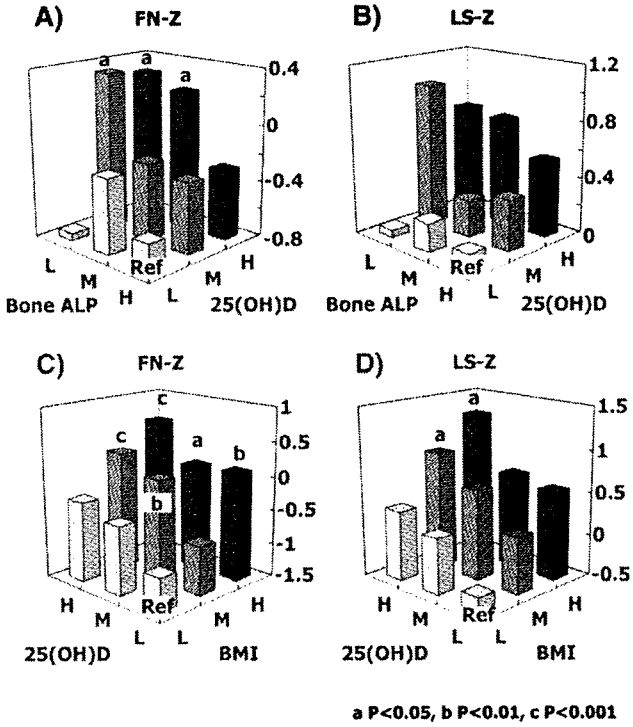

Fig. 4. BMD Z-scores for the nine different tertile combinations for (A, B) 25(OH)D and bone ALP, and (C, D) 25(OH)D and BMI. Patients were categorized and then placed into nine groups based on the tertiles of 25(OH)D, bone ALP, and BMI. The group with the lowest 25(OH)D and highest bone ALP, and the group with lowest 25(OH)D and lowest BMI were used as the reference groups in each figure. H, high; M, middle; L, low. a: $P<0.05$, b: $P<0.01$, c: $P<0.001$ as compared with the reference group (Dunnett's test).

action of 25(OH)D on bone metabolism through auto/paracrine actions in osteoblast might explain the mechanism for the association of 25(OH)D and bone turnover [27].

One of the limitations of our study was that it was a cross-sectional observational one, and thus, it was not possible to elucidate the causal relationship. To truly clarify the contribution of 25(OH)D to CKD-MBD, a further longitudinal interventional study with 25(OH)D supplementation is required. While Ziyad Al-aly et al. performed a retrospective study that followed this format [28], they did not have data with regard to changes in BMD or in bone turnover markers when administering ergocalciferol to predialysis patients. Hernandez et al. reported that administration of ergocalciferol completely improved the clinical symptoms associated with osteomalacia in a young hemodialysis patient, despite discontinuation of paricalcitol. In this patient, reduction of PTH and ALP, an increase in BMD, and a remarkable improvement in the overall bone histology were observed [29]. The findings of their case report are in agreement with our current cross-sectional study.

Intriguingly, in our study, a multivariate analysis showed that 1-84 PTH was positively associated with calcitriol even in the late stages of CKD. This result is compatible with the physiological role of PTH, which upregulates 1α-hydroxylase in the proximal tubules [30]. In fact, in primary hyperparathyroidism, serum calcitriol levels are usually high because of this unregulated, elevated hormone [31]. However, in CKD patients, 1-84 PTH fails to upregulate 1α-hydroxylase enough, because of the reduced number of viable nephrons. In hemodialysis patients, the phosphate load is roughly reflected in the serum phosphate levels, and the serum phosphate levels are positively correlated with 1-84 PTH [32]. As in the multivariate model that did not include FGF-23, this positive association also applies to the predialysis patients. However, after adjustment for FGF-23, the positive association of phosphate with 1-84 PTH was extinguished. Given that FGF-23 is also upregulated by the oral phosphate load [2,32,33], our results imply that 1-84 PTH can be upregulated not only by serum phosphate but also by the phosphate load. Measurement of urinary phosphate or phosphate intake would likely have revealed this, but we did not measure these levels, which is another limitation of our study. Acute injections of recombinant FGF-23 was shown to reduce PTH in rats without renal failure [34]. However, we did not observe a negative association between PTH and FGF-23. The main reason for this might be that PTH, in turn, can upregulate FGF-23 [18]. High PTH even under the condition of extremely high levels of FGF-23 could be attributed to some FGF-23 resistance of the parathyroid cells due to reduced Klotho expression, which was previously reported in human primary HPT [35].

Diverse difference of the skeletal sensitivity to PTH across the CKD stages might explain the reason for the lack of association between 1-84 PTH and BMD. This difference is possibly due to the down-regulation of the PTH-1 receptor caused by the uremic toxins [36], and is reflected in our finding of a positive association of bone ALP/1-84 PTH with eGFR in the current study. In contrast to osteocalcin, renal function did not interfere with the levels of bone ALP [37]. Moreover, our data showed that the postmenopausal high bone turnover state was also reflected in bone ALP [25]. These should be the main reason why bone ALP not 1-84 PTH is a determinant of BMD Z-score. A recent study has found that in hemodialysis patients, full-length biologically active FGF-23 had no significant correlation with bone mass or bone turnover markers [38]. This observation was also noted in the predialysis CKD patients in the present study.

Among the nine groups based on the 25(OH)D and bone ALP, the mean BMD Z-score was unexpectedly low in the group that exhibited a low bone ALP and low 25(OH)D. In this group, 1-84 PTH was the lowest among all of the nine groups. Thus, the poor response of this anabolic hormone to the low vitamin D status might lead to impaired bone formation, which in turn could lead to the low BMD in this group.

In summary, this study demonstrated the importance of the vitamin D status in non-diabetic CKD-MBD with regard to II HPT and BMD. Since the serum calcitriol level itself was the consequence of the regulations by endogenous 1-84 PTH and FGF-23, we propose that it is not the serum calcitriol but its precursor 25(OH)D that should be monitored in predialysis patients.

Conflict of interest statement
None declared.

References

[1] Slatopolsky E, Caglar S, Gradowska L, Canterbury J, Reiss E, Bricker NS. On the prevention of secondary hyperparathyroidism in experimental chronic renal disease using "proportional reduction" of dietary phosphorus intake. Kidney Int 1972;2:147–51.
[2] Slatopolsky E, Mercado A, Morrison A, Yates J, Klahr S. Inhibitory effects of hypermagnesemia on the renal action of parathyroid hormone. J Clin Invest 1976; 58:1273–9.
[3] Kates DM, Sherrard DJ, Andress DL. Evidence that serum phosphate is independently associated with serum PTH in patients with chronic renal failure. Am J Kidney Dis 1997;30:809–13.
[4] Pitts TO, Piraino BH, Mitro R, Chen TC, Segre GV, Greenberg A, et al. Hyperparathyroidism and 1,25-dihydroxyvitamin D deficiency in mild, moderate, and severe renal failure. J Clin Endocrinol Metab 1988;67:876–81.
[5] Segersten U, Correa P, Hewison M, Hellman P, Dralle H, Carling T, et al. 25-hydroxyvitamin D(3)-1alpha-hydroxylase expression in normal and pathological parathyroid glands. J Clin Endocrinol Metab 2002;87:2967–72.
[6] Lundgren S, Carling T, Hjalm G, Juhlin C, Rastad J, Pihlgren U, et al. Tissue distribution of human gp330/megalin, a putative Ca(2+)-sensing protein. J Histochem Cytochem 1997;45:383–92.
[7] Gonzalez EA, Sachdeva A, Oliver DA, Martin KJ. Vitamin D insufficiency and deficiency in chronic kidney disease. a single center observational study. Am J Nephrol 2004;24:503–10.
[8] Gordon CM, DePeter KC, Feldman HA, Grace E, Emans SJ. Prevalence of vitamin D deficiency among healthy adolescents. Arch Pediatr Adolesc Med 2004;158: 531–7.
[9] K/DOQI clinical practice guidelines for bone metabolism and disease in chronic kidney disease. Am J Kidney Dis 2003;42:S1–201.

