non-functioning tumor, *Malig* malignant PNETs. (), number of patients; unknown, histological diagnosis was unknown

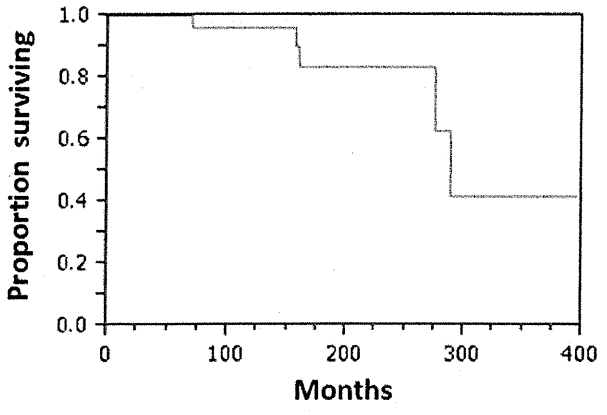

Fig. 1 Overall survival after initial parathyroidectomy in patients with MEN 1

living before the 1960s, whereas metastases of pancreatic carcinoids became the leading cause of death in the 1990s. Goudet et al. [16] also reported similar observations in MEN1 patients: the death rate due to GI hemorrhage was 14.5 % before the 1990s and fell to 2.8 % after the 1990s. Mortality due to tumor progression also changed from 47.8 % before the 1990s to 65.7 % thereafter [16]. Consequently, PNETs and carcinoids have been the predominant causes of death among patients with MEN1.

After the isolation of the *MEN1* gene, many investigators searched for genotype–phenotype or genotype–prognosis correlations. Kouvaraki et al. [17] reported that mutations in exon 2 are frequent in MEN1 patients with PNETs and mutations in exon 3 are frequent in those with thymic carcinoids. Ferolla et al. [18] reported that truncated

mutations are frequently present in patients with MEN1-associated thymic carcinoids. Kouvaraki et al. [17] found no relationship between genotype and prognosis.

The issues addressed in this retrospective study of MEN 1 patients were: (1) whether there are any genotype–phenotype correlations and (2) whether there are any prognostic predispositions depending on the *MEN1* genotype.

With regard to the first issue, we did not find any significant genotype–phenotype correlations. However, our experience is unique from several viewpoints. First, functional PNETs were observed in patients with *MEN 1* mutations in exons 2, 3 and 10 only. Second, thymic tumors were observed in patients with mutations in exon 3 and in the patient with the large deletion. Third, malignant diseases unrelated to MEN1, such as papillary thyroid carcinoma, lung cancer and osteomyelodysplasia, were observed only in patients with mutations in exons 2 and 10. Bartsch et al. [19] reported that patients with mutations in exons 2, 9 or 10 have a significantly higher rate of malignant PNETs (55 %) than those with mutations elsewhere (10 %). Kouvaraki et al. [17] reported that PNETs are frequently observed when the mutation is present in exon 2, whereas carcinoids are frequent when the mutation is in exon 10.

With regard to the second issue, MEN1-associated deaths were identified only among patients with mutations of exons 2 and 3 and in the one patient with a large deletion. However, it is premature to conclude that these mutations are specifically related to mortality. In fact, Vierimaa et al. [20] observed MEN1-related deaths in patients with mutations of exon 10.

Mortality associated with MEN1 in the present series was 16 %, which is quite different from figures reported previously [7, 14, 21]. One of the reasons for this difference may be advances in both knowledge and management of MEN1. In fact, in all of the cohort studies reported previously, the patients had been observed since the early 1900s.

The present study had several limitations. First, the study population included patients who had undergone parathyroidectomy. Such selection may have led to biased estimates of the phenotypic prevalence and disease-specific survival. Second, the number of patients (32) was too small to draw firm conclusions from the results. Third, the follow-up period was limited. Fourth, neither diagnostic nor follow-up examinations were uniform among the patients. The variation in work-up may have compromised the validity of the estimated disease frequency and prognosis.

In conclusion, no significant genotype–phenotype or genotype–prognosis correlations were observed in this study. The fact that the study involved a single institution may also have been a limiting factor. In addition, a longer follow-up period in the patients is needed. In 2008, researchers in Japan established a MEN study group, the "MEN Consortium of Japan," to construct a nationwide database of Japanese MEN patients. It is anticipated that this database will yield data on many unanswered questions related to MEN.

