that OXT knockout mice consume larger quantities of sweet and nonsweet carbohydrates than wild-type mice [74]. In the Prader–Willi syndrome, characterized by extreme hyperphagia leading to morbid obesity in human, the number of OXT-containing neurons is decreased in the PVN [75].

Some studies showed that nerve fibers containing the feeding-inhibitory peptides, such as cocaine- and amphetamine-regulated transcript (CART) [76] and prolactin-releasing peptide (PrRP) [77], have synaptic contact with hypothalamic OXT neurons. OXT neurons are activated by the administration of CART or PrRP [78]. It has also been suggested that α-melanocyte stimulating hormone (α-MSH), a feeding inhibition factor released by proopiomelanocortin (POMC) neurons, activates OXT neurons [79]. It is therefore possible that OXT contributes to feeding inhibition by CART, PrRP and α-MSH.

OXT has a short-term feeding-inhibitory effect. However, when OXT is administered for a long period, it has been reported that food intake becomes increased after an initial decrease in feeding [80]. In addition, OTR antagonists also do not block feeding inhibition by α_1 receptor agonists [81].

The site of OXT action upon the feeding inhibition is not completely understood. OXT neurons in the PVN project to the medullary dorsal nucleus of the vagus nerve, and microinjection of OXT into the dorsal nucleus of the vagus nerve inhibits gastric motility, suggesting that OXT neurons of the PVN projecting to the medulla oblongata may act to inhibit feeding [82]. OXT neurons in the PVN also project to sympathetic preganglionic neurons of intermediolateral nuclei in the spinal columns. OXT has been suggested to excite these sympathetic preganglionic neurons. Consequently, it is possible that activation of the sympathetic nervous system may cause inhibition of feeding. OXT reduces binding affinity in the hypothalamus of α_2NA receptor agonists that have a feeding promotion effect [81]. Thus, modification of NA receptors may also contribute to feeding inhibition by OXT. In addition to feeding inhibition, OXT released in the CNS after various stress stimuli has been proposed to modify neuroendocrine stress responses, such as ACTH secretion, and to affect anxiety behaviors [83]. More recently, Maejima et al. showed that peripheral OXT treatment reduced food intake and visceral fat mass, and ameliorates obesity, fatty liver and glucose intolerance [84]. Peripheral OXT treatment provides a new therapeutic avenue for treating obesity and hyperphagia.

In human, the overnight secretion of OXT in women with anorexia nervosa is decreased compared with healthy women [85]. In underweight anorexia nervosa patients, estrogen- or insulin-induced hypoglycemia results in an impaired response in plasma OXT level [86]. In recovered anorexia nervosa patients, cerebrospinal fluid OXT level was normal [87]. Although it is still unclear the pathophysiological mechanism of OXT in anorexic nervosa, OXT maybe have an important role in hypopahagia, including anorexia nervosa and cachexia.

3.4. Salt appetite

OXT appears to play an important role in salt appetite. Icv administration of OXT inhibited hypovolemia-induced salt appetite but had little effect on water intake [88]. Icv administration of OXT also inhibited angiotensin-induced salt appetite [89]. The salt intake was increased in the OXT knockout mice [90]. Moreover, hypovolemia-induced saline intake was increased in rats where OTR bearing neurons were selectively ablated by the application of OXT conjugated to the A chain of ricin [91]. In the OXT knockout mice, the hypovolemia- and dehydrated-induced sodium intake was increased [92,93]. By contrast, sodium intake did not decrease in OXT knockout mice [94]. These studies suggested that OXT pathways are not the only regulator of salt intake, OXT may be more critical in controlling salt intake over brief intervals when an animal is quickly compensating for a dehydrating stimulus [94].

3.5. Social recognition

Social recognition is necessary for the development of all social relationships and requires the appropriate processing of social cues and the activation of processes related to learning and memory. OXT plays an important role in the neural processing of social information and in social recognition. Low doses of central administration of OXT facilitate social recognition in rats, however, higher doses of OXT can be amnestic [95]. Both male and female OXT knockout mice had a profound disruption of social recognition [96,97]. OXT facilitated social recognition through its actions on OTR in the medial amygdala during memory formation. The administration of OXT into the medial amygdala, prior to but not after the initial social exposure to a stimulus female, completely rescues social recognition in OXT knockout mice [98]. In female wild-type mice, infusion of OTR antisense DNA into the medial amygdala decreases OTR protein and blocks social recognition [99].

4. Peptides to stimulate oxytocin release

4.1. Adrenomedullin family

Adrenomedullin (AM) is a 52-amino acid neuropeptide that was originally isolated from tissue extracts of human pheochromocytoma and later found to be widely distributed in peripheral organs and the CNS [100] (Fig. 2). A similar 47-amino acid neuropeptide, adrenomedullin 2 (AM2), identical to intermedin, was first isolated from pufferfish [101,102] and later from mammals [102,103] by the search in the genomic databases [101,102] (Fig. 2). AM2 is identical to intermedin, which was discovered by Roh et al. [102]. AM, AM2/intermedin, and amylin belong to the calcitonin gene-related peptide (CGRP). Each member of AM family has an N-terminal ring structure and an amidated carboxyl

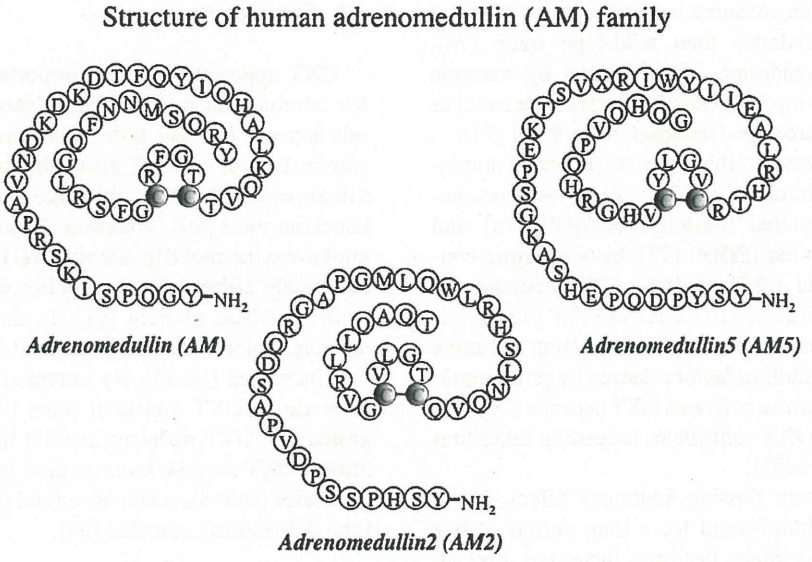

Fig. 2. Structure of adrenomedullin (AM) family. Each member of AM family has an N-terminal ring structure and an amidated carboxyl terminus.

terminus (Fig. 2). Both of these structures are critical for receptor binding and subsequent signaling [104,105].