[10] Yan L, Zhou B, Wang X, D'Ath S, Laidlaw A, Laskey MA, Prentice A. Older people in China and the United Kingdom differ in the relationships among parathyroid hormone, vitamin D, and bone mineral status. Bone 2003;33:620–7.
[11] Aloia JF, Talwar SA, Pollack S, Feuerman M, Yeh JK. Optimal vitamin D status and serum parathyroid hormone concentrations in African American women. Am J Clin Nutr 2006;84:602–9.
[12] Nakamura K, Nashimoto M, Tsuchiya Y, Saito T, Nishiwaki T, Ueno K, et al. Threshold value of serum 25-hydroxyvitamin D concentration in relation to elevated serum parathyroid hormone concentrations in elderly Japanese women. J Bone Miner Metab 2006;24:395–400.
[13] Nakamura K, Tsugawa N, Saito T, Ishikawa M, Tsuchiya Y, Hyodo K, et al. Vitamin D status, bone mass, and bone metabolism in home-dwelling postmenopausal Japanese women: Yokogoshi study. Bone 2008;42:271–7.
[14] Stavroulopoulos A, Cassidy MJ, Porter CJ, Hosking DJ, Roe SD. Vitamin D status in renal transplant recipients. Am J Transp 2007;7:2546–52.
[15] Kazama JJ, Omori T, Ei I, Ei K, Oda M, Maruyama H, et al. Circulating 1–84 PTH and large C-terminal PTH fragment levels in uremia. Clin Exp Nephrol 2003;7:144–9.
[16] Kurajoh M, Inaba M, Yamada S, Imanishi Y, Tsuchida T, Ishimura E, et al. Association of increased active PTH(1-84) fraction with decreased GFR and serum Ca in predialysis CRF patients: modulation by serum 25-OH-D. Osteoporos Int 2008;19:709–16.
[17] Shimada T, Hasegawa H, Yamazaki Y, Muto T, Hino R, Takeuchi Y, et al. FGF-23 is a potent regulator of vitamin D metabolism and phosphate homeostasis. J Bone Miner Res 2004;19:429–35.
[18] Kawata T, Imanishi Y, Kobayashi K, Miki T, Arnold A, Inaba M, et al. Parathyroid hormone regulates fibroblast growth factor-23 in a mouse model of primary hyperparathyroidism. J Am Soc Nephrol 2007;18:2683–8.
[19] Wang H, Yoshiko Y, Yamamoto R, Minamizaki T, Kozai K, Tanne K, et al. Overexpression of fibroblast growth factor 23 suppresses osteoblast differentiation and matrix mineralization in vitro. J Bone Miner Res 2008;23(6):939–48.
[20] Krakauer JC, McKenna MJ, Buderer NF, Rao DS, Whitehouse FW, Parfitt AM. Bone loss and bone turnover in diabetes. Diabetes 1995;44:775–82.
[21] Fujii H, Hamada Y, Fukagawa M. Bone formation in spontaneously diabetic Torii-newly established model of non-obese type 2 diabetes rats. Bone 2008;42:372–9.
[22] Payne RB, Little AJ, Williams RB, Milner JR. Interpretation of serum calcium in patients with abnormal serum proteins. Br Med J 1973;4:643–6.
[23] Imai E, Horio M, Nitta K, Yamagata K, Iseki K, Tsukamoto Y, et al. Modification of the modification of diet in renal disease (mdrd) study equation for Japan. Am J Kidney Dis 2007;50:927–37.
[24] Shimada T, Mizutani S, Muto T, Yoneya T, Hino R, Takeda S, et al. Cloning and characterization of FGF23 as a causative factor of tumor-induced osteomalacia. Proc Natl Acad Sci U S A 2001;98:6500–5.
[25] Urena P, Hruby M, Ferreira A, Ang KS, de Vernejoul MC. Plasma total versus bone alkaline phosphatase as markers of bone turnover in hemodialysis patients. J Am Soc Nephrol 1996;7:506–12.
[26] Coen G, Mantella D, Manni M, Balducci A, Nofroni I, Sardella D, et al. 25-hydroxyvitamin D levels and bone histomorphometry in hemodialysis renal osteodystrophy. Kidney Int 2005;68:1840–8.
[27] van Driel M, Koedam M, Buurman CJ, Hewison M, Chiba H, Uitterlinden AG, et al. Evidence for auto/paracrine actions of vitamin D in bone: 1alpha-hydroxylase expression and activity in human bone cells. FASEB J 2006;20:2417–9.
[28] Al-Aly Z, Qazi RA, Gonzalez EA, Zeringue A, Martin KJ. Changes in serum 25-hydroxyvitamin D and plasma intact PTH levels following treatment with ergocalciferol in patients with CKD. Am J Kidney Dis 2007;50:59–68.
[29] Hernandez JD, Wesseling K, Boechat MI, Gales B, Salusky IB. Osteomalacia in a hemodialysis patient receiving an active vitamin D sterol. Nat Clin Pract Nephrol 2007;3:227–32.
[30] Booth BE, Tsai HC, Morris Jr RC. Vitamin D status regulates 25-hydroxyvitamin D3-1 alpha-hydroxylase and its responsiveness to parathyroid hormone in the chick. J Clin Invest 1985;75:155–61.
[31] Need AG, Horowitz M, Morris HA, Nordin BC. Vitamin D status: effects on parathyroid hormone and 1, 25-dihydroxyvitamin D in postmenopausal women. Am J Clin Nutr 2000;71:1577–81.
[32] Ferrari SL, Bonjour JP, Rizzoli R. Fibroblast growth factor-23 relationship to dietary phosphate and renal phosphate handling in healthy young men. J Clin Endocrinol Metab 2005;90:1519–24.
[33] Antoniucci DM, Yamashita T, Portale AA. Dietary phosphorus regulates serum fibroblast growth factor-23 concentrations in healthy men. J Clin Endocrinol Metab 2006;91:3144–9.
[34] Ben-Dov IZ, Galitzer H, Lavi-Moshayoff V, Goetz R, Kuro-o M, Mohammadi M, et al. The parathyroid is a target organ for FGF23 in rats. J Clin Invest 2007;117:4003–8.
[35] Bjorklund P, Krajisnik T, Akerstrom G, Westin G, Larsson TE. Type I membrane klotho expression is decreased and inversely correlated to serum calcium in primary hyperparathyroidism. J Clin Endocrinol Metab 2008;93(10):4152–7.
[36] Nii-Kono T, Iwasaki Y, Uchida M, Fujieda A, Hosokawa A, Motojima M, et al. Indoxyl sulfate induces skeletal resistance to parathyroid hormone in cultured osteoblastic cells. Kidney Int 2007;71:738–43.
[37] Yamada S, Inaba M, Kurajoh M, Shidara K, Imanishi Y, Ishimura E, et al. Utility of serum tartrate-resistant acid phosphatase (TRACP5b) as a bone resorption marker in patients with chronic kidney disease: independence from renal dysfunction. Clin Endocrinol (Oxf) 2008;69(2):189–96.
[38] Urena Torres P, Friedlander G, de Vernejoul MC, Silve C, Prie D. Bone mass does not correlate with the serum fibroblast growth factor 23 in hemodialysis patients. Kidney Int 2008;73:102–7.

RESEARCH ARTICLE

A method for assessment of *Helicobacter pylori* genotype using stool specimens

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Keywords
Helicobacter pylori; stool specimen; noninvasive genotyping; virulence factor; healthy individual.

Abstract

Helicobacter pylori infection has been regarded as a major factor associated with the development of gastric diseases. The characterization of infected *H. pylori* in asymptomatic individuals is important for the prediction of the onset of such diseases. However, because of the difficulty in obtaining gastric biopsy samples, *H. pylori* in healthy subjects have not been studied sufficiently. Therefore, we tested a noninvasive method for the characterization of *H. pylori* using stool specimens. This method involved *H. pylori* antigen detection in stool specimens by immunochromatography; confirmation of *H. pylori* DNA by real-time PCR that involved the detection of its 16S rRNA gene in the DNA extracted from stool specimens; and nested PCR with genotype-specific primer pairs. A total of 80 samples obtained from asymptomatic subjects were assessed using this method. The results showed that the prevalence of *H. pylori* in asymptomatic Japanese individuals was 37.5%. The detection rate of the virulence factor gene *cagA* was 18.8%. Furthermore, all the detected *cagA* belonged to the highly virulent East-Asian type. These data suggest that the method used in this study is valuable for studying the molecular epidemiology of *H. pylori* infection in asymptomatic people.

Introduction

Helicobacter pylori has been recognized as a group I carcinogen by the International Agency for Research on Cancer, because infection with this pathogen causes persistent gastritis and is directly linked to the development of peptic ulcer, gastric cancer, and gastric lymphoma of the mucosa-associated lymphoid tissue (Fox & Wang, 2007; Amieva & El-Omar, 2008). Various virulence factors of *H. pylori*, such as VacA, CagA, and urease, have been implicated in the pathology of *H. pylori* infection (Maeda & Mentis, 2007). Among these factors, *cagA*, which is located in the pathogenicity island (*cag*PAI) region, has been identified as a critical virulence factor with regard to the initiation of cancer. There are many genotypes of the *cagA* gene, including the East-Asian and Western types. The East-Asian type is more virulent than the Western type because of its strong potency in stimulating signal transduction pathways in the host (Higashi *et al.*, 2002; Hatakeyama, 2006). Therefore, genotyping of the *cagA* gene of clinical *H. pylori* isolates obtained from patients with a gastric disease has been widely conducted. In contrast, the genotype of the virulence factors of *H. pylori* isolates from asymptomatic individuals has rarely been qualitatively and quantitatively assessed due to the difficulty in specimen collection. There are two possible types of specimens for isolating and characterizing the infected bacteria in asymptomatic individuals: one is an endoscopic biopsy specimen and the other is a stool specimen. Although using an endoscopic biopsy specimen is more reliable for isolating the infected *H. pylori*, this method of collection has several disadvantages, including unnecessary invasion into healthy individuals. Consequently, such disadvantages render the carrying out of an epidemiological study difficult, especially with large cohorts. On the other hand, studies using stool specimens are noninvasive and the specimens are easy to collect. These advantages facilitate epidemiological analysis, such as a case–control study, a factor control study, or a prospective study. However, stool has not been considered as a good material for the isolation of *H. pylori*, because the bacteria might be in a 'viable but

nonculturable' state. In fact, only a few reports have described the direct isolation of the bacteria from stool specimens (Kabir, 2001), although many attempts have been made to isolate the bacteria. In addition, the presence of inhibitors and the considerable complexity of the bacteria in stool specimens may also hinder detailed analysis of the *H. pylori* genes using molecular biological techniques such as PCR amplification (Abu Al-Soud & Radstrom, 2000). Molecular characterization of *H. pylori* in asymptomatic individuals is considered as one of the most valuable factors for predicting the onset of diseases associated with silent *H. pylori* infection. Therefore, in this study, in order to assess the virulence of *H. pylori*, we used a noninvasive genotyping method for genotyping the *cagA* gene from stool specimens of asymptomatic individuals.

Materials and methods

Clinical samples

The study was conducted from July to August 2007 in Osaka, Japan. Informed consent was given by all participants. Stool specimens were collected from 80 asymptomatic volunteers aged 40 years and above. The research protocols were approved by the ethics committee of the Division of Health Sciences at the Osaka University Graduate School of Medicine (Osaka, Japan).

Detection of *H. pylori* antigen in stool specimens and extraction of the bacterial DNA

A commercially available rapid-test kit (Testmate Rapid Pylori Antigen, BD, Tokyo, Japan) was used for the detection of the *H. pylori* antigen catalase (Suzuki *et al.*, 2002) in stool specimens. The detection was performed by following the manufacturer's instruction manual.