References

1. Marini F, Falchetti A, Del Monte F, Sala SC, Gozzini A, Luzi E, et al. Multiple endocrine neoplasia type 1. Orphanet J Rare Disease. 2006;1:38.
2. Brandi ML, Gagel RF, Angeli A, Bilezikian JP, Beck-Peccoz P, Bordi C, et al. Consensus: guidelines for diagnosis and therapy of MEN type 1 and type 2. J Clin Endocrinol Metab. 2001;86: 5658–71.
3. Larsson C, Skogseid B, Oberg K, Nakamura Y, Nordenskjold M. Multiple endocrine neoplasia type 1 gene maps to chromosome 11 and is lost in insulinoma. Nature. 1988;332:85–7.
4. Chandrasekharappa SC, Guru SC, Manickam P, Olufemi SE, Collins FS, Emmert-Buck MR, et al. Positional cloning of the gene for multiple endocrine neoplasia-type 1. Science. 1997;276: 404–7.
5. Agarwal SK, Kester MB, Debelenko LV, Hepper C, Emmert-Buck MR, Skarulis MC, et al. Germline mutations of the *MEN1* gene in familial multiple endocrine neoplasia type 1 and related states. Hum Mol Genet. 1997;6:1169–75.
6. Wermer P. Genetic aspects of adenomatosis of endocrine glands. Am J Med. 1954;16:363–71.
7. Dean P, van Heerden JA, Farley DR, Thompson GB, Grant CS, Harmsen WS, et al. Are patients with multiple endocrine neoplasia type prone to premature death? World J Surg. 2000;24:1437–41.
8. Erdheim J. Zur normalen und pathologischen Histologie der Glandula thyreoidea, parathyreoidea und Hypophysis. Beitr Pathol Anat. 1903;33:158–236.
9. Underdahl LO, Woolner LB, Black MB. Multiple endocrine adenomas: report of 8 cases in which the parathyroids, pituitary and pancreatic islets were involved. J Clin Endocrinol Metab. 1953;13:20–47.
10. Hai N, Aoki N, Matuda A, Mori T, Kosugi S. Germline *MEN1* mutations in sixteen Japanese families with multiple endocrine neoplasia type 1. Eur J Endocrinol. 1999;141:475–80.
11. Turner JJO, Leotleta PD, Pannett AAJ, Forbes SA, Bassett JHD, Harding B, et al. Frequent occurrence of an intron 4 mutation in multiple endocrine neoplasia type 1. J Clin Endocrinol Metab. 2002;87:2688–93.
12. Lemos MC, Takker RV. Multiple endocrine neoplasia type 1 (MEN1): analysis of 1336 mutations reported in the first decade following identification of the gene. Hum Mutat. 2008;29: 22–32.
13. Morelli A, Falchetti A, Martineti V, Becherini L, Mark M, Friedman E, et al. *MEN1* gene mutation analysis in Italian patients with multiple endocrine neoplasia type 1. Eur J Endocrinol. 2000;142:131–7.
14. Wilkinson S, Teh BT, Davey KR, McArdle JP, Young M, Shepherd JJ. Cause of death in multiple endocrine neoplasia type 1. Arch Surg. 1993;128:683–90.
15. Wilson SD, Krzywda EA, Zhu Y, Yen TWF, Wang TS, Sugg SL, et al. The influence of surgery in MEN-1 syndrome: observations over 150 years. Surgery. 2008;144:695–702.
16. Goudet P, Murat A, Binquet C, Cardot-Bauters C, Costa A, Ruszniewski P, et al. Risk factors and cause of death in MEN1 disease. A GTE (Group d'Etude des Tumeurs Endocrines) cohort study among 758 patients. World J Surg. 2010;34:249–55.

17. Kouvaraki MA, Lee JE, Shapiro SE, Gagel RF, Sherman SI, Sellin RV, et al. Genotype-phenotype analysis in multiple endocrine neoplasia type 1. Arch Surg. 2002;137:641–7.
18. Ferolla P, Falchetti A, Filosso P, Tomassetti P, Tamburrano G, Avenia N, et al. Thymic neuroendocrine carcinoma (carcinoid) in multiple endocrine neoplasia type 1 syndrome: the Italian series. J Clin Endocrinol Metab. 2005;90:2603–9.
19. Bartsch DK, Langer P, Wild A, Schilling T, Celik I, Rothmund N, et al. Pancreaticoduodenal endocrine tumors in multiple endocrine neoplasia type 1: surgery or surveillance? Surgery. 2000;128:958–66.
20. Vierimaa O, Ebeling TML, Kytola S, Bloigu R, Eloranta E, Salmi J, et al. Multiple endocrine neoplasia type 1 in northern Finland; clinical features and genotype-phenotype correlation. Eur J Endocrinol. 2007;157:285–94.
21. Doherty GM, Olson JA, Frisella MM, Lairmore TC, Wells SA, Norton JA. Lethality of multiple endocrine neoplasia type 1. World J Surg. 1998;22:581–7.