AM and AM2 are both considered potent vasodilators because peripheral administration of either peptide decreases arterial blood pressure, inhibits urine flow, decreases food intake, and suppresses gastric activity [102,103,106–109]. By all accounts, the effects of the two peptides are qualitatively and quantitatively similar [102,103,106,107]. The actions of AM or AM2 given centrally are quite different from their actions when given peripherally. Central administration of AM2 inhibits food intake and drinking in rats in much the same manner as it does when given peripherally, but centrally administered AM2 elevates arterial blood pressure and heart rate [110]. Icv administration of either AM2 or AM caused hypertension and tachycardia [103,108,110]. We showed that central administration of AM activated OXT neurons [111,112] and caused an elevation of plasma OXT levels in rats [111]. We also showed similar activation of OXT neurons and circulating OXT levels after central administration of AM2 [113] (Fig. 3). These effects of AM2 are similar to those of AM [108,109,111,114–117] and may be mediated by both AM and CGRP receptors [110]. Moreover, we showed that centrally effects of AM2 were stronger than those of AM in the expression of the c-*fos* gene in the SON and PVN, plasma OXT level, and blood pressure in rats [118]. Interestingly, combined AM and CGRP receptor blockade was incomplete for central effects of AM2 [118]. These results suggested that the more potent central effects of AM2 and only partial blockade by AM/CGRP receptor antagonists may result from its action on an additional, as yet unidentified, specific receptor in the CNS.

More recently, in teleost fish, AM peptides were identified as five AMs (AM1–5), and they form an independent subfamily [101]. Takei et al. searched the orthologs of the AMs in the genome and established sequence tag databases and identified AM2 and AM5 genes in mammals [103,119] (Fig. 2). Since AM and AM2 have many effects on the CNS in mammals, AM5, which is a newly discovered 50-amino acid peptide identical to fish AM5, may also have similar actions on the CNS through the CLR/CTR–RAMPs complexes. We showed that centrally administered AM5 induced the expression of c-*fos* gene in the SON and the PVN, and this induction was significantly reduced, incomplete, by pretreatment with both the CGRP and AM receptor antagonists [120]. Therefore, we presume that central AM5 activates OXT-secreting neurons in the SON and the PVN partly through the CGRP and/or AM receptor. Further study is required to explore the possibility that unknown specific receptors for AM5 and/or AM2 may exist in the CNS.

What is the relationship between AM family and OXT? We showed that coexistence of AM- and OXT-LI was identified in the SON and PVN in rats [121]. Although we don't know whether other AMs would be co-existed with OXT, we suggested that AM family might play a role as autocrine/paracrine functions. Further study is required to explore the relationship between AM family and OXT functions.

4.2. Apelin

Apelin, a 36-amino acid peptide, originally has been isolated from bovine stomach tissue extracts as the endogenous ligand of the human orphan G protein-coupled receptor APJ [122,123]. APJ is now therefore commonly referred to as the apelin receptor [124]. Apelin and its receptor are widely distributed throughout the rat nervous system [125–132] and are particularly strongly expressed in the SON and PVN [126–129]. Both AVP and OXT neurons produce apelin