Bacterial DNA was extracted from all stool specimens using the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions, with minor modifications. In brief, bacterial DNA was extracted from *c.* 1 g of stool specimen and was obtained in a volume of 200 μL with DNA concentrations of up to 150 ng μL^{-1}. The extracted DNA was stored at −20 °C until further use.

PCR analysis

A solution with an extracted DNA concentration of 50 ng μL^{-1} or a 10-fold diluted solution of the extracted DNA was used as a template for all PCR analyses. In order to detect 16S rRNA gene of *H. pylori*, real-time PCR was performed with gene-specific primers, a probe (Table 1), and TaqMan Universal PCR Master Mix (Applied Biosystems, Foster, CA), according to the manufacturer's instruction, whereby the existence of *H. pylori* DNA in the extracted DNA could be confirmed.

Table 1. Oligonucleotide primers and probe used for PCR analysis of *Helicobacter pylori* 16S rRNA gene and *cagA*

Gene	Primer	Primer sequence
16S rRNA gene (Yamazaki *et al.*, 2005)	Forward	5′-TGC GAA GTG GAG CCA ATC TT-3′
	Reverse	5′-GGA ACG TAT TCA CCG CAA CA-3′
	Probe	5′-(FAM) CCT CTC AGT TCG GAT TGT AGG CTG CAA C (TAMRA)-3′
cagA detection (This study)	Forward (common)	5′-GGA ACC CTA GTC AGT AAT GGG TT-3′
	Reverse (JR)	5′-AAT TCT TGT TCC CTT GAA AGC CC-3′
	Reverse (WR)	5′-GCT TTA GCT TCT GAT ACC GCT TGA -3′
cagA-Western (Yamazaki *et al.*, 2005)	Forward	5′-AGG CAT GAT AAA GTT GAT GAT-3′
	Reverse	5′-AAA GGT CCG CCG AGA TCA T-3′
cagA-East-Asian (Yamazaki *et al.*, 2005)	Forward	5′-AAA GGA GTG GGC GGT TTC A-3′
	Reverse	5′-CCT GCT TGA TTT GCC TCA TCA-3′

Nested PCR was performed for *cagA* genotyping. The first round of PCR was performed with a common forward primer and any one of the two reverse primers (Table 1). The PCR conditions were as follows: 95 °C for 2 min, followed by 40 cycles consisting of 94 °C for 15 s, 55 °C for 30 s, and 68 °C for 1 min. The second round of PCR was then performed with 1 μL of the first-round PCR products as the template. In second-round PCR, primers specific to these two types were used in separate reactions. The conditions in the second round of PCR were as follows: 94 °C for 2 min, followed by 50 cycles of 98 °C for 10 s and 65 °C for 2 s. The PCR products were visualized by 2% agarose gel electrophoresis and staining with ethidium bromide.

Detection limit of PCR for *H. pylori* DNA in stool samples

The detection limit of PCR was determined by a spike test. The *cagA* genotype-defined clinical isolates of *H. pylori* were used for the spike test. A series of 10-fold dilutions of *H. pylori* culture in phosphate-buffered saline, ranging from 0 to 10^5 CFU g^{-1} of stool, were added to the antigen-negative stool samples. The bacterial DNA was extracted from the stool sample, and the extracted DNA was applied to PCR.

Results and discussion

The prevalence of *H. pylori* in an asymptomatic Japanese population has been studied by a serological test (Asaka *et al.*, 1992; Osawa *et al.*, 1996; Fujisawa *et al.*, 1999; Yamagata *et al.*, 2000). However, the serological test utilized in these studies has several disadvantages, for example, it

Table 2. Summary of the detection of *Helicobacter pylori* and genotyping of *cagA*

	Tested #	Positive # (%)
H. pylori antigen test	80	30 (37.5)
Real-time PCR		
16S rRNA gene	80	26 (32.5)
Conventional PCR		
Detection of *cagA*	80	15 (18.8)
Genotyping of *cagA*	15	
East-Asian type		15 (100.0)

also detects past and cured infections. In this regard, a novel detection method that could directly detect the bacterial antigen in stool specimens using a monoclonal antibody against *H. pylori* catalase has been recently developed, and its sensitivity and specificity are comparable to those of the breath test (Cardenas *et al.*, 2008). Therefore, we applied this detection method to assess the prevalence of *H. pylori* in asymptomatic Japanese individuals.

It is known that the prevalence of *H. pylori* infection increases with age, and a higher prevalence can be found in a population aged over 40 years (Asaka *et al.*, 1992). Therefore, asymptomatic Japanese individuals aged 40 years and above were selected as a source of stool specimens in this study. As is evident in Table 2, the *H. pylori* antigen was detected in 30 (37.5%) of the 80 specimens collected from asymptomatic adult individuals. This rate was lower than that in previous reports, wherein the antigens were determined using the above-mentioned serological method (Asaka *et al.*, 1992; Fujisawa *et al.*, 1999; Yamagata *et al.*, 2000). The reasons for the lower prevalence of *H. pylori* in healthy people examined in this study are not clear. However, it seems that the difference in the present results and previous reports are probably due to the different detection methods used; that is, the antigen detection method in the present study could reveal the active and current *H. pylori* infection, while the serological method used in the previous studies also detected past and cured infection.

In order to determine the genotype of *H. pylori* in asymptomatic individuals, a PCR analysis of the virulence factor gene *cagA* was performed. *Helicobacter pylori* DNA was extracted from all stool specimens. The existence of *H. pylori* DNA in the extracted DNA was confirmed by real-time PCR specific for *H. pylori* 16S rRNA gene (Yamazaki *et al.*, 2005). *cagA* genotyping was achieved by nested PCR that was specific for either East-Asian or Western type. In general, it can be expected that the contents of *H. pylori* DNA may not be high in the DNA extracted from stool specimens. In addition, the DNA extracted from stool samples may not be a good template for PCR because of the existence of PCR inhibitors and its higher level of complexity (Cavallini *et al.*, 2000). Therefore, before the analysis of the DNA extracted from stool specimens, the sensitivity of the PCR used in this study was evaluated using the spike test. The results showed that the detection limit of the real-time PCR specific for 16S rRNA gene and nested PCR for *cagA* genotype was $2.1 \pm 0.1 \times 10^2$ and 1.0×10^4 CFU g^{-1}, respectively. As shown in Table 2, *H. pylori* 16S rRNA gene was detected in 26 of the 80 extracted DNA samples from stool specimens. The low detection rate (32.5%) of *H. pylori* DNA by PCR was probably due to the different targets (antigen vs. DNA) used for the detection and the presence of PCR inhibitors in the extracted DNA samples (Kabir, 2004). Nevertheless, the results of *H. pylori* DNA detection were consistent with a previous report on *H. pylori* DNA detection in stool specimens (Monteiro *et al.*, 2001).

After confirmation of the existence of *H. pylori* DNA, the virulence factor gene *cagA* was detected and genotyped. The *cagA* gene was detected in 15 (18.8%) of the 80 samples. The *cagA* detection rate obtained in this study was relatively lower than that in the previous studies, which showed detection rates ranging from 53.8% to 70.8% (Russo *et al.*, 1999; MacKay *et al.*, 2003; Sicinschi *et al.*, 2003a, b). The reasons for the low detection rate observed in this study may be due to the different target population utilized such as healthy people.

The analysis of *cagA* showed that all of the detected *cagA* belonged to the highly virulent East-Asian genotype. That is, 18.8% of Japanese individuals tested were infected with highly virulent *H. pylori*. In this regard, our recent study of *cagA* genotype in asymptomatic Thai people showed that the East-Asian genotype was present in $< 2\%$ of the samples tested (unpublished data). Therefore, it seems likely that the prevalence of the highly virulent East-Asian *cagA* genotype of *H. pylori* in asymptomatic people may differ across countries. Such a different prevalence, if any, may be related to the incidence of gastric diseases, including stomach cancer. Nevertheless, it can be conjectured from the results obtained in this study that a significant number of Japanese healthy people may be infected with the highly virulent *H. pylori*.

Thus, the results obtained in this study indicate that the method used in this study is useful for the assessment of *H. pylori* infections in healthy people.

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References

Abu Al-Soud W & Radstrom P (2000) Effects of amplification facilitators on diagnostic PCR in the presence of blood, feces, and meat. *J Clin Microbiol* **38**: 4463–4470.

Amieva MR & El-Omar EM (2008) Host–bacterial interactions in *Helicobacter pylori* infection. *Gastroenterology* **134**: 306–323.

Asaka M, Kimura T, Kudo M, Takeda H, Mitani S, Miyazaki T, Miki K & Graham DY (1992) Relationship of *Helicobacter pylori* to serum pepsinogens in an asymptomatic Japanese population. *Gastroenterology* **102**: 760–766.

Cardenas VM, Dominguez DC, Puentes FA, Aragaki CC, Goodman KJ, Graham DY & Fukuda Y (2008) Evaluation of a novel stool native catalase antigen test for *Helicobacter pylori* infection in asymptomatic North American children. *J Pediatr Gastroenterol Nutr* **46**: 399–402.

Cavallini A, Notarnicola M, Berloco P, Lippolis A & De Leo A (2000) Use of macroporous polypropylene filter to allow identification of bacteria by PCR in human fecal samples. *J Microbiol Meth* **39**: 265–270.

Fox JG & Wang TC (2007) Inflammation, atrophy, and gastric cancer. *J Clin Invest* **117**: 60–69.

Fujisawa T, Kumagai T, Akamatsu T, Kiyosawa K & Matsunaga Y (1999) Changes in seroepidemiological pattern of *Helicobacter pylori* and hepatitis A virus over the last 20 years in Japan. *Am J Gastroenterol* **94**: 2094–2099.

Hatakeyama M (2006) *Helicobacter pylori* CagA – a bacterial intruder conspiring gastric carcinogenesis. *Int J Cancer* **119**: 1217–1223.

Higashi H, Tsutsumi R, Muto S, Sugiyama T, Azuma T, Asaka M & Hatakeyama M (2002) SHP-2 tyrosine phosphatase as an intracellular target of *Helicobacter pylori* CagA protein. *Science* **295**: 683–686.