Review

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Regulation of Gastroduodenal Motility: Acyl Ghrelin, Des-Acyl Ghrelin and Obestatin and Hypothalamic Peptides

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Key Words
Gastroduodenal motility, regulation · Acyl ghrelin · Des-acyl ghrelin · Obestatin · Hypothalamic peptides

Abstract
Real-time measurements for gut motility in conscious rats or mice combined with intracerebroventricular or intravenous injection of peptide agonists or antagonists allow us to understand the regulatory mechanism of gastrointestinal motility. Neuropeptide Y (NPY) in the arcuate nucleus in the hypothalamus stimulates the fasted motility in the duodenum, while urocortin in the paraventricular nucleus inhibits fed and fasted motility in the antrum and duodenum. Acyl ghrelin exerts stimulatory effects on the motility of the antrum and duodenum in both the fed and fasted state of animals. NPY Y2 and Y4 receptors in the brain may mediate the action of acyl ghrelin, and vagal afferent pathways might be involved in this mechanism. Des-acyl ghrelin exerts inhibitory effects on the motility of the antrum but not on the motility of the duodenum in the fasted state of animals. CRF type 2 receptor in the brain may mediate the action of des-acyl ghrelin, and vagal afferent pathways might not be involved in this mechanism. Obestatin exerts inhibitory effects on the motility of the antrum and duodenum in the fed state but not in the fasted state of animals. CRF type 1 and type 2 receptors in the brain may mediate the action of obestatin, and vagal afferent pathways might be partially involved in this mechanism.

Experimental Design for Studying Gastrointestinal Motility and Brain-Gut Axis

Although the regulation of hypothalamic peptides on feeding behavior or energy expenditure has been well established, the regulation on the gastrointestinal (GI) motility has not been fully understood. The detailed studies on the brain-gut axis are somewhat hampered by the methodological difficulties in directly measuring the GI motility in conscious animals with intracerebroventricular (ICV) injection of peptide agonists or antagonists.

We developed a freely moving conscious animal model to measure the GI motility in rats [1] and mice [2], combined with intravenous (IV) or ICV injection. This model permits the real-time measurements of GI motility in

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animals in the physiological fed and fasted states under stimulation of the brain or peripheral administration of peptide agonists or antagonists. In the fasted state, the cyclic change of pressure waves were detected in both antrum and duodenum, including the quiescence period during which relatively low amplitude contractions occur (phase I-like contractions), followed by a grouping of strong contractions (phase III-like contractions). After food intake, such a fasted motor pattern was disrupted and replaced by a fed motor pattern, which consisted of irregular contractions of high frequency. By using this method the effects of brain-gut peptides on the GI motility can be examined.

Neuropeptide Y and Urocortin in the Hypothalamus Regulate the Gastroduodenal Motility

Neuropeptide Y (NPY) is a potent feeding-stimulatory peptide that expresses in the arcuate nucleus of the hypothalamus and projects predominantly to the paraventricular nucleus. Because few previous studies have examined the effects of centrally administered NPY-related peptide on GI motility, we investigated the role of NPY in the control of GI motility using a variety of NPY analogs [1]. ICV injection of Y2 and Y4 receptor agonists induced the phase III-like contractions in the duodenum when given in the fed state of rats; however, Y1 and Y5 receptor agonists had no effects on the motility despite their potent feeding-stimulatory effects [1]. More interestingly, immunoneutralization of NPY by ICV injection of NPY antibody completely blocked the phase III-like contractions in the duodenum [1]. This finding suggests that the fasted motor activity in the upper GI tracts is regulated by brain NPY neurons but not regulated by peripheral mechanism.

CRF and endogenous CRF receptor ligand urocortin are feeding-inhibitory peptides localized at the paraventricular nucleus in the hypothalamus. Urocortin binds both CRF type 1 and 2 receptors but shows a higher affinity for CRF type 2 receptor than type 1 receptor. CRF type 2 receptors are related to the stress-induced alterations of GI functions. ICV or IV injection of urocortin disrupted fasted motor activity in both antrum and duodenum, which were replaced by fed-like motor patterns [3]. When urocortin was given ICV in the fed state, the % motor index (%MI) was decreased in the antrum and increased in the duodenum [3]. Increase the %MI in the duodenum was non-propagated and therefore urocortin suppressed the transit of intestinal contents [3].

Colocalization of Acyl Ghrelin, Des-Acyl Ghrelin and Obestatin in Endocrine Cells in the Stomach

Acyl ghrelin was first isolated from rat and human stomach [4], and the localization of acyl ghrelin in the stomach was studied in various animals by using the specific antibody for acyl ghrelin [4, 5]; however, the localization of des-acyl ghrelin in the stomach has scarcely been examined. Our group developed antibodies specific for acyl ghrelin (anti-rat octanoyl ghrelin (1–15)-cys-KLH serum) and for des-acyl ghrelin (anti-rat des-octanoyl ghrelin (1–15)-cis-KLH serum) and successfully detected the different localization of acyl ghrelin and des-acyl ghrelin in the rat stomach [6].