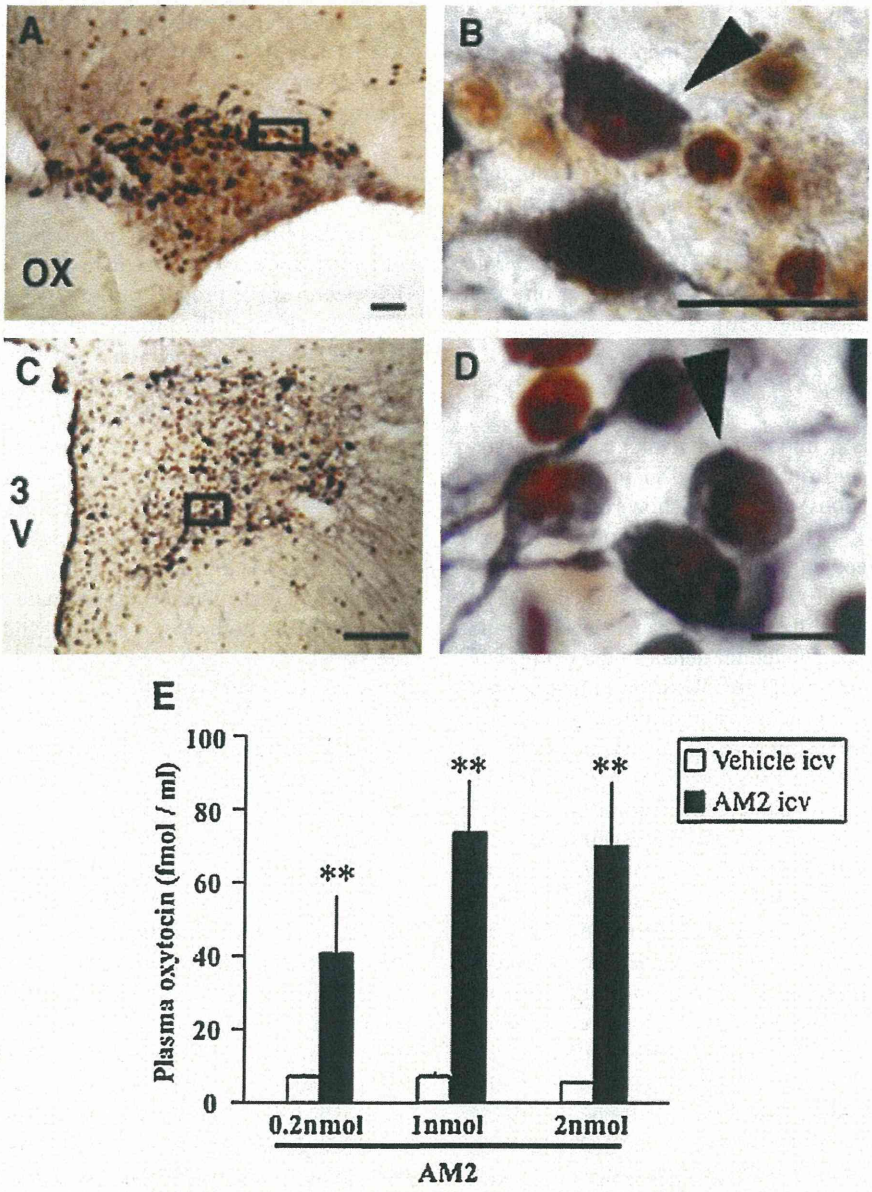

Fig. 3. Central effects of adrenomedullin 2 (AM2) in the OXT release.A–D: Coexistence of Fos-like immunoreactivity (LI) and OXT-LI in the supraoptic nucleus (SON; A and B) and the paraventricular nucleus (PVN; C and D) of rats 90 min after icv administration of AM2 (2 nmol/rat). A and C: Coexistence of Fos-LI (brown, in round structures) and OXT-LI (violet, in spindle-shaped structures). B and D: Enlargements from the boxed areas in A and C. Black arrowheads indicate coexistence of nuclear Fos-LI and OXT-LI. White arrowheads indicate OXT-LI without Fos-LI. 3V, third ventricle; OX, optic chiasma. Bars indicate 50 μm. E: Effects of icv administration of AM2 (0.2, 1, and 2 nmol/rat) or saline (vehicle) on plasma concentrations of oxytocin in conscious rats. All rats were decapitated 30 min after icv administration of the AM2 (0.2, 1, and 2 nmol/rat) or vehicle. Data for plasma concentrations of OXT are expressed as means ± SE ($n = 6$ rats). $^{**}P < 0.01$ compared with vehicle-administered rats. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)
Modified with permission from Figs. 1 and 2 in ref. [110].

receptor mRNA in the SON in rats [127,133]. In immunohistochemistry study, OXT neurons contain apelin in the SON and PVN in male and virgin female rats [134]. APJ immunoreactivity cell were also seen in the somata, dendrites, axon fibers, the ventral glial lamina, and axon terminals of magnocellular OXT and AVP neurons [135]. Apelin-13 increased the firing rates of AVP cells but had no effect on the firing rate of OXT neurons in extracellular electrophysiological recordings from the transpharyngeally exposed SON of urethane-anaesthetized rats [135].

Recently, Bodineau et al. showed that apelin modulated the activity of magnocellular and parvocellular OXT neurons in the lactating rat [136]. They revealed that the colocalization of apelin with OXT in about 20% of the hypothalamic

OXT neurons by double immunofluorescence study. They also showed that icv administered apelin inhibited the activity of magnocellular and parvocellular OXT neurons by immunohistochemistry for c-*fos* and electrophysiological study. These central effects of apelin were correlated with a decrease in the amount of milk ejected. Thus, apelin may inhibit the activity of OXT neurons through a direct action on apelin receptors expressed by these neurons in an autocrine and paracrine manner. They suggested that the inhibitory role of apelin as an autocrine/paracrine peptide acting on OXT neurons during breastfeeding [136].

4.3. Cholecystokinin (CCK)

CCK is regarded as an important physiological satiety signal [137]. The pathway from CCK to OXT release has been well investigated. Previous studies have shown that peripheral administration of CCK-8 stimulated secretion of OXT but not AVP and inhibited feeding [138–141]. Otsuka Long-Evans Tokushima Fatty (OLETF) rats have been established as an animal model of non-insulin dependent diabetes mellitus and obesity [142,143], has a congenital defect in the expression of the CCK-A receptor gene [144]. We showed that peripheral administration of CCK-8 does not activate hypothalamic OXT neurons and the brainstem neurons in the nucleus of the solitary tract (NTS) and the area postrema (AP) in OLETF rats [145]. We suggested that systemic administration of CCK-8 might selectively activate the hypothalamic OXT neurons and brainstem neurons through CCK-A receptor in rats. OXT release induced by peripheral administration of CCK-8 was abolished by subdiaphragmatic vagotomy and chemical destruction of vagal afferents [146,147], and also by administration of the selective CCK-A receptor antagonist, but not by the CCK-B receptor antagonist [148]. Moreover, systemic administration of CCK stimulates gastric vagal afferents via CCK-A receptor and activates noradrenergic neurons in the NTS [149]. It is postulated that these noradrenergic inputs activate magnocellular OXT neurons in the SON and the PVN and cause secretion of OXT into the systemic circulation in rats [150,151]. In addition, selective gastric vagotomy eliminates the OXT response to CCK, and lesions of the NTS abolish the behavioral effects of CCK-8 on food intake [148,152]. Therefore, CCK-A receptors in the stomach are stimulated, the abdominal vagus nerve is activated, NA neurons in the A2 region of the NTS are excited, and NA is released in the hypothalamus, which activates magnocellular OXT neurons [153–155].

In addition to the A2 NA neurons [156], NA neurons in the medullary ventrolateral A1 region also play an important role in OXT secretion after stressful stimuli such as noxious stimuli [157]. It is unlikely that OXT in the peripheral blood controls feeding directly. At the time when OXT release from the posterior pituitary is promoted, OXT release within the hypothalamus was increased, and OXT in the CNS induced to inhibit feeding [82]. Icv administration of an OXT receptor antagonist attenuates feeding reduction in response to LiCl or CCK [158,159] and blocked feeding reduction in response to CRH [160]. These studies suggest that intrinsic OXT may play an important physiological role in inhibition of feeding during satiety and stress.

4.4. Kisspeptin

Kisspeptin, a placental polypeptide secreted throughout pregnancy, is suggested to play a role at parturition. Kisspeptin is the product of the *kiss1* gene and its receptor, GPR54, which is the product of *kiss1r*. Kisspeptin stimulate the release of GnRH and gonadotrophin and advance vaginal opening in rodents, sheep and primates [161–166]. Kisspeptin is found in both the periphery and the CNS. In the periphery, kisspeptin has been identified in the testis, ovary, anterior pituitary gonadotrophs, pancreas and small intestine [167–169]. However, peripheral expression of kisspeptin is highest in the placenta with maternal plasma levels of kisspeptin in the third trimester of pregnancy rising to 7000-fold greater than in the non-pregnant state [167,170,171]. In the CNS, both *Kiss1* mRNA and kisspeptin protein are particularly highly expressed in the Arc, anteroventral periventricular nucleus (AVPV) and periventricular nucleus [162] in the mice. In primates including humans, hypothalamic *KISS1* mRNA is predominantly found within the infundibular nucleus, which is the equivalent of the Arc in this order of mammals [172].

Previous studies showed that intravenous (iv) administered kisspeptine-10 increased plasma OXT level in female rats [173], whereas icv administered kisspeptin-10 increased plasma AVP level in male rats [174]. Recently, in vivo extracellular single unit recording, peripheral administered kisspeptin increased plasma OXT level and icv administered kisspeptin-10 increases AVP levels [175]. Iv administered kisspeptin-10 significantly increased the firing rate of OXT neurons from 3.7 ± 0.8 to 4.7 ± 0.8 spikes/s, but only a quarter of AVP neurons responded to iv administered kisspeptin-10, showing a short (<3 s) high-frequency (>15 spikes/s) burst of firing. By contrast, icv administered kisspeptin-10 (2 and 40 μg) did not alter OXT or AVP neuron firing rate. This effect of peripheral administered kisspeptin-10 in OXT neurons on firing rate blocked by pretreatment of capsaicin which desensitize vagal afferents. Kisspeptin may activate on magnocellular neuron via the vagus, and presumably NTS during pregnancy and lactation, when circulating kisspeptin levels are increased.