Kabir S (2001) Detection of *Helicobacter pylori* in faeces by culture, PCR and enzyme immunoassay. *J Med Microbiol* **50**: 1021–1029.

Kabir S (2004) Detection of *Helicobacter pylori* DNA in feces and saliva by polymerase chain reaction: a review. *Helicobacter* **9**: 115–123.

MacKay WG, Williams CL, McMillan M, Ndip RN, Shepherd AJ & Weaver LT (2003) Evaluation of protocol using gene capture and PCR for detection of *Helicobacter pylori* DNA in feces. *J Clin Microbiol* **41**: 4589–4593.

Maeda S & Mentis AF (2007) Pathogenesis of *Helicobacter pylori* infection. *Helicobacter* **12** (suppl 1): 10–14.

Monteiro L, Gras N, Vidal R, Cabrita J & Megraud F (2001) Detection of *Helicobacter pylori* DNA in human feces by PCR: DNA stability and removal of inhibitors. *J Microbiol Meth* **45**: 89–94.

Osawa H, Inoue F & Yoshida Y (1996) Inverse relation of serum *Helicobacter pylori* antibody titres and extent of intestinal metaplasia. *J Clin Pathol* **49**: 112–115.

Russo F, Notarnicola M, Di Matteo G, Leoci C, Caruso ML, Pirrelli M, Caradonna M, Morandi L & Di Leo A (1999) Detection of *Helicobacter pylori cagA* gene by polymerase chain reaction in faecal samples. *Eur J Gastroen Hepat* **11**: 251–256.

Sicinschi LA, Correa P, Bravo LE & Schneider BG (2003a) Detection and typing of *Helicobacter pylori cagA/vacA* genes by radioactive, one-step polymerase chain reaction in stool samples from children. *J Microbiol Meth* **52**: 197–207.

Sicinschi LA, Correa P & Schneider BG (2003b) Comparison of genotyping of *Helicobacter pylori cagA* and *vacA* virulence genes from gastric biopsies and stool specimens. *Helicobacter* **8**: 601–607.

Suzuki N, Wakasugi M, Nakaya S *et al.* (2002) Production and application of new monoclonal antibodies specific for a fecal *Helicobacter pylori* antigen. *Clin Diagn Lab Immun* **9**: 75–78.

Yamagata H, Kiyohara Y, Aoyagi K *et al.* (2000) Impact of *Helicobacter pylori* infection on gastric cancer incidence in a general Japanese population: the Hisayama study. *Arch Intern Med* **160**: 1962–1968.

Yamazaki S, Kato S, Matsukura N *et al.* (2005) Identification of *Helicobacter pylori* and the *cagA* genotype in gastric biopsies using highly sensitive real-time PCR as a new diagnostic tool. *FEMS Immunol Med Mic* **44**: 261–268.

エビデンスに基づく CKD 診療ガイドライン 2009

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要　旨

・本年 3 月，日本腎臓学会より「エビデンスに基づく CKD 診療ガイドライン」が発刊された。2007 年 9 月に日本腎臓学会より上梓され，現在広く普及している「CKD 診療ガイド」との違いは何か？　という質問をよくいただく。

・本ガイドラインは，日本腎臓学会学術委員会が主体となり，上記「CKD 診療ガイド」が発刊される前，その形をほぼ整えた 2007 年 7 月頃より構想を開始し，意欲的な若手医師をワーキンググループとして公募し，以後約 1 年半の期間をかけて集中的に論文を読み込み，議論を重ねて作成されたものである。

・診療ガイドと診療ガイドラインは対象の読者や目的とするところが異なり，同時に存在して差し支えないものである。

本稿ではガイドラインについての一般的基本事項，「CKD 診療ガイド 2009」との位置づけの違いについて述べ，本ガイドライン作成にあたっての考え方，ステートメント作成プロセス，また利用にあたって留意すべき点について解説する。また，本ガイドラインの概要を紹介し，ポイントとして「CKD と生活習慣病」を例示し，最後に今後の展望についても触れる。

はじめに

米国腎臓財団(NKF)により 2002 年に Kidney Disease Outcome Quality Initiative(K/DOQI)からガイドラインが提示され[1]，米国循環器学会は心血管疾患(CVD)のリスクとしての慢性腎臓病(CKD)の重要性を踏まえて，2002 年には CVD リスクとしての CKD に関する Scientific Statement[2]を，また 2006 年には CVD 患者における CKD の早期発見の重要性についての Science Advisory[3]を発表している。

このような国際的動向を背景として，日本腎臓学会においても CKD の総合的対策を担う組織として，2005 年に慢性腎臓病対策委員会が発足し活動を開始した。その成果の一つとして 2007 年 9 月には，「CKD 診療ガイド」が上梓され，診療現場で広く活用されている。さらに本年 3 月，「エビデンスに基づく CKD 診療ガイドライン」[4]が発刊され，CKD 対策の一層の展開が期待される。

本稿では，本ガイドライン作成のねらい，経緯について述べる。また「CKD 診療ガイド 2009」[5]との位置づけの違いについて言及する。そして，本ガイドラインの項立てを紹介しポイントについて述べる。

CKD の定義と診断基準

CKD は以下のように診断される。

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CKD の診断基準

(1) GFR の値にかかわらず，腎障害を示唆する所見(検尿異常，画像異常，血液異常など)が 3 カ月以上存在すること

(2) GFR 60 ml/min/1.73 m^2未満が 3 カ月以上持続すること

この片方または両方を満たす場合に CKD と診断される。

わが国における CKD の日常診療において，血清クレアチニン，年齢，性別の三つのデータから eGFR を算出する下記の推算式を日本腎臓学会として作成し推奨している[6]。

GFR(ml/min/1.73 m^2)
$=194\times Cr^{-1.094}\times Age^{-0.287}\times 0.739$(女性の場合)

EBM に基づいた診療ガイドラインの役割

近年，臨床の現場でエビデンスに基づいた医療(EBM)が重要視されるようになり，診療ガイドラインも EBM に立脚した記述で構成されることが一般化した。この EBM に基づいた診療ガイドラインの考え方は欧米で発達してきたもので，その定義は，「医療者と患者が特定の臨床現場で適切な決断を下せるよう支援する目的で，体系的な方法に則って作成された文書。EBM の手順で作成することに最大の特徴がある」(Institute of Medicine, National Academy 1990，米国)とされている。

EBM は重要であり，診療方針選択にあたって一定の科学的根拠を提供することの意義に疑いの余地はない。しかし，EBM は個々の医師の専門的技量に取って代わるものではなく，あくまで治療の主体は医師であり，EBM で得られた情報をどのように実地診療の場で個々の患者に適応していくかは医師の専門家としての能力に依存している。

すなわち，診療ガイドラインとは，決して診療行為を直接規定するものではなく，あくまでも医師の診療の裁量の中でよりよい診療実践を支援するものである。さらに，ガイドラインは医事紛争や医療訴訟における判断基準を示すものと受け取られがちであるが，本来の目的に照らせばガイドラインとはそのようなものではなく，この点について理解が広まることを期待したい。

CKD 診療ガイドと CKD 診療ガイドライン

日本腎臓学会 CKD 対策委員会より 2007 年 9 月に刊行された「CKD 診療ガイド」は，CKD 対策の重要性と治療・管理の包括的方針について，一般医を含む医療従事者全般に啓発するために作成されたものであり，現在広く普及し CKD 診療のレベルアップに貢献している。なお，「CKD 診療ガイド」はその後の学問的進展を盛り込むため，2009 年 3 月に改訂版として「CKD 診療ガイド 2009」が刊行された。

一方，「CKD 診療ガイドライン」は，その読者として専門医レベルを想定したものである。一般に，診療ガイドは作成委員のコンセンサスを土台として記述されるが，診療ガイドラインは EBM(根拠に基づいた医療)を拠り所として作成される。近年の臨床医学領域における EBM の集積に伴い，EBM に準拠したガイドライン作成が可能となり，それを作成し公表するのは臨床医学系の学会の責務とされるようになった。CKD 診療ガイドラインは CKD 診療にかかわるエビデンスを広く収集し，それらを吟味，解釈し，それに基づいた診療指針を提示したものであり，日本腎臓学会が公表するガイドラインとしては初めての EBM に立脚したガイドラインである。

CKD 診療ガイドラインにおけるエビデンスとステートメント

CKD 診療ガイドラインでは，引用した文献のエビデンスレベルを研究遂行のデザインで分け，水準の高いものから，次のレベル 1〜5 に分類した。

1．システマティックレビュー/メタアナリシスによる，

2．一つ以上のランダム化比較試験による，

3．非ランダム化比較試験による，
4．分析疫学的研究(コホート研究や症例対照研究)による，
5．記述研究(症例報告やケース・シリーズ)による，コンセンサスとなっている。

さらに，エビデンスの質やわが国における診療現場に応じた判断も含め，各項目にステートメント(推奨)のグレードを記した。ステートメントは，ある事項についてのエビデンスの集大成であり，その推奨の強さをグレードAからグレードDまでに分けた。

グレードA：行うよう強く勧められる
グレードB：行うよう勧められる
グレードC：行うよう勧めるだけの根拠が明確でない
グレードD：行わないよう勧められる

本ガイドラインではグレードDは該当なしであった。

なお，本診療ガイドラインを参考にする場合は，エビデンスレベルの高さよりもステートメントのグレードを重視していただきたい。エビデンスは欧米において得られたものが少なからずあり，わが国の医療の実情とそぐわないものも散見される。

エビデンスレベルは上述の基準に則って決められるが，推奨レベルは日本の医療の実情を勘案しながら執筆者，グループリーダー，学術委員会の各レベルにおける確認，合意を経て決定した。エビデンスが乏しいが日本の医療現場では，標準となっているような治療法(IgA腎症，ネフローゼ症候群においてみられる)の取り扱いについては，学術委員会での議論を経て，なるべくステートメントに取り上げるが，解説においてエビデンスがないことを明記することで，記載が実臨床と大きく乖離しないこと，また読者がevidence-practice gapを意識し，今後の実地診療に役立つエビデンスの集積へのモチベーションが高まる一助となることを期した。