Both acyl ghrelin- and des-acyl ghrelin-immunoreactive cells were distributed in the oxyntic and antral mucosa of the rat stomach, with higher density in the antral mucosa than oxyntic mucosa. Immunofluorescence double staining showed that acyl ghrelin- and des-acyl ghrelin-positive reactions overlapped in closed-type round cells, whereas des-acyl ghrelin-positive reaction was found in open-type cells in which acyl ghrelin was negative (fig. 1a). Acyl ghrelin/des-acyl ghrelin-positive closed-type cells contain obestatin (fig. 1b), on the other hand, des-acyl ghrelin-positive open-type cells contain somatostatin [6].

It is possible that open-type cells may react to luminal stimuli more than closed-type cells. Therefore, we investigated the effects of different intragastric pH levels on the release of acyl ghrelin and that of des-acyl ghrelin from the ex vivo perfused rat stomach [6]. The results showed that the release of acyl ghrelin was not affected by intragastric pH, whereas the release of des-acyl ghrelin was increased at intragastric pH 2 compared to that at intragastric pH 4 [6]. Therefore, the release of des-acyl ghrelin is stimulated after meals by lowering the intragastric pH. The release of acyl ghrelin, on the other hand, is stimulated before meals and the release is regulated by plasma levels of glucose and insulin [7].

Regulation of Acyl Ghrelin on the Gastroduodenal Motility

We examined the effects of acyl ghrelin on the gastroduodenal motility and involvement of hypothalamic peptides mediating this action. Acyl ghrelin stimulated the %MI in the antrum and induced the fasted motor activity in the duodenum when given in the fed state of animals [8]. Acyl ghrelin increased the frequency of phase

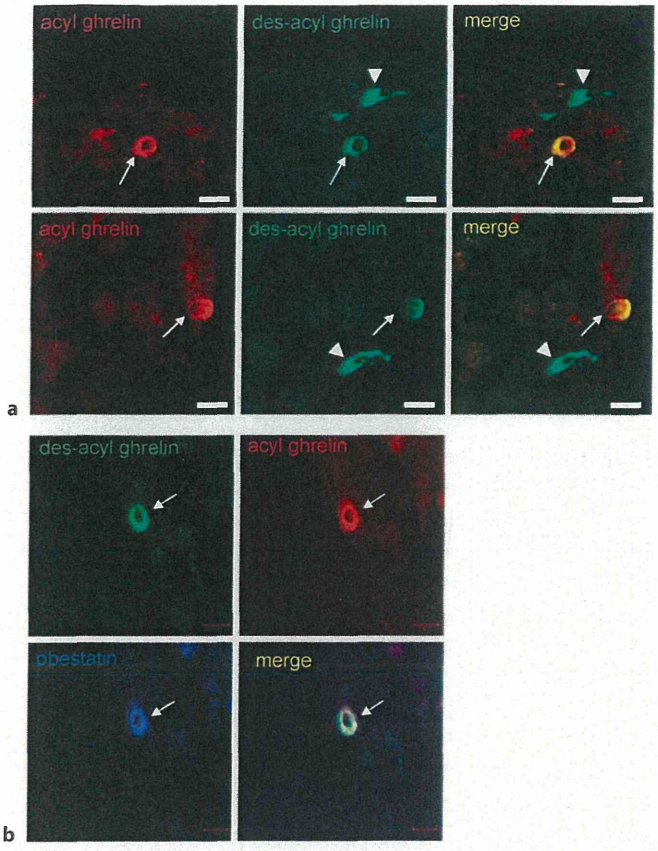

Fig. 1. Localization of acyl ghrelin, des-acyl ghrelin and obestatin in the rat stomach. **a** Immunofluorescence double staining for acyl ghrelin- (red) and des-acyl ghrelin-positive (green) reaction in the antral mucosa of rat stomach. Acyl ghrelin-positive reaction and des-acyl ghrelin-positive reaction are colocalized in closed-type cells (arrows), whereas des-acyl ghrelin-positive reaction is localized in open-type cells (arrowheads). **b** Immunofluorescence triple staining for des-acyl ghrelin (green), acyl ghrelin (red) and obestatin (blue) in the antral mucosa of rat stomach. Three peptides are colocalized in the closed-type cells (arrows). Bars = 10 μm [from 6].