4.5. Nesfatin-1

Nesfatin-1 is a recently discovered, 82-amino acid protein derived from the cleavage of a precursor, NEFA/nucleobindin2 (NUCB2) [176]. Nesfatin-1 is produced in several hypothalamic nuclei, such as the SON, PVN, arcuate nucleus (Arc), and lateral hypothalamic area (LHA) [176], and in extra-hypothalamic areas as well, including the raphe pallidus, the Edinger–Westphal nucleus, and the NTS

[177]. Nesfatin-1 has been shown to colocalize with several well-described peptides, including CART, CRH, OXT, and AVP [178]. Double-labeling immunohistochemistry in these areas has revealed that nesfatin-1 is colocalized with feeding-related factors such as CRH, OXT, POMC and CART [177–179]. Central administration of α-MSH increases NUCB2 mRNA in the hypothalamus [180]. Anorectic effect by icv administration of nesfatin-1 was mediated by OXT in the PVN [181,182]. Icv administered nesfatin-1 decreased food intake and inhibited gastroduodenal motility in mice [183]. Nesfatin-1 and OXT both suppresses food intake in *fa/fa* Zucker rats, and leptin-induced satiety is unaltered by immunoneutralizing nesfatin-1 IgG [176,182]. These results suggest that nesfatin-1 induces anorexia in a leptin-independent and melanocortin-dependent manner [176,182].

4.6. Prolactin-releasing peptide (PrRP)

Prolactin-releasing peptide (PrRP) was isolated as an endogenous ligand of an orphan G-protein-coupled receptor (GPR10/hGR3) and belongs to the RFamide peptide [183]. Initial studies showed that PrRP could stimulate prolactin release both in vitro [184] and in vivo [185,186], giving rise to the name of this peptide. However, recent morphological and physiological studies have shown that PrRP is not a hypophysiotropic prolactin-releasing factor [187–189], but have suggested rather that PrRP was involved in a wider range of neuroendocrine and autonomic functions [190,191].

PrRP-synthesizing cells have been identified in the dorsomedial hypothalamic nucleus (DMH), the A1 region of the ventrolateral medulla (VLM) and the A2 region of the NTS in the medulla oblongata [192–196]. Icv administration of PrRP significantly increased plasma OXT and AVP levels [197] and to stimulate ACTH secretion via CRH from the parvocellular cells in the PVN [198]. As stress activates medullary and hypothalamic PrRP neurons, PrRP and NA may both function cooperatively in neuroendocrine responses to stress [180,199]. Icv administration of anti-PrRP antibodies to rats attenuates OXT secretion in response to conditioned fear [199]. Our previous study showed that central administration of PrRP induced the expression of c-*fos* gene in the PVN and increased plasma corticosterone levels in conscious rats [200]. Moreover, we showed that the restraint stress and acute inflammatory stress upregulated the expression of PrRP gene in the NTS and the VLM. The nociceptive stimulus upregulated the expression of PrRP gene in the ventrolateral medulla. We also showed that pretreatment with an anti-PrRP antibody significantly attenuated nociceptive stimulus induced the expression of the c-*fos* gene in the PVN. These results indicate that PrRP may be potent and important mediator of stress responses.

PrRP neurons in the brainstem were activated by CCK [77] and PrRP mediates CCK-induced satiety [201]. Icv or microinjection of PrRP inhibits feeding [180,202] but does not induce nausea [203]. Icv co-administration of PrRP and leptin resulted in additive reduction in food intake and body weight gain, and that PrRP mRNA levels were reduced in Zucker (*fa/fa*) rats with mutated leptin receptor and in fasted rats [78]. Thus, PrRP is regulated by leptin. PrRP promotes release of the feeding inhibition factors, α-MSH and neurotensin [203]. It is possible that α-MSH and neurotensin contribute to the inhibitory effect of PrRP. Icv administration of PrRP also increased the core temperature and oxygen consumption in male rats [204]. These results indicate that PrRP may affect energy homeostasis by the reduction of food intake and the increase in energy expenditure. Icv administration of PrRP activated OXT neurons at the PVN in mice, which was significantly reduced in GPR10 knockout mice, which is the phenotype of PrRP knockout mice [199]. The roles of PrRP on energy homeostasis were supported by studies on GPR10 knockout mice, which became hyperphagic and obese [205]. More recent study showed icv administration of RFamide-related peptides (RFRP-1 and RFRP-3), which are belong to RFamide peptide such as PrRP, increased the plasma OXT level and activated the OXT neurons [206]. RFamide peptide, including PrRP and RFRP, may play a role in the control of energy metabolism.

4.7. Secretin

Secretin is best known for its role as a duodenal hormone released in response to acidification of the intestinal lumen [207]. Secretin, however, can also activate vagal sensory nerves [208,209]. Secretin is synthesized within the brain and can activate hypothalamic neurons [210–213]. Peripheral administration of secretin induced Fos expression in the SON [209,214]. Icv administration of secretin also increases Fos expression in SON neurons and increases secretion of OXT and AVP, and secretin receptors are found in the SON and the magnocellular area of the PVN [215]. Secretin also activates vagal primary afferent neurons [210]. Furthermore, lacking secretin receptors mice exhibit defects in social and cognitive behaviors [216]. Although the treatment of secretin was beneficial in autism and associated gastrointestinal abnormalities [217], its efficacy was not confirmed in subsequent clinical trials [218]. Moreover, these studies have suggested on the existence of a specific relationship between autism and inflammatory bowel disease [218]. Recently, the combined administration of secretin and OXT inhibited chronic colitis in rats [219]. These results suggested that the administration of both secretin and OXT would develop a novel treatment of inflammation-associated intestinal disorder.

5. Perspective

Although OXT was discovered over 60 years ago, the primary role of OXT has not been known yet. In this review, we know that OXT has relationship with various physiological and pathophysiological functions. OXT works as a hormone in the periphery and as a neurotransmitter in the CNS. The importance of OXT in milk ejection and uterine

contraction is well known. Recently, we showed the central effects of some neuropeptides, such as adrenomedullin family and other peptides in OXT release in rats. OXT is also involved in lots of physiological and pathological functions such as appetite, anxiety, antinociception, social recognition and stress, with many neuropeptides. In each function, the relationship between OXT and neuropeptides is not fully understood. OXT may be an important key in some disease and develop a novel treatment for them. We anticipate that further studies can clarify the relationship with between OXT and neuropeptides.