CKD診療ガイドラインの概要

表1に本ガイドラインの項立てを示す。CKDという語で包含される病態，原因疾患は多岐にわたり，またCKD自体が原因疾患を問わない，機能異常のみを拠り所とする概念である。これらにより，CKDについてのガイドライン作成は一本道のプロセスとはなり難い。

表1 項目

1．CKDの診断
2．CKDの意義
3．CKDと生活習慣
4．CKDと栄養
5．CKDと高血圧・心血管合併症
6．腎性貧血
7．CKDに伴う骨ミネラル代謝異常
8．糖尿病性腎症
9．IgA腎症
10．ネフローゼ症候群
11．腎硬化症
12．動脈硬化性腎動脈狭窄症
13．常染色体優性多発性嚢胞腎(ADPKD)
14．CKDと脂質代謝異常
15．肥満・メタボリックシンドローム
16．小児CKDの診断
17．小児CKDの治療
18．透析療法
19．腎移植
20．高齢者のCKD診療
21．薬物投与

本ガイドライン作成にあたっては，「CKD」の趣旨に照らして腎機能悪化とCVDのリスク増加に関係する共通メカニズムについての病態解明と治療法選択に主眼を置くべき，という議論もあった。しかし，本書が専門医を主な読者と想定していることより，従来腎臓病学が対象としてきた専門医が診療するべき疾患を含めることには意義があるとの観点から，代表的な腎疾患として糖尿病性腎症，IgA腎症，ネフローゼ症候群(特発性膜性腎症，一次性巣状分節性糸球体硬化症)，常染色体優性多発性嚢胞腎が取り上げられた。これに加え，腎硬化症，腎動脈硬化性腎動脈狭窄症について別項を立てた。

CKDに随伴する異常(腎性貧血，骨ミネラル代謝異常)についても記している。特筆すべき点として，小児のCKDについて，診断，治療に分けて章を設けた。今回の項立ては一つの考え方であり，今後の改訂において新たな視点からの方向性が打

表2　ステートメント

1 喫　煙　グレードA　レベル④

喫煙はCKDの発症および進行に関連する独立した危険因子であり[1~10]，CVDの発症リスクを増加させることから[11~13]，CKD患者は禁煙すべきである。

2 飲　酒　グレードB　レベル④

中等量の飲酒(エタノール20~40 g/日)はCKDのリスクとはならず，むしろ進行を抑制し[2]，CVDの発症も抑制する[13]。一方，大量飲酒(エタノール60 g/日以上)はCKDのリスクとなり，CVDの発症も増加させるため，避けるべきである[14]。

3 運動・身体活動度

① 身体活動度の維持　グレードA　コンセンサス

CKD患者に安静・運動制限を一律に行うべきではなく，肥満の是正，糖尿病新規発症の予防，高血圧の治療，CVD予防のために身体活動度を維持すべきである。

② 運動強度　グレードB　レベル③

運動疲労を起こさない程度の運動(5 METs前後)が安定したCKDを悪化させるという根拠はなく，合併症などの身体状況が許す限り，定期的施行が推奨される[15,16]。

4 ワクチン接種　グレードB　レベル④

CKD患者には，インフルエンザワクチンの接種が推奨される[17]。

5 癌スクリーニング　グレードB　コンセンサス

CKD患者の癌スクリーニングは，一般人と同様の対応が推奨される。腫瘍マーカーの評価に際しては，偽陽性などに注意が必要である。

ち出されることも十分想定される。

本ガイドラインは新規であり，ポイントの紹介は恣意的にならざるを得ないが，個人的には第3章「CKDと生活習慣」は，これまであまり注目されず，エビデンスの有無すらあまり知られていなかった領域について，現時点でのエビデンスの集大成がなされた点に大きな意義を感じている。参考までに，ステートメントをそのまま引用させていただく(**表2**)。

CKD診療ガイドラインの今後の展望

EBMに基づいたガイドライン作成には，良質なエビデンスが不可欠である。また，良質なガイドラインは，最新のEBMを取り入れ定期的に改訂されるべきである。今回，個人的にはCKD診療ガイドラインの作成にかかわる機会に恵まれ大変よい経験をさせていただいたが，同時に，CKD領域のエビデンス，とりわけ，日本人を対象としたデータが不足していることを痛感する機会ともなった。腎臓領域での初の本格的ガイドラインとなった本ガイドラインが嚆矢となり，CKD領域で「日本人の，日本人による，日本人のための」オリジナリティーの高いエビデンスが構築され，それに基づきCKD診療ガイドラインが進化していくことを期待したい。

文　献

1) K/DOQI Clinical Practice Guidelines for chronic kidney disease : evaluation, classification, and stratification. Am J Kidney Dis **39** : 1-266, 2002
2) Sarnak MJ, Levey AS, Schoolwerth AC, et al ; American Heart Association Councils on Kidney in Cardiovascular Disease, High Blood Pressure Research, Clinical Cardiology, and Epidemiology and Prevention : Kidney disease as a risk factor for development of cardiovascular disease : a statement from the American Heart Association Councils on Kidney in Cardiovascular Disease, High Blood Pressure Research, Clinical Cardiology, and Epidemiology and Prevention. Circulation **108** : 2154-2169, 2003
3) Brosius FC 3rd, Hostetter TH, Kelepouris E, et al ; American Heart Association Kidney and Cardiovascular Disease Council ; Council on High Blood Pressure Research ; Council on Cardiovascular Disease in the Young ; Council on Epidemiology and Prevention ; Quality of Care and Outcomes Research Interdisciplinary Working Group : Detection of chronic kidney disease in patients with or at increased risk of cardiovascular disease : a science advisory from the American Heart Association Kidney And Cardiovascular Disease Council ; the Councils on High Blood Pressure Research, Cardiovascular Disease in the Young, and Epidemiology and Prevention ; and the Quality of Care and Outcomes Research Interdisciplinary Working Group : developed in collaboration with the National Kidney Foundation. Circulation **114** : 1083-1087, 2006
4) 日本腎臓学会編：エビデンスに基づくCKD診療ガイドライン，東京医学社，東京，2009
5) 日本腎臓学会編：CKD診療ガイド2009，東京医学社，東京，2009
6) Matsuo S, Imai E, Horio M, et al ; on behalf of the collaborators developing the Japanese equation for estimated GFR : Revised equations for estimated GFR from serum creatinine in Japan. Am J Kidney Dis **53** : 982-992, 2009

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ORIGINAL ARTICLE

Negative effects of anemia on quality of life and its improvement by complete correction of anemia by administration of recombinant human erythropoietin in posttransplant patients

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Abstract
Background Anemia is a common complication in posttransplant patients (posttransplant anemia: PTA). We tested the hypothesis that targeting hemoglobin (Hb) over 13.3 g/dl by administration of recombinant human erythropoietin (rHuEPO-ad) has positive impact on quality of life (QOL).
Methods Twenty-four patients, whose initial Hb and estimated glomerular filtration rate (eGFR) were 10.5 ± 0.2 g/dl and 48.5 ± 2.7 ml/(min 1.73 m^2), respectively, were enrolled in the present study. Physical and mental QOL in these patients before and after rHuEPO-ad were acquired and summarized as physical summary sore (PSC) and mental summary sore (MSC), respectively, by the 36-item Short Form (SF-36), an international questionnaire for analysis of QOL.
Results Before rHuEPO-ad, posttransplant patients had preserved MSC (54.1 ± 2.3) but impaired PSC (32.6 ± 3.2). rHuEPO-ad for 6 months increased their Hb to 13.7 ± 0.3 g/dl. This was accompanied by improvement of PSC (49.1 ± 2.1: $P < 0.01$ versus before rHuEPO-ad). MSC was preserved during rHuEPO-ad (54.4 ± 1.6: NS versus before rHuEPO-ad). There was inverse correlation between initial PSC or MSC and responses of these parameters to rHuEPO-ad (PSC, $P = 0.007$; MSC, $P = 0.009$). Patients whose initial PSC was lower than 39.6 or whose initial MSC was lower than 39.4 were expected to improve their PSC or MSC by more than 10 by rHuEPO-ad.
Conclusions Anemia in posttransplant patients has negative impacts on their QOL. Scoring mental and physical QOL by SF-36 in posttransplant patients is useful to identify groups of patients whose QOL could be improved by rHuEPO-ad.

Keywords SF-36 · QOL · EPO · Posttransplant

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Introduction

Anemia is a common complication in patients with chronic kidney diseases, including posttransplant patients (posttransplant anemia: PTA). Prevalence of PTA during the first 5-year posttransplant period has been reported to be 30–40% [1]. Lower GFR has been identified as the major risk factor among the various factors for PTA, including low serum erythropoietin, younger age, female gender, iron deficiency, systemic illnesses, acute and chronic infections, immunosuppressive regimens, and use of angiotensin I converting

enzyme inhibitor/angiotensin II receptor blocker (ACE-I/ARB) [2–4]. Impact of PTA on mortality and graft failure in posttransplant patients has been clearly demonstrated by Molnar [5], who has shown higher rates of mortality and graft failure in groups of patients whose hemoglobin (Hb) levels were lower than 11.0 g/dl. Administration of recombinant human erythropoietin (rHuEPO-ad) has been shown to be effective in correcting PTA [6]. In our previous study, we showed that elevation of hematocrit (Ht) from 31.7 ± 1.9% to 33.6 ± 3.6% by rHuEPO-ad in posttransplant patients improved left-ventricular hypertrophy [6]. An identical conclusion was also reported by Hayashi [7], and these results may represent the potential benefit of rHuEPO-ad on the cardiovascular system in posttransplant patients. In the same study, we investigated the impact of rHuEPO-ad on QOL of posttransplant patients, scored by the 36-item Short Form (SF-36) [8], an international questionnaire for analysis of QOL. To our surprise, rHuEPO-ad failed to improve QOL in posttransplant patients. This opposes the established beneficial role of rHuEPO-ad on QOL in subjects with cancer or chronic renal failure [9]. Apparently, a possible explanation for our negative result is that the targeted level of Ht was not sufficient to improve QOL. Accordingly, the aim of this study was to test whether improvement of Hb to 13.3 g/dl by rHuEPO-ad can improve QOL in posttransplant patients.