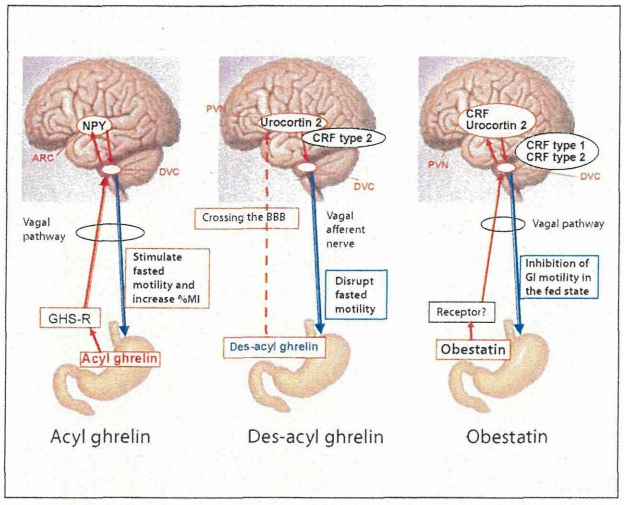

Fig. 2. Different pathways of acyl ghrelin, des-acyl ghrelin and obestatin. Acyl ghrelin released from the endocrine cells in the stomach may act on the ghrelin receptor on vagal afferent nerve terminals, and NPY neurons in the arcuate nucleus may mediate the action of acyl ghrelin to stimulate gastroduodenal motility. Des-acyl ghrelin released from endocrine cells in the stomach may activate urocortin-2 neurons in the paraventricular nucleus by crossing the BBB, and exert inhibitory effects on the antral motility via CRF type 2 receptor in the brain. Obestatin may act on the obestatin receptor on vagal afferent nerve terminals, and CRF and urocortin-2 neurons in the paraventricular nucleus may mediate the action of obestatin to inhibit the gastroduodenal motility via CRF type 1 and type 2 receptors in the brain.

III-like contractions in both antrum and duodenum when given in the fasted state of animals [8]. The effects of IV injection of acyl ghrelin on gastroduodenal motility were blocked by IV injection of GHS-R antagonist [8]. Immunoneutralization of NPY in the brain blocked the stimulatory effects of acyl ghrelin on the gastroduodenal motility [8]. These results indicate that acyl ghrelin released from the stomach may act on the ghrelin receptor on vagal afferent nerve terminals and NPY neurons in the brain may mediate the action of acyl ghrelin on the gastroduodenal motility. Taken together, in normal animals, acyl ghrelin may stimulate gastroduodenal motility by activating the GHS-R on vagal afferent nerve terminals and affect NPY neurons in the hypothalamus, Y2 and/or Y4 receptors in the brain may mediate the action of acyl ghrelin (fig. 2; table 1).

Regulation of Des-Acyl Ghrelin on the Gastroduodenal Motility

Des-acyl ghrelin disrupted fasted motility in the antrum but not in the duodenum; however, des-acyl ghrelin did not alter fed motor activity in both the antrum and duodenum [9]. Capsaicin treatment did not alter the disruptive effect of IV injection of des-acyl ghrelin on fasted motility in the antrum [9]. These results suggest that peripherally administered des-acyl ghrelin may cross the blood-brain barrier (BBB) and act directly on

Table 1. Comparison of the effects of acyl ghrelin, des-acyl ghrelin and obestatin on the gastroduodenal motility

	Acyl ghrelin		Des-acyl ghrelin		Obestatin	
	fasted motility	fed motility	fasted motility	fed motility	fasted motility	fed motility
Stomach	↑	↑	↓	–	–	↓
Duodenum	↑	↑	–	–	–	↓
Hypothalamic neuron	NPY		Urocortin-2		CRF, urocortin-2	
Brain receptor	Y2, Y4		CRF type 2		CRF type 1, type 2	
Vagal afferent pathway	+		–		+	

the brain receptor and disrupt the fasted motility in the antrum.

The centrally administered CRF type 2 receptor antagonist, but not the CRF type 1 receptor antagonist, blocked the effects of centrally and peripherally administered des-acyl ghrelin on gastric motility [9]. The density of c-*Fos*-positive cells in the PVN was significantly increased by intraperitoneal injection of des-acyl ghrelin compared to vehicle-injected controls [9]. These data suggest that peripherally administered des-acyl ghrelin may activate neurons in the PVN by crossing the BBB, and exert inhibitory effects on the antral motility via CRF type 2 receptor in the brain (fig. 2; table 1).

Regulation of Obestatin on the Gastroduodenal Motility

Most of the previous studies have shown the negative effects of obestatin on the GI motility; in those studies, however, only the gastric emptying or MMC cycle time has been used as indices for motor activity. We obtained the positive effects of obestatin on the gastroduodenal motility by analyzing the motor activity in fed and fasted states, and measuring the time taken to the initiation of phase III-like contractions in the antrum and duodenum of conscious rats [10].