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ORIGINAL

A novel splice site mutation of the *MEN1* gene identified in a patient with primary hyperparathyroidism

Yuko Nagamura[1], Masanori Yamazaki[2], Satoko Shimazu[1], Kenji Sano[3], Toshihiko Tsukada[1] and Akihiro Sakurai[2, 4]

[1] *Division of Familial Cancer Research, National Cancer Center Research Institute, Tokyo 104-0045, Japan*
[2] *Division of Diabetes, Endocrinology and Metabolism, Department of Internal Medicine, Shinshu University School of Medicine, Matsumoto 390-8621, Japan*
[3] *Department of Laboratory Medicine, Shinshu University School of Medicine, Matsumoto 390-8621, Japan*
[4] *Department of Medical Genetics, Shinshu University School of Medicine, Matsumoto 390-8621, Japan*

Abstract. Heterozygous germline mutation of the tumor suppressor gene *MEN1* is responsible for multiple endocrine neoplasia type 1 (MEN1), a familial cancer syndrome characterized by pituitary, parathyroid and enteropancreatic tumors. Various mutations have been identified throughout the entire gene region in patients with MEN1 and its incomplete forms often manifested as familial isolated hyperparathyroidism and apparently sporadic parathyroid tumor. Mutation analysis of the *MEN1* gene is a powerful tool for the early diagnosis of MEN1; however, the clinical significance of the identified mutations is not always obvious. In this study, a previously unreported missense *MEN1* mutation, c.824G>T was identified in a patient with primary hyperparathyroidism and evaluated for its pathogenicity. This mutation was predicted to generate a putative missense menin protein, R275M. A stability test of the menin protein demonstrated that the stability of R275M mutant was reduced only slightly as compared with wild type menin, and therefore could not preclude the possibility that it was a rare benign polymorphism. However, further analysis of leukocyte mRNA and minigene experiments indicated that the mutant c.824G>T allele gives rise to abnormally spliced menin mRNA, and thereby confirmed that c.824G>T mutation is causative for MEN1. Thus, leukocyte mRNA analysis has been demonstrated useful to identify a splicing mutation of the *MEN1* gene.

Key words: MEN1, Menin, Splicing, Minigene, Stability

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]. Primary hyperparathyroidism (PHPT) is the most common disorder, and is usually the initial manifestation in MEN1. Its prevalence in MEN1 patients during lifetime is nearly 100%, and the average age of onset is during the third decade of life, which is much earlier than that of sporadic primary hyperparathyroidism [2, 3]. Anterior pituitary tumors are seen in 40-60% of MEN1 patients. Among those, prolactinomas are the most common followed by nonfunctioning tumors and growth hormone producing tumors. Gastroenteropancreatic tumors develop in about 60% of the patients and gastrinoma is the most frequent functioning tumor followed by insulinoma. Other manifestations include adrenal cortex adenomas, which are mostly nonfunctioning, foregut carcinoid tumors and cutaneous tumors.

Germline mutations of the causative gene, *MEN1*, which is localized to human chromosome 11q13 and encodes a 610-amino acid nuclear protein, menin, can be identified in most of the affected subjects [4, 5]. To date, more than 500 different germline *MEN1* mutations have been identified in patients with MEN1. The majority of mutations identified in affected subjects are nonsense and frameshift mutations, which predict premature protein truncations. Splice mutations and large deletions of the *MEN1* gene have also been reported in several families.

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Correspondence to: Akihiro Sakurai, M.D., Ph.D., Department of Medical Genetics, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621 Japan.
E-mail: aksakura@shinshu-u.ac.jp

Menin shows no significant homology to other known proteins, and its physiological function is not yet fully understood. Moreover, despite its widespread expression, the molecular basis of its role in tissue-specific tumorigenesis remains elusive [6-10]. Generally, when a missense mutation is identified in an affected subject, examination of the physiological function of the encoded mutant protein is necessary to determine whether the mutation is indeed pathogenic. For menin, however, there are no established parameters that can adequately represent its diverse physiological functions. In the event that no functional assays are available, a linkage study within the affected family may be informative. However, in order to draw a reliable conclusion, it requires a number of affected subjects within the family [11-13], and such analysis is rarely performed in practice. There was a report of a mutation, which was initially considered pathogenic but later turned out to be a rare benign polymorphism [14]. Conversely, a missense mutation initially thought to be a rare polymorphism may be characterized later as a pathogenic mutation with low penetrance.

As is the case for the majority of other hereditary cancer-related genes, *MEN1* is categorized as a tumor suppressor gene. Tumor occurrence by mutation of the *MEN1* gene can be explained by Knudson's two-hit theory [3]. In cells containing a heterozygous *MEN1* mutation, the function of one allele has already been lost through a germline mutation and cells acquire a tumor phenotype when the function of the remaining wild-type allele is lost somatically during cell division. Indeed, in tumors from *MEN1* patients, the wild-type allele is usually deleted and identified as loss of heterozygosity [15-17]. As a result, there should be no functioning menin protein in tumor cells arising in patients with *MEN1* mutations.

Genetic analysis of a patient with PHPT revealed a previously unknown single nucleotide substitution in the *MEN1* gene, c.824G>T, which can be interpreted as a missense mutation causing an amino acid substitution of arginine by methionine at codon 275. To determine whetherthe mutation is pathogenic, we examined the characteristics of mRNAs and protein encoded by the mutated *MEN1* gene.

Case Presentation

A 33 year-old woman at the 24th week of gestation was referred to our department due to severe hypercalcemia (Ca 17.5 mg/dL). Based on a markedly elevated level of plasma intact PTH (1425 pg/mL, normal range; 10-65 pg/mL), a diagnosis of PHPT was made. Cervical ultrasonography and MRI revealed a large parathyroid nodule with cystic change. An enlarged parathyroid gland and right lobe of thyroid gland were surgically removed. The removed parathyroid tumor was 5.5 × 2.5 × 2.5 cm in size, and microscopically, chief cells were massively proliferated. After surgery, her serum calcium level normalized and intact PTH decreased to 19.2 pg/mL. Imaging studies for pituitary and enteropancreas performed after parathyroidectomy revealed no abnormal findings. Results of biochemical studies are summarized in Table 1. Based on the young age of onset of PHPT, genetic testing for the *MEN1* mutation was proposed [18, 19]. Written informed consent was obtained from the patient before genetic testing. The full sequence of the coding region of the *MEN1* gene showed a heterozygous single nucleotide substitution, c.824G>T (Fig. 1A). This nucleotide substitution occurred at the last nucleotide of exon 5, and if it does not affect splicing, this mutation was predicted to substitute amino acid codon 275 of menin from arginine (AGG) to methionine (ATG). Screening of family members revealed that her father, 68 years old, had hypercalcemia (10.9 mg/dL) and an increase in intact PTH level (125.3 pg/mL). Imaging studies revealed an enlarged parathyroid nodule, but he declined any treatment beyond regular screening. He refused genetic testing.