Patients, materials, and methods

This was a prospective cohort study on patients recruited over a 2-month period starting from 1 September 2003, which included a follow-up period over 6 months. The subjects were patients who met the following inclusion criteria: (1) having undergone renal transplantation at the Department of Urology of Osaka University Medical School and subsequently followed up at Inoue Hospital, (2) without serious rejection, defined by resistance to the conventional treatments and progressive loss of renal function, (3) with Hb level less than 12.0 g/dl with or without administration of erythropoietin, (4) with either an iron saturation level over 16% or a serum ferritin level over 50 ng/ml, and (5) who consented to participate in the study. The following patients were excluded: (1) those who were hypersensitive to erythropoietin, (2) those who had a history of gelatin allergy, (3) women who were pregnant, breast feeding or had child-bearing potential, (4) those with severe heart failure, (5) those with nephrotic syndrome or severe edema, (6) those with severe hypertension that was resistant to antihypertensive treatments, (7) those with a history of myocardial or cerebral infarction, or pulmonary embolism, (8) those with certain predispositions such as drug allergies, (9) those with serious complications such as pancytopenia, malignant neoplasms, systemic amyloidosis, severe infections or severe hemorrhagic lesions, and (10) those using antipsychotic drugs or with dementia. The endpoint of the present study was QOL. A self-reported questionnaire survey (version 2.0 of the Japanese edition [10, 11] of SF-36) was used to score the QOL before and 3 and 6 months after administration of rHuEPO. The present study was conducted according to Declaration of Helsinki principles and was approved by the local ethical committee of our institution. All patients provided written informed consent prior to participation.

Treatment

rHuEPO preparation Epoetin-α (Kyowa Hakko Kirin Co., Ltd, Tokyo, Japan) was administered subcutaneously at dose of 6,000 IU once a week in order to increase Hb level to 13.3 g/dl. For safety, increase of Hb was maintained below 0.3 g/dl per week. Once Hb reached 13.3 g/dl, administration of rHuEPO was adjusted within the range of 6,000–12,000 IU once every 2 weeks or 6,000 IU once a week, as required.

Observations and tests

QOL was evaluated at the start of follow-up, and at 3 and 6 months after the commencement of the study, and the changes in the eight subscales of SF-36 [physical function (PF), limitations due to physical problems (RP), bodily pain (BP), general health perceptions (GH), vitality (VT), social function (SF), limitations due to emotional problems (RE), and mental health (MH)] were obtained to calculate the physical and mental summary score (PSC and MSC) to evaluate improvement of QOL by administration of rHuEPO. Hemoglobin (Hb) and serum creatinine (sCr) were also measured and eGFR was calculated at time of QOL evaluation. sCr was measured by enzymatic method, and GFR-estimating equations for Japanese eGFR, i.e., eGFR [ml/(min 1.73 m^2)] = 194 × $sCr^{-1.094}$ × $age^{-0.287}$ × 0.739 (if female), were applied to calculate eGFR in this study [12]. Differences in laboratory findings were tested by one-way analysis of variance (ANOVA) (repeated). r^2 values given by correlation analysis of two factors were converted to P value according to sample numbers. Results are expressed as mean ± standard error on the mean (SEM) and $P < 0.05$ was considered significant. All analyses were performed using SAS software version 6.12.

Results

Twenty-four patients were recruited and analyzed in the 2 months from 1 September to 31 October in 2003. The

patients included 12 men and 12 women with mean age of 36.6 ± 2.2 years (27–59 years) and whose mean time since renal transplantation was 7.1 ± 0.8 years (5.0–20 years). Mean Hb before administration of rHuEPO (rHuEPO-ad) was 10.5 ± 0.2 g/dl and improved significantly to 13.7 ± 0.6 g/dl after 3 months of rHuEPO-ad ($P < 0.01$). Thereafter, values were maintained throughout the rest of the observation period (6 months: 13.7 ± 0.3 g/dl; $P < 0.01$ versus before, NS versus 3 months). Mean serum creatinine (sCr) and eGFR before rHuEPO-ad were 1.30 ± 0.07 mg/dl and 48.5 ± 2.7 ml/(min 1.73m^2), respectively, and were not affected by rHuEPO-ad (Table 1). Among the eight subscales of SF-36, posttransplant patients had preserved body pain (BP: 49.3 ± 2.4), vitality (VT: 52.4 ± 2.6), general health perception (GH: 47.5 ± 1.4), and mental health (MH: 48.5 ± 2.8), but impaired physical function (PF: 45.3 ± 2.6), limitations due to physical problem (RP: 30.7 ± 4.0), limitations due to emotional problems (RE: 34.6 ± 4.3), and social function (SF: 39.8 ± 3.1) (Fig. 1). The summary scores calculated from these eight subscales showed preserved mental summary score (MSC: 54.1 ± 2.3) but impaired physical summary score (PSC: 32.6 ± 3.2) (Fig. 2). No correlation was found between initial Hb and initial PSC or MSC (data not shown). Three months of rHuEPO-ad improved PCS, accompanied by improvement of PF, RP, SF, and RE (Figs. 1, 2). Thereafter, scores were maintained throughout the rest of the observation period. There were no correlations between initial PSC and initial MSC, or between changes in PSC and MSC with rHuEPO-ad, which demonstrates the independence of these two parameters (Fig. 3). Interestingly, inverse correlation was observed between initial PSC or MSC and their responses to rHuEPO-ad for 6 months (Fig. 4). Accordingly, patients whose initial PSC was lower than 39.6 or whose initial MSC was lower than 39.4 were expected to improve their PSC or MSC by more than 10 after rHuEPO-ad. When patients were divided into two subgroups (group A: initial PSC > 39.6, $n = 8$; group B: initial PSC ≤ 39.6, $n = 16$), rHuEPO-ad significantly improved PSC in group B, whereas it failed to show any impact in group A (Fig. 5a). Only two patients had MSC lower than 39.4, which was not sufficient for further analysis (Fig. 5b).

Discussion

We investigated the effect on QOL of increase of Hb over 13.3 g/dl by rHuEPO-ad in anemic posttransplant patients. As shown in Table 1, we successfully improved Hb to over 13.3 g/dl by rHuEPO-ad in this study. Although the initial level of Ht did not correlate with initial QOL in agreement with the previous study [13], significant improvements of physical QOL were observed after increase of Hb over 13.3 g/dl. No relationships were observed among magnitude of correction of Hb and degree of improvement of PSC or MSC. The clear contrast between the present study, which succeeded in improving QOL by rHuEPO-ad, and our previous study, which failed to improve QOL by rHuEPO-ad [6], demonstrates the importance of the target level of Hb for QOL in posttransplant patients. In our previous study, target level of Ht (33–36%, corresponding to Hb of 11.0–12.0 g/dl) was determined on the basis of The National Kidney Foundation Kidney Disease Outcomes Quality Initiative (NKF-K/DOQI) recommendation and related factors [14], but this recommendation is developed for chronic renal failure subjects including those on hemodialysis and may not be sufficient to improve QOL of transplant patients, probably due to the positive impact of renal transplantation itself on QOL [15]. This idea is supported by the study of Ogutmen [16], which showed better QOL in transplant patients than in hemodialysis or peritoneal dialysis patients.

Although the present study demonstrated the positive impact of rHuEPO-ad on QOL, negative aspects of this treatment have also been reported in recent studies. Accordingly, in patients with chemotherapy-induced anemia, rHuEPO-ad increases risk of thromboembolic events and hypertension [9]. rHuEPO-ad also increases risk of cardiovascular events in anemic subjects with renal insufficiency [17]. We did not record any vascular embolic side-effects in the present study. We also did not record adverse effects on renal function (Table 1). Furthermore, it has been speculated from recent study that cardiovascular side-effects of rHuEPO-ad might be specific to subjects who are resistant to rHuEPO therapy [18]. However, we cannot neglect the potential risk of vascular embolism due to the higher target level of Hb. One strategy to minimize this risk is

Table 1 Hemoglobin, serum creatinine, and eGFR

Total ($n = 24$)	Baseline (BL)	After 3 months (3 M)	After 6 months (6 M)	P value		
				BL versus 3 M	BL versus 6 M	3 M versus 6 M
Hb (g/dl)	10.5 ± 0.2	13.7 ± 0.6	13.7 ± 0.3	$P < 0.01$	$P < 0.01$	NS
sCr (mg/dl)	1.30 ± 0.07	1.28 ± 0.07	1.27 ± 0.07	NS (by ANOVA)		
eGFR [ml/(min 1.73 m^2)]	48.5 ± 2.7	49.1 ± 2.5	49.7 ± 2.6	NS (by ANOVA)		