Obestatin decreased the %MI of fed motility in the antrum and prolonged the time before the return of fasted motility in the duodenum [10]. IV injection of obestatin induced a significant increase in the number of c-*Fos*-positive cells in the PVN compared to saline-injected controls [10]. Immunofluorescence overlap staining showed that the PVN neurons activated by IV injection of obestatin contain CRF or urocortin-2 [10]. The inhibitory action of IV injection of obestatin on the motor activities in the antrum and duodenum were blocked by ICV injection of CRF type 1 and type 2 receptor antagonists, suggesting that both types of CRF receptors in the brain may mediate the action of peripherally injected obestatin on gastroduodenal motility [10]. Combined together, obestatin inhibits gastroduodenal motility in the fed state but not in the fasted state of conscious rats. In the brain, CRF- and urocortin-2-containing neurons might be activated by IV injection of obestatin, and at the level, CRF type 1 and type 2 receptors might be involved in the inhibitory action of obestatin on antral and duodenal motility. Vagal afferent pathways might be involved partially, but not entirely, in these actions of obestatin (fig. 2; table 1).

Conclusions

Acyl ghrelin, des-acyl ghrelin and obestatin are included in the endocrine cells in the stomach and regulate the upper GI motility by activating hypothalamic peptides [11, 12]. Since hypothalamic peptides are strongly affected by stress or anxiety, such brain-gut interaction seems to be important to understand the pathogenesis of functional disorder in the GI tracts.

Disclosure Statement

The authors declare that no financial or other conflict of interest exists in relation to the content of the article.

References

1 Fujimiya M, Itoh E, Kihara N, Yamamoto I, Fujimura M, Inui A: Neuropeptide Y induces fasted pattern of duodenal motility via Y2 receptors in conscious fed rats. Am J Physiol Gastrointest Liver Physiol 2000;278:G32–G38.

2 Tanaka R, Inui A, Asakawa A, Atsuchi K, Ataka K, Fujimiya M: New method of manometric measurement of gastroduodenal motility in conscious mice: effects of ghrelin and Y2 depletion. Am J Physiol Gastrointest Liver Physiol 2009;297:G1028–G1034.

3 Kihara N, Fujimura M, Yamamoto I, Itoh E, Inui A, Fujimiya M: Effect of central and peripheral urocortin on fed and fasted gastroduodenal motor activity in conscious rats. Am J Physiol Gastrointest Liver Physiol 2001; 280:G406–G419.

4 Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K: Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 1999;402:656–660.

5 Sakata I, Mori T, Kaiya H, Yamazaki M, Kangawa K, Inoue K, Sakai T: Localization of ghrelin-producing cells in the stomach of the rainbow trout *(Oncorhynchus mykiss)*. Zoolog Sci 2004;21:757–762.

6 Mizutani M, Atsuchi K, Asakawa A, Matsuda N, Fujimura M, Inui A, Kato I, Fujimiya M: Localization of acyl ghrelin- and des-acyl ghrelin-immunoreactive cells in the rat stomach and their responses to intragastric pH. Am J Physiol Gastrointest Liver Physiol 2009;297:G974–G980.

7 Chen CY, Asakawa A, Fujimiya M, Lee SD, Inui A: Ghrelin gene products and the regulation of food intake and gut motility. Phamacol Rev 2009;61:430–481.

8 Fujino K, Inui A, Asakawa A, Kihara N, Fujimura M, Fujimiya M: Ghrelin-induced fasted motor activity of the gastrointestinal tract in conscious fed rats. J Physiol 2003; 550:227–240.

9 Chen CY, Inui A, Asakawa A, Fujino K, Kato K, Chen CC, Ueno N, Fujimiya M: Des-acyl ghrelin acts by CRF type 2 receptors to disrupt fasted stomach motility in conscious rats. Gastroenterology 2005;129:8–25.

10 Ataka K, Inui A, Asakawa A, Kato I, Fujimiya M: Obestatin inhibits motor activity in the antrum and duodenum in the fed state of conscious rats. Am J Physiol Gastrointest Liver Physiol 2008;294:G1210–G1218.

11 Fujimiya M, Asakawa A, Ataka A, Kato I, Inui A: Different effects of ghrelin, des-acyl ghrelin and obestatin on gastroduodenal motility in conscious rats. World J Gastroenterol 2008;14:6318–6326.

12 Fujimiya M, Asakawa A, Ataka K, Chen CY, Kato I, Inui A: Ghrelin, des-acyl ghrelin, and obestatin: regulatory roles on the gastrointestinal motility. Int J Pept 2010;2010. pii: 305192.