Materials and Methods

Stability analysis of variant menin

The intracellular stability of missense menin variants was evaluated using a quantitative fluorescent immunohistochemical method as described previously [20, 21]. Briefly, WI38VA13 cells were transfected with a bicis-

Table 1 Results of biochemical studies

			Reference range
GH	(ng/mL)	0.7	<1.0
IGF-1	(ng/mL)	264	121-436
PRL	(ng/mL)	10.8	1.4-10.8
Insulin (fasting)	(μU/mL)	5.0	<10
Glucose (fasting)	(mg/dL)	88	<110
Gastrin	(pg/mL)	35	37-172
Glucagon	(pg/mL)	68	23-197

GH, Growth Hormone; IGF-1, Insulin-like Growth Factor-1; PRL, Prolactin

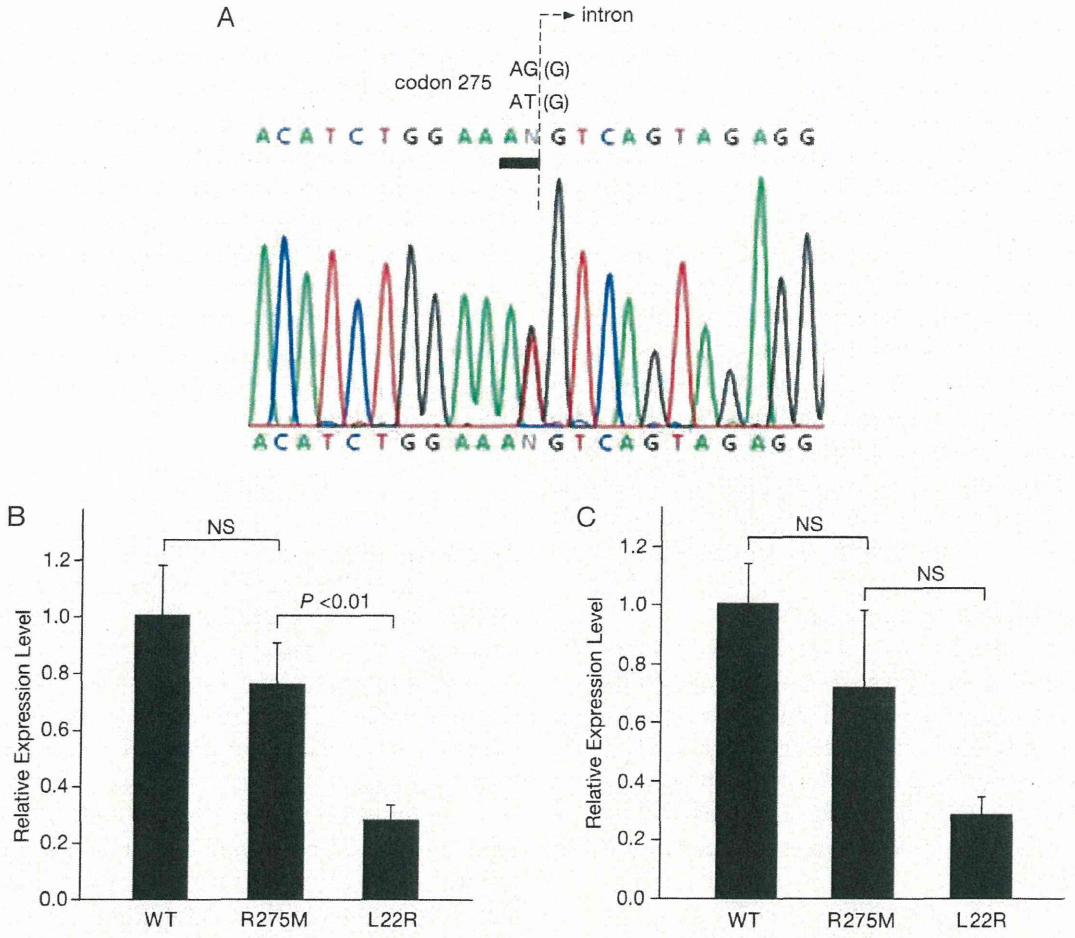

Fig. 1 Stability of menin missense mutant
Genomic DNA was isolated from whole blood of the patient for genetic testing. Sequencing analysis of *MEN1* gene identified c.824G>T mutation (A). This nucleotide substitution is predicted to generate putative missense menin protein, R275M. Mutant menin protein 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 (B) or FLAG-tagged mutant and Myc-tagged wild type menin (C). 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). The thin bars represent standard error of the mean of three independent transfection experiments. NS, not statistically significant ($P>0.05$)

tronic 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. 48 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. The mean of analyzed nuclei number was 24 per transfection and the minimum was 9 per transfection. Mutant menin was located mainly in the nucleus although the cytoplasm was also faintly stained in some cells. Only nuclear staining was analyzed.

Analysis of menin mRNA in blood cells

RNA was isolated from whole blood with the LeukoLOCK^{TM} total RNA isolation system (Ambion,

Austin, TX, USA), and treated with RNase-Free DNase set (QIAGEN, Hilden, Germany). cDNA was synthesized with oligo dT primer using SuperScript III (Invitrogen, Carlsbad, CA, USA). The cDNA was amplified by PCR with primers 3-3 (5'-acctggcacggcaagggcaacga-3') and 7-3 (5'-gtagccagccaggtacatgtagg-3'), which were designed on the basis of the sequences of exon 3 and exon 7 of the *MEN1* gene, respectively. The PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. The DNA fragments were excised from the gel and purified with UltraClean 15 DNA purification kit (MO BIO Laboratories, Carlsbad, CA, USA), then sequenced directly, or cloned into pCR2.1-TOPO TA vector (Invitrogen, Carlsbad, CA, USA) and sequenced with a BigDye terminators v1.1 cycle sequencing kit (Applied biosystems, Foster City, CA, USA).

Sequencing analysis of tumor DNA

Tumor DNA was extracted with DEXPAT^{TM} (TAKARA BIO, Shiga, Japan) and amplified by PCR with primers 56-1 (5'-aaggacccgttctcctccctgttcc-3') and 56-2 (5'-ggcccctgcctcagccactgttag-3'), which were designed on the basis of intron sequences upstream of exon 5 and downstream of exon 6, respectively. The PCR product was sequenced directly as described above.