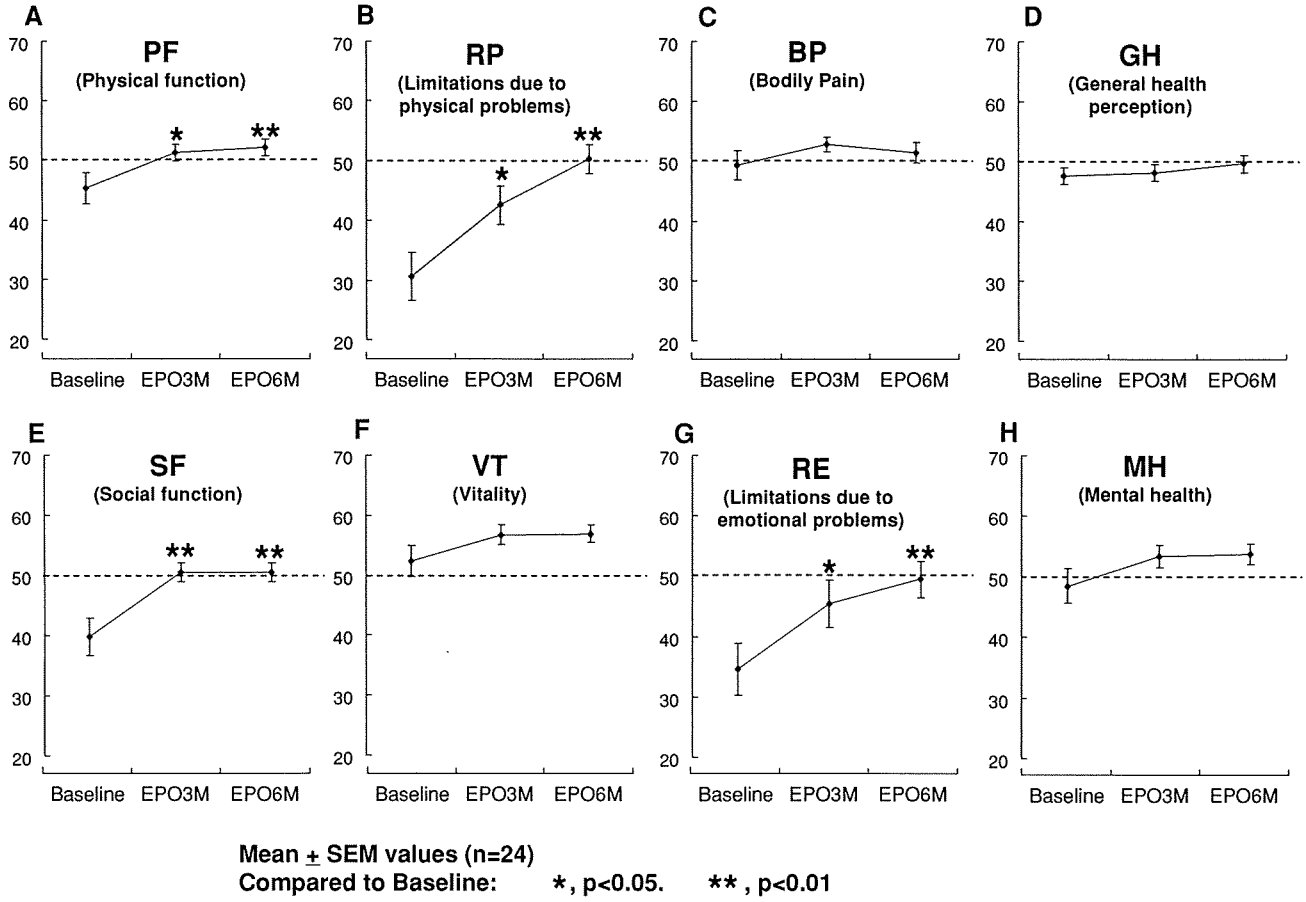

Fig. 1 The eight subscales of SF-36 in posttransplant patients before and after administration of rHuEPO. **a** physical function (PF), **b** limitations due to physical problem (RP), **c** body pain (BP), **d** general health perception (GH), **e** social function (SF), **f** vitality (VT), **g** limitations due to emotional problems (RE), and **h** mental health (MH)

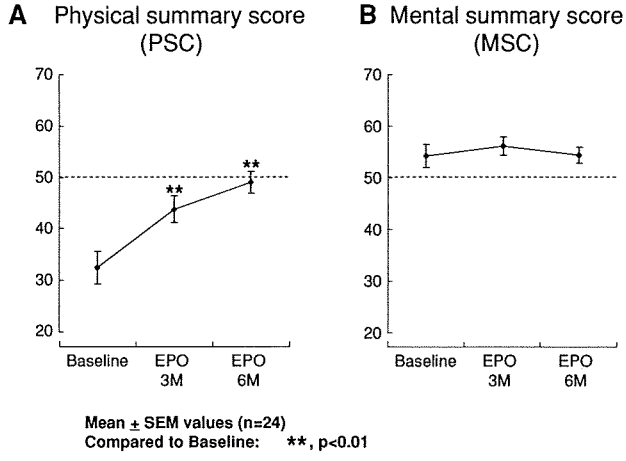

Fig. 2 Calculated physical (**a**) and mental summary scores (**b**) in posttransplant patients before and after administration of rHuEPO

identification of the groups of subjects whose QOL may or may not be improved by rHuEPO-ad. If rHuEPO-ad could be applied only to QOL responders, this would not only reduce the risk of side-effects due to unnecessary rHuEPO-ad, but would also have a beneficial impact on medication cost. In the present study, we identified that improvement of QOL by rHuEPO-ad is highly dependent on initial level of QOL before rHuEPO-ad (Fig. 4). The correlation study indicated that rHuEPO-ad is expected to improve PSC or MSC by more than 10 when initial PSC is lower than 39.6 or initial MSC is lower than 39.4. Indeed, the group of patients whose initial PSC was lower than 39.6 showed significant increase in PSC with rHuEPO-ad, whereas those who had initial PSC higher than 39.6 failed to show improvement of PSC with rHuEPO-ad. Although further investigations are necessary to conclude the exact values of initial PSC and MSC that can predict QOL responders, the present study firstly identifies the usefulness of QOL scoring of post-transplant subjects by SF-36 for the prediction of QOL responders with rHuEPO-ad. The limitation of this study is the small number of samples ($n = 24$) and the short period of observation (6 months), and further investigations are necessary to conclude the impact of rHuEPO-ad on QOL and safety of rHuEPO-ad in posttransplant patients.

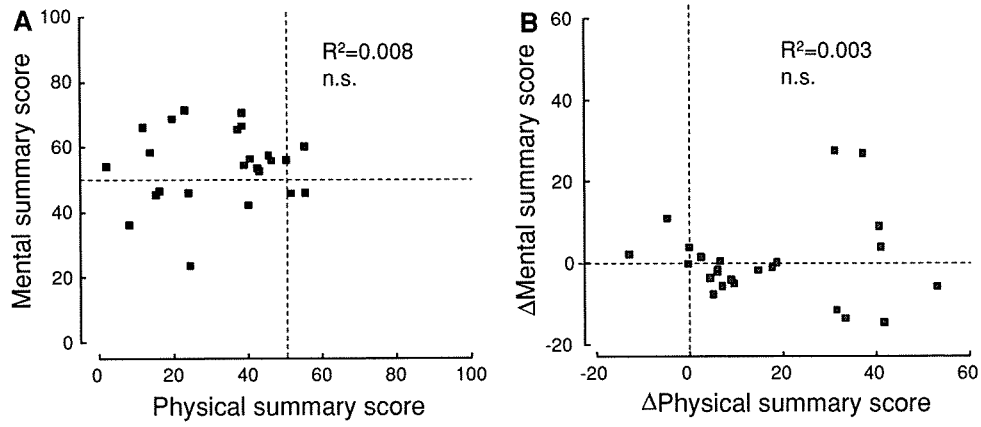

Fig. 3 No correlation between initial physical and initial mental summary score (**a**) or between changes in physical and mental summary score (**b**) with administration of rHuEPO

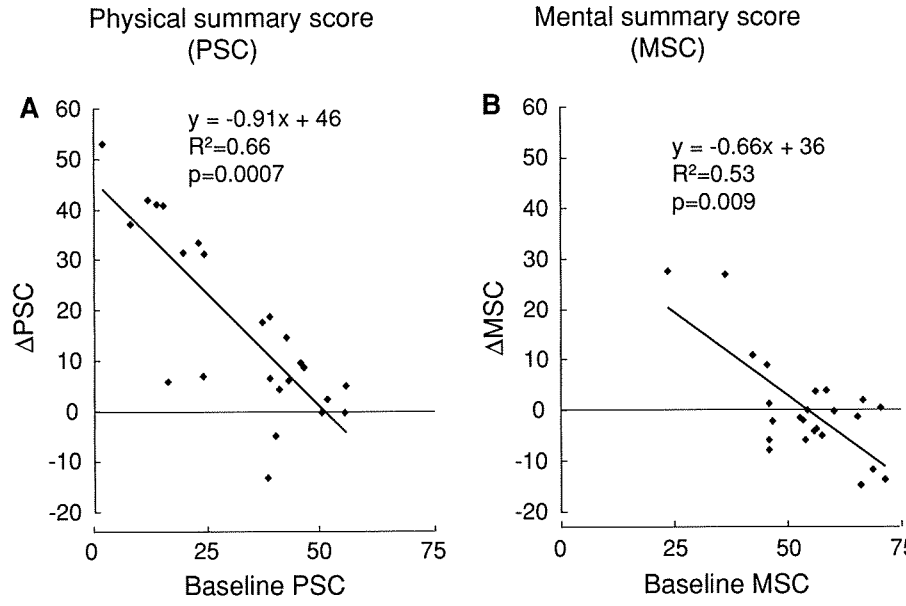

Fig. 4 **a** Correlation between physical summary score and change in physical summary score with administration of rHuEPO. **b** Correlation between mental summary score and the change in mental summary score with administration of rHuEPO