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シンポジウム：摂食障害の新たな展開

脂質代謝の変化からみたカロリー制限による抗老化・寿命延長作用のメカニズム

樋上賀一*

抄録：カロリー制限（caloric restriction：CR）は，老化過程を抑制，加齢に伴う疾患の発生を遅延し，平均および最大寿命を延長する唯一の簡便な再現性の高い方法として，老化研究に広く応用されている．しかし，そのメカニズムはいまだ解明されていない．一般にCRは，成長を抑制し，身体を小さく保ち，脂肪組織量を減少させ，高血糖および高インスリン血症を抑制，炎症を抑制，低体温で，脂質やエネルギー代謝を修飾し，内因性および外因性ストレスに対する抵抗性を増強，ミトコンドリア・バイオジェネーシスを亢進，サーチュインを活性化することが知られている．われわれは，CR動物では，食餌不足に対する適応反応として脂肪組織のリモデリングを介して脂質を効率的に利用していること，このような代謝の変化に脂肪酸合成関連遺伝子群発現の主要転写因子であるsterol regulatory element binding protein 1c（SREBP1c）が重要である可能性を示した．SREBP1cを介した*de novo*脂肪酸合成系の活性化がCRの主要なメカニズムの1つと考えられる．

Key words：カロリー制限，老化，脂質代謝，脂肪組織リモデリング，SREBP1c

カロリー制限の抗老化・寿命延長効果とは

1935年，米国のMcCayらは，離乳直後からの摂取カロリーの制限（caloric restriction：CR）により，ラットの寿命が延長することを報告した．それ以来75年以上にわたり，CRは，食餌制限（dietary restriction，food restriction），エネルギー制限（energy restriction）とも呼ばれ，唯一で簡便な再現性の高い寿命延長法として広く老化研究に応用されてきた[1)2)]．CRによる寿命延長効果は，酵母や線虫といった下等生物からげっ歯類にいたるまで広く観察されることから，進化の過程で保存されたメカニズムが関与することが示唆される．1989年Holliday[3)]は，CRによる抗老化・寿命延長作用のメカニズムとして，以下のように適応反応仮説を提唱し，食餌不足に対する神経内分泌および代謝の変化の重要性を進化論的観点から説明した．食餌が豊富な時期には，個体は成長し，強い大きな個体で積極的に生殖することで子孫を増やし，さらに過剰なエネルギーを脂肪組織に貯蔵する．一方，食餌が不足する時期には，個体の成長や生殖を抑制し，脂肪組織に貯えたエネルギーを使いながら，寿命を延長し，食餌が十分に得られる時期を待つ．このような食餌不足に対する適応能力の発達した動物が，進化の過程で選択されてきた．CRは，この食餌不足に対する適応反応を活性化し，抗老化・寿命延長をもたらすのではないかと考えられる．

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ほ乳類では，げっ歯類を中心に研究され，CRはさまざまな生理的加齢現象を抑制，老化に伴って発症する種々の疾患発症を遅延もしくは抑制し，寿命を延長することが明らかとなってきた．CRは活動性を維持し，平均および最大寿命を延長すること，CRの効果は，その期間や程度に比例すること，またエネルギー摂取の抑制にのみ依存しており，エネルギー制限のない各栄養素の摂取制限（糖質，脂質制限，タンパク質の摂取制限など）では，効果は得られないことが報告されている[1)2)]．一般的に，CR動物では，高血糖や高インスリン血症の抑制，酸化ストレスを含む内因性ストレスおよび外因性環境ストレスに対する抵抗性の増強，炎症の抑制，低体温，エネルギー代謝の効率化，ミトコンドリア・バイオジェネシスの活性化，サーチュインの活性化などが観察されており，これらがCRによる抗老化・寿命延長効果に重要であろうと考えられている．しかしながら，その詳細なメカニズムはいまだ解明されていない[4)〜6)]．

米国では1980年代の後半から数施設において，霊長類においてもCRの効果が有効であるか，サルを用いて検証されている．サルの寿命が長いことから最終的な結論は出ていないが，げっ歯類で観察されるCR動物の表現型はサルにおいても観察されることなどから，CRは霊長類においても有用であろうと考えられている[7)8)]．また，CRされたサルで観察された低体温，低血糖および年齢に伴って低下するdehydroepiandrosterone（DHEAS）の減少率が低いヒトの集団は，そうでない集団に比べて平均余命が長いことも報告されている[9)]．それゆえ，CRの有益な効果は，げっ歯類のみならず，ヒトを含む霊長類においても有効であろうと考えられる．