Minigene analysis of c.824G>T mutant

DNA fragment containing the sequence between the 5' end of exon 3 and 3' end of exon 7 of the *MEN1* gene was amplified by PCR with primers containing *Eco*RI or *Sal*I recognition sites (5'-gaattcgcaccaaattggacagctccggtgtgg-3' and 5'-gtcgactcctggatgacagtggccgtgtcctcc-3'), using human genomic DNA (Clontech, Mountain View, CA, USA) as a template. The PCR products were cloned into pCR-Blunt II-TOPO vector (Invitrogen) prior to confirmation by nucleotide sequencing that the insert sequence was identical to the published *MEN1* gene sequence (GenBank accession No. U93237), then excised and transferred to the mammalian expression vector, pCMV-Tag2 (Stratagene, La Jolla, CA, USA). A c.824G>T mutant minigene was constructed by introducing the mutation into the wild-type minigene using the QuikChange Site-Directed Mutagenesis kit (Stratagene).

Minigene was introduced into WI38VA13 cells with FuGENE6 (Roche Diagnostics, Indianapolis, IN, USA). Total RNA was extracted 24 hr after transfection with QIAshredder and RNeasy Mini kit (QIAGEN), treated with DNase, and subjected to cDNA synthesis as described above. The cDNAs were amplified by PCR with primers (5'-gattacaagcatgacgacgataag-3' and 5'-ggcgaattgggtacacttacctgg-3') designed to anneal to the 5' and 3' minigene-specific regions of the transcripts. The PCR products were separated on a 3% agarose gel, visualized by ethidium bromide staining, and excised and directly sequenced as described above.

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 R275M

The intracellular stability of the putative products of the c.824G>T mutation, R275M was examined by comparing the relative expression levels of mutant *vs.* wild-type menin protein 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, 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 stability test showed that the stability of the R275M mutant was not significantly different from that of wild type menin (Fig. 1 B, C). The stability of the R275M mutant suggests that the c.824G>T mutation may not cause MEN1 if its primary effect was the amino acid substitution [21].

Menin mRNA in blood cells of the patient with c.824G>T mutation

Given that the c.824G>T mutation occurred at an exon-intron junction, this mutation could act as a splicing mutation rather than a simple missense mutation. The menin mRNA of the patient was therefore examined for evidence of abnormal mRNA splicing. PCR amplification with primers on exons 3 and 7 of the patient's blood cell cDNA generated several fragments in addition to the predicted wild type 400-bp cDNA (Fig. 2A). Direct sequencing of the normal-sized fragment with a primer on exon 5 showed only normal sequence and the mutation identified in the germline was not detected (Fig. 2B). The three additional fragments of 360 bp,

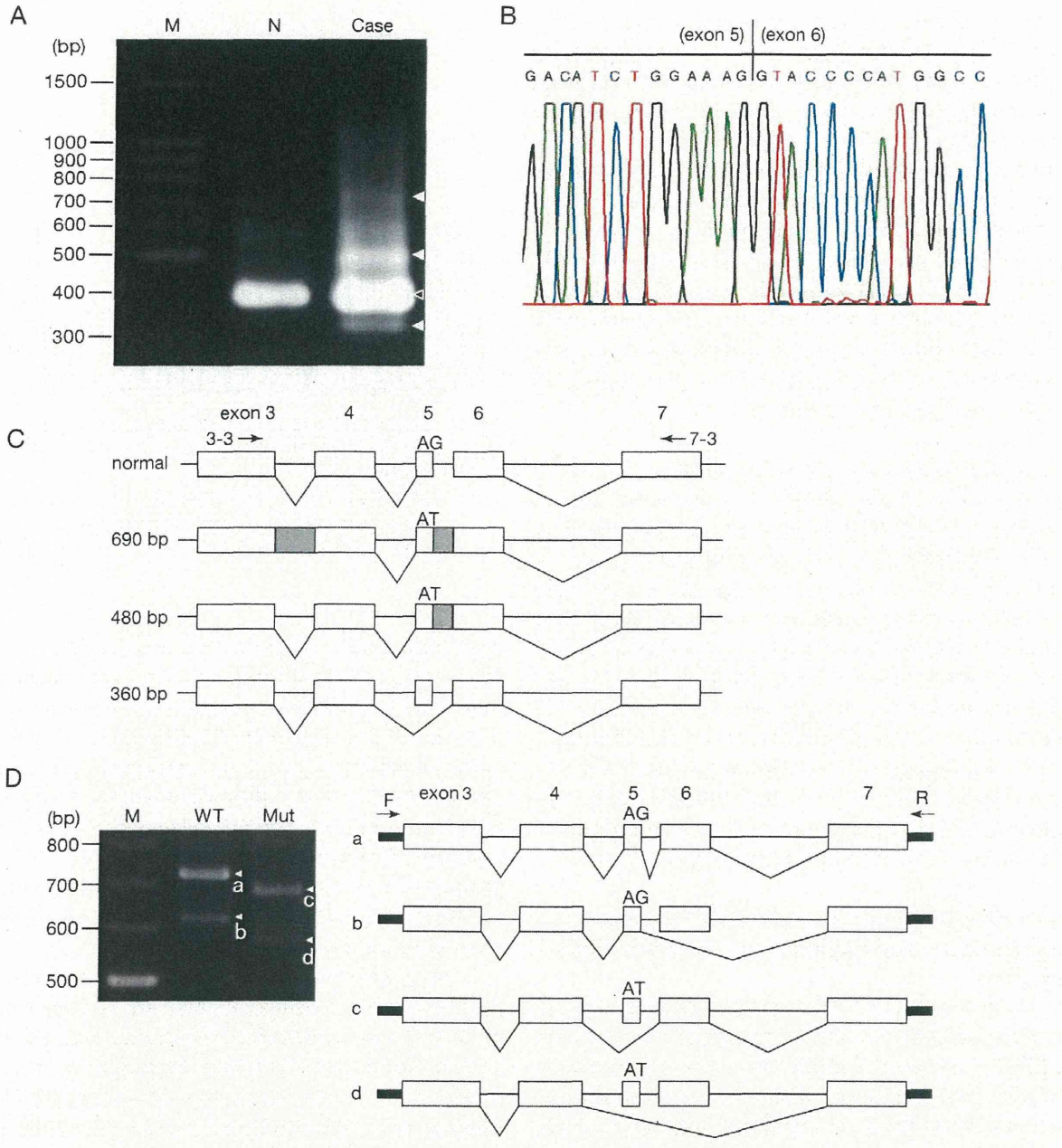

Fig. 2 *MEN1* mRNA in the patients with c. 824G>T mutation

A. The patient's blood cell cDNA was amplified with primers on *MEN1* exons 3 and 7, and separated on agarose gel (Case) along with that from a normal subject (N). The PCR product of the normal size (open triangle) and abnormal PCR products (solid triangles) were excised and subjected to either direct sequencing (normal fragment) or sequencing after cloning (690-bp, 480-bp and 360-bp abnormal fragments). M: size marker. B. Direct sequencing of the normal-sized cDNA fragment analyzed with a sequencing primer on exon 5. The mutated sequence at the 3' end of exon 5 was not detectable. C. Structures of normal-sized, 690-, 480- and 360-bp cDNAs. Open boxes and closed boxes indicate exons and unspliced introns, respectively. V-shaped lines below each diagram indicate the splicing events that give rise to each mRNA. Normal-sized cDNA contained only the wild type sequence (AG) at the exon-intron junction while the 690- and 480-bp cDNAs contained only the mutant sequence (AT). The positions of the PCR primers used are shown above as arrows. D. mRNA from the wild type (WT) and c.824G>T mutant (Mut) minigenes. The structures of PCR products a, b, c and d identified on agarose gel (left) were analyzed by sequencing and shown in the right. The wild type and mutant sequences at the 3' end of exon 5 is shown as AG and AT, respectively. Thick lines represent minigene-specific regions of the transcripts where PCR primers anneal (F and R, shown by arrows).