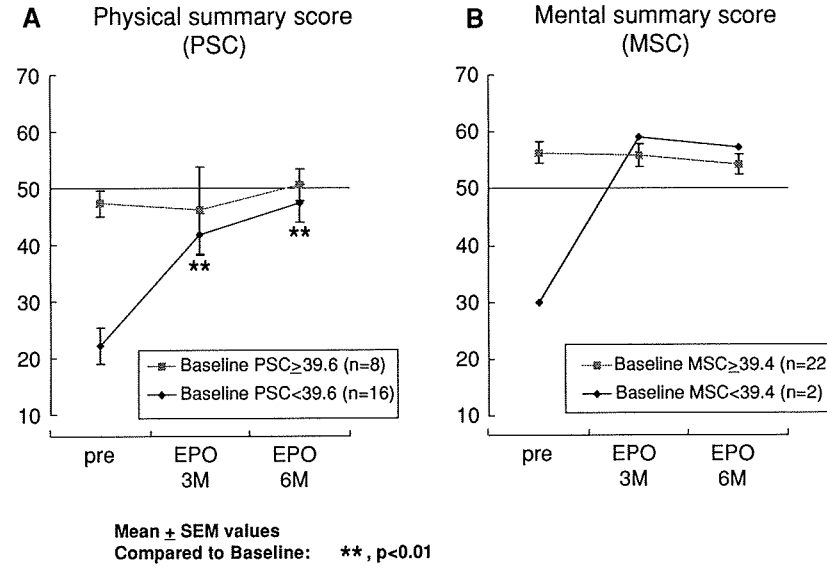

Fig. 5 Calculated physical and mental summary score in posttransplant patients before and after administration of rHuEPO. **a** Physical summary score in groups of patients whose initial physical summary score was over 39.6 ($n = 8$) or under 39.6 ($n = 16$). **b** Mental summary score in groups of patients whose initial mental summary score was over 39.4 ($n = 22$) or under 39.4 ($n = 2$)

In conclusion, anemia in posttransplant patients has negative impacts on their QOL. Scoring of posttransplant patient's QOL by SF-36 is useful to identify the group of patients whose QOL will be improved by administration of rHuEPO. Posttransplant anemic patients whose initial PSC was lower than 39.6 or whose initial MSC was lower than 39.4 are strongly expected to experience improved QOL by targeting Hb over 13.3 g/dl by administration of rHuEPO.

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References

1. Zadrazil J, Horák P, Horcicka V, Zahálková J, Strébl P, Hrubý M. Endogenous erythropoietin levels and anemia in long-term renal transplant recipients. Kidney Blood Press Res. 2007;30:108–16.
2. Sinnamon KT, Courtney AE, Maxwell AP, McNamee PT, Savage G, Fogarty DG. Level of renal function and serum erythropoietin levels independently predict anaemia post-renal transplantation. Nephrol Dial Transplant. 2007;22:1969–73.
3. Shah N, Al-Khoury S, Afzali B, Covic A, Roche A, Marsh J, et al. Posttransplantation anemia in adult renal allograft recipients: prevalence and predictors. Transplantation. 2006;8:1112–8.
4. Montanaro D. Anemia after renal transplantation. G Ital Nefrol. 2007;24:13–22.
5. Molnar MZ, Czira M, Ambrus C, Szeifert L, Szentkiralyi A, Beko G, et al. Anemia is associated with mortality in kidney-transplanted patients—a prospective cohort study. Am J Transplant. 2007;7:818–24.
6. Kawaguchi T, Moriyama T, Suzuki K, Hatori M, Tanaka T, Takahara S, et al. Pilot study of the optimum hematocrit for patients in the predialysis stage after renal transplantation. Transplant Proc. 2004;36:1293–6.
7. Hayashi T, Suzuki A, Shoji T, Togawa M, Okada N, Tsubakihara Y, et al. Cardiovascular effect of normalizing the hematocrit level during erythropoietin therapy in predialysis patients with chronic renal failure. Am J Kidney Dis. 2000;35:250–6.
8. Ware JE Jr, Sherbourne CD. The MOS 36-item short-form health survey (SF-36). I. Conceptual framework and item selection. Med Care. 1992;30:473–83.
9. Bokemeyer C, Aapro MS, Courdi A, Foubert J, Link H, Osterborg A, et al. European Organisation for Research and Treatment of Cancer (EORTC) Taskforce for the elderly. EORTC guidelines for the use of erythropoietic proteins in anaemic patients with cancer: 2006 update. Eur J Cancer. 2007;43:258–70.
10. Fukuhara S, Bito S, Green J, Hsiao A, Kurokawa K. Translation, adaptation, and validation of the SF-36 Health Survey for use in Japan. J Clin Epidemiol. 1998;51:1037–44.
11. Fukuhara S, Ware JE Jr, Kosinski M, Wada S, Gandek B. Psychometric and clinical tests of validity of the Japanese SF-36 Health Survey. J Clin Epidemiol. 1998;51:1045–53.
12. Matsuo S, Imai E, Horio M, Yasuda Y, Tomita K, Nitta K, Yamagata K, Tomino Y, Yokoyama H, Hishida A. Revised equations for estimating glomerular filtration rate (GFR) from serum creatinine in Japan. Am J Kidney Dis 2009 (in press).
13. Shah VS, Ananth A, Sohal GK, Bertges-Yost W, Eshelman A, Parasuraman RK, et al. Quality of life and psychosocial factors in renal transplant recipients. Transplant Proc. 2006;38:1283–5.
14. National Kidney Foundation. IV. NKF-K/DOQI clinical practice guidelines for anemia of chronic kidney disease: update 2000. Am J Kidney Dis. 2001;37:S182–238.
15. Fiebiger W, Mitterbauer C, Oberbauer R. Health-related quality of life outcomes after kidney transplantation. Health Qual Life Outcomes. 2004;2:2.
16. Ogutmen B, Yildirim A, Sever MS, Bozfakioglu S, Ataman R, Erek E, et al. Health-related quality of life after kidney transplantation in comparison intermittent hemodialysis, peritoneal dialysis, and normal controls. Transplant Proc. 2006;38:419–21.
17. Singh AK, Szczech L, Tang KL, Barnhart H, Sapp S, Wolfson M, et al. Correction of anemia with epoetin alfa in chronic kidney disease. N Eng J Med. 2006;355:2085–98.
18. Kilpatrick RD, Critchlow CW, Fishbane S, Besarab A, Stehman-Breen C, Krishnan M, et al. Greater epoetin alfa responsiveness is associated with improved survival in hemodialysis patients. Clin J Am Soc Nephrol. 2008;3:1077–83.

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Assessment of East Asian-type *cagA*-positive *Helicobacter pylori* using stool specimens from asymptomatic healthy Japanese individuals

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Recent investigations have suggested that CagA, a virulence factor of *Helicobacter pylori* and known to have multiple genotypes, plays a critical role in the development of stomach cancer. However, the prevalence of *cagA*-positive *H. pylori* strains and the *cagA* genotypes have not been well studied in healthy individuals because of the difficulty in collecting gastric specimens. In the present study, we assessed the prevalence of infection with *H. pylori*, particularly the strains with the East Asian *cagA* genotype (which is more potent in causing gastric diseases), among healthy asymptomatic Japanese individuals by a noninvasive method using stool specimens. The *H. pylori* antigen was detected in 40.3 % of healthy asymptomatic adult individuals (n=186) enrolled in the study. For the detection and genotyping of the *cagA* gene, DNA was extracted from the stool specimens of these individuals and analysed by PCR. We detected the East Asian *cagA* genotype in the DNA samples of a significantly high number (63.1 %) of healthy asymptomatic Japanese individuals. These results indicate that a significant number of asymptomatic healthy Japanese individuals were infected with highly virulent *H. pylori*.

INTRODUCTION

The significance of various virulence factors of *Helicobacter pylori* has been studied with regard to the pathology of gastric diseases, such as peptic ulcers and gastric cancer. Among these, CagA is one of the well-studied virulence factors of *H. pylori*. The CagA protein is classified into two major types – the East Asian type and the Western type – depending on the combination of the four domains (A, B, C and D) present on the variable region of the C-terminal domain of this protein (Higashi *et al.*, 2002a; Yamaoka *et al.*, 2000b). Each domain contains a single Glu-Pro-Ile-Tyr-Ala (EPIYA) motif (Covacci *et al.*, 1993). Recent studies have revealed that EPIYA motifs are potential targets of the Src family of protein tyrosine kinases. Furthermore, of the four EPIYA motifs in each domain, EPIYA-D of the East Asian CagA protein has a stronger transforming activity than that of the Western type because of the stimulation of signal transduction cascades (Higashi *et al.*, 2002b; Naito *et al.*, 2006). Therefore, the East Asian CagA is more virulent than the Western type, which contains the EPIYA-C domain. In addition, it has been reported that the East Asian CagA probably plays a more effective pathophysiological role than the Western type in the development of gastric diseases caused by *H. pylori* infection (Azuma, 2004). Therefore, extensive genotyping of the *cagA* gene has been carried out in many countries using *H. pylori* strains clinically isolated from patients with gastric diseases (Devi *et al.*, 2006; Kanada *et al.*, 2008; Vilaichone *et al.*, 2004; Yamaoka *et al.*, 2000b, 2008; Yamazaki *et al.*, 2005b).

In contrast, there have been only few studies on the different *cagA* genotypes of *H. pylori* in healthy asymptomatic individuals, mainly because of the difficulty in collecting gastric biopsy samples from healthy individuals (Chattopadhyay *et al.*, 2002; Molnar *et al.*, 2008; Yamaoka *et al.*, 2000a). Therefore, we established a genotyping method that involves the use of stool specimens, which were collected from healthy asymptomatic individuals (Hirai *et al.*, 2009). In the present study, we determined the incidence of *H. pylori* infection by using a noninvasive method to analyse stool specimens and detected the East Asian *cagA* genotype in healthy asymptomatic Japanese individuals.

METHODS

Participants and stool specimens. This study was conducted in Osaka, Japan, from June 2007 to October 2007. Initially, a total of 235 individuals were enrolled in this study. These individuals were screened for age (>39 years) and medical history. The exclusion

criteria included any antibiotic treatment in the past 3 months, eradication therapy for *H. pylori*, and a confirmed diagnosis of digestive tract diseases. Finally, 186 individuals (65 women, 121 men; age range 40–63 years) participated in this study. Stool specimens were collected from the participants, and they were also asked to fill out questionnaires. This study was approved by the ethics committee of the Osaka University Graduate School of Medicine, Osaka, Japan.

Detection of the *H. pylori* antigen and DNA extraction. The individuals' stool specimens were tested for catalase, i.e. an *H. pylori* antigen, by immunochromatographic analysis using a commercially available rapid test kit (TestMate Rapid Pylori Antigen; BD