単一遺伝子の変異により長寿命を示すマウスやラット

CR以外寿命を延長する方法がなかったが，米国のBartkeら[10)]は1996年，Ames矮小マウスが長寿を示すと報告した．Ames矮小マウスは下垂体の発生や分化にかかわる転写因子であるProp1遺伝子に変異があり，成長ホルモンなどの下垂体前葉ホルモンの分泌に障害がある．Prop1遺伝子と類似した機能を有するPit1遺伝子に変異のあるSnell矮小マウス，次いで成長ホルモン放出ホルモン受容体遺伝子に変異のあるLittleマウスが長寿であることも報告された．その後，分子生物学および分子遺伝学の進歩と相まって，成長ホルモン受容体遺伝子やインスリン様成長因子1受容体遺伝子をノックアウトしたマウスなどが次々に作られ，筆者の知る限り現在まで報告されている単一遺伝子の改変により長寿を示すマウスやラットは約20種以上に及ぶ[11)]．これらを分類すると，その半数は成長ホルモン（GH）/インスリン様成長因子1（IGF-1）シグナルに関連する遺伝子を修飾したものであり，酸化ストレス/レドックス制御に関連するもの，脂肪細胞もしくはアディポサイトカインに関連するもの，およびその他の4つに大別できる．

われわれも，アンチセンス成長ホルモン遺伝子を成長ホルモン産生細胞に発現するようなトランスジェニック（tg）ラットにおいて，寿命を検討した．するとホモラット（tg/tg）の寿命はかえって短縮したが，ヘテロ（tg/−）ラットでは平均および最大寿命とも5〜10%延長した[12)]．CRにおいてもGH/IGF-1シグナルは抑制されるため，CRの有益な作用はGH/IGF-1シグナルの抑制に関連すると示唆された．そこで，野生型ラットと寿命が延長した（tg/−）ラットにCRを行った．すると予想に反して，野生型および（tg/−）ラットとも平均および最大寿命が同程度延長した[13)]．このことは，GH/IGF-1シグナルの抑制はCRの主要なメカニズムでないことを示唆している．

Table 1　CR と GH/IGF-1 抑制との比較

	CR による変化		GH/IGF-1 抑制による変化
	摂食後	摂食前	
血液生化学データ			
総脂質	↓	↓	↓
トリグリセリド	↓	↓	↓
総コレステロール	↓	↓	→
遊離コレステロール	↓	↓	↓
リン脂質	↓	↓	↓
遊離脂肪酸	↓	→	→
ケトン体	→	↑	→
グルコース負荷試験	↑		↑
インスリン負荷試験	↑		↑
肝臓の遺伝子発現			
β酸化関連遺伝子	→	↑	→
脂肪酸合成関連遺伝子	↑	↓	→
ストレス応答遺伝子	→	↑	↑
白色脂肪組織の遺伝子発現			
脂肪酸合成関連遺伝子	↑		→
炎症関連遺伝子	↓		→

成長ホルモン（GH）/インスリン様成長因子 1（IGF-1）シグナル非依存的な CR のメカニズム

われわれは，同程度に寿命が延長した野生型（−/−）CR ラット（以下，CR ラット）および（tg/−）自由摂食ラット（以下，Tg ラット）のさまざまなパラメーターを野生型（−/−）自由摂食ラット（以下，AL ラット）と比較することで，GH/IGF-1 シグナル非依存的な CR の影響を明らかにしようと試みた．

血清総脂質，トリグリセリド，遊離コレステロール，リン脂質レベルは，AL ラットに比較して，摂食状態にかかわらず CR ラットおよび Tg ラットで，同様に有意に低値を示した．一方，CR による摂食後の遊離脂肪酸の低下および摂食後のケトン体の増加は，Tg ラットでは観察できなかった[14]．また，グルコース負荷試験およびインスリン負荷試験では，CR ラットでも Tg ラットでも，グルコース耐性およびインスリン高感受性を示した[15]．肝臓の網羅的遺伝子発現解析では，CR により摂食前ではミトコンドリアβ酸化関連遺伝子の発現が亢進した．また摂食後では脂肪酸合成関連遺伝子の発現が顕著に亢進した．しかしながら，これら遺伝子発現の変化は Tg ラット肝臓では観察できなかった．一方，MDR2 や OCT1A などストレス耐性遺伝子発現の亢進は CR ラットでも Tg ラットでも，同様に観察された（**Table 1**）．以上の結果から，CR により摂食前にはミトコンドリアβ酸化を介して脂質をエネルギー源とし，摂食後には脂質を貯蔵するシステムが活性化していること，この CR による代謝の変化は GH/IGF-1 非依存性に制御されていることが示唆された[14]．

前述したように，単一遺伝子の改変により長寿を示すと報告されているマウスやラットに，脂肪細胞特異的にもしくはアディポサイトカイン分泌を修飾したマウスが含まれる．具体的には脂肪特異的にインスリン受容体をノックアウトしたマウス，脂肪細胞の分化因子である c/EBPβ を c/EBPα locus にノックインした c/EBPβ/β マウス，さらに脂肪細胞特異的なサイトカインでありインスリン感受性を正に制御し，抗炎症性サイトカインでもあるアディポネクチンを肝臓で過剰発現させたトランスジェニックマウスが長寿命であることが報告されて