480 bp and 690 bp were cloned and sequenced (Fig. 2A, C). The 360-bp fragment lacked exon 5; the 480-bp fragment contained an unspliced 80-bp intron sequence following the mutated exon 5; and the 690-bp fragment contained a 210-bp intron sequence following exon 3 as well as the previously observed 80-bp intron sequence following the mutated exon 5. Similar intron retention between exons 3 and 4 in menin mRNA induced by a distant splicing mutation has been reported previously [22]. These findings suggest that the c.824G>T mutation causes aberrant mRNA splicing, and that all detectable menin mRNA splicing variations potentially cause protein truncation by frame-shift or a cryptic stop codon within unspliced intron sequence.

Minigene analysis of c.824G>T mutation

The effect of the c.824G>T mutation on mRNA splicing was examined by minigene experiments (Fig. 2D). The wild type minigene construct generated a normally spliced transcript containing all of exons 3-7 and a splicing variant which lacked exon 6. The mutant construct generated a transcript lacking exon 5 and its variant which lacked both exons 5 and 6, and failed to generate a normally spliced transcript. The deletion of exon 6 in the transcripts of both constructs may be a consequence of artificial gene structure and experimental conditions. These findings strongly suggest that normally spliced mRNA is not generated from the c.824G>T mutant allele of the patient.

Loss of wild type allele of the MEN1 gene in parathyroid tissue obtained from a patient with c.824G>T mutation

We next examined whether the wild type allele is lost by a second hit in the tumor cells of a patient with c.824G>T mutation. DNA was isolated from tumor cells as described in the Materials and Methods and sequenced. As shown in Fig. 3, only the mutant allele was detectable in tumor cells, confirming the loss of the wild type allele.

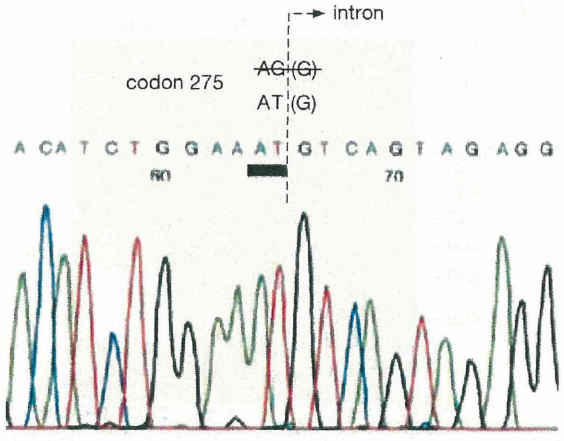

Fig. 3 Loss of the wild type allele of the *MEN1* gene in the parathyroid gland obtained from a patient with c.824G>T mutation
DNA isolated from parathyroid tissue was amplified and sequenced. Note that only mutant allele was seen in the tumor. Compare the sequence to that shown in Fig. 1A.

Discussion

Identification of the *MEN1* gene in 1997 enabled early diagnosis of MEN1 even when patients had developed only a single tumor [4]. Moreover, early or presymptomatic diagnosis of at risk relatives became possible. In the case of frameshift mutation, nonsense mutation or large deletion, it is relatively straightforward to consider those lesions as pathogenic because *MEN1* gene is a tumor suppressor gene. However, when identified mutations are missense mutations or in-frame deletions, molecular diagnosis of MEN1 is not so simple, since the pathogenicity of these mutations is not clear *per se*. Furthermore, when the mutation exists near exon-intron junction, possible deleterious effects of the mutation on splicing have to be considered. Indeed, pathogenic aberrant splicing caused by point mutations are often overlooked as routine genetic testing examines only genomic DNA [23].

In the present report, we examined the pathogenicity of nucleotide substitution of the *MEN1* gene which exists at the last nucleotide of the exon 5. Using analysis of leukocyte mRNA and minigene experiments, our present study clearly demonstrated that the c.824G>T mutation is a splice site mutation causing protein truncation, rather than a missense mutation. Because of nonsense-mediated mRNA decay, it is often difficult to detect aberrantly spliced mRNAs transcribed from a mutant tumor suppressor gene in leukocytes. Nevertheless, the leukocyte mRNA analysis in our case proved useful in demonstrating a splicing mutation of the *MEN1* gene. Analysis of the *MEN1* mutation database revealed that 9% and 14% of *MEN1* germline mutations identified in patients with MEN1 and familial isolated hyperparathyroidism, respectively, were splice mutations [5]. Also in our recent report on

Japanese patients with MEN1, 5.6% (10/180) of germline *MEN1* mutations were splice mutations [24]. However, evidence of aberrant splicing has not always been demonstrated.

In our case, the patient had PHPT but no other MEN1-related tumors. Screening of family members revealed that her father also had PHPT. Since her father declined any further examination, it is unknown whether he had other MEN1-related diseases. Results of our mRNA analysis gave us a rationale to survey the patient with the same protocol as that for patients with typical MEN1.

Menin is considered to function as a scaffold protein for other cellular proteins, and its physiological function appears to be diverse including regulation of cell cycle, transcription, DNA repair, chromatin remodeling, and apoptosis [6-10]. Tissue-specific regulation of endocrine function and cellular proliferation by menin has also been reported [25-29]. There have been studies that examined molecular and physiological function of menin, but these studies examined only specific functions among diverse roles of menin and none of the methods used in these reports are capable of evaluating the function of menin as a whole. In this regard, lack of wild type protein in tumor cells may be the most reliable information which suggests pathogenicity of the mutation. In our present study, we could clearly demonstrate that tumor cells have only mutant allele (Fig. 3), and that mutant allele does not produce normally spliced mRNA, indicating no functional menin protein in tumor cells (Fig. 2).

In conclusion, we examined the pathogenicity of novel nucleotide substitution in the *MEN1* gene identified in a patient with PHPT using a menin stability test and analysis of menin mRNA. Our results clearly demonstrated that the mutation, c.824G>T, is indeed pathogenic.

Acknowledgments

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Disclosure Summary

All authors have nothing to disclose.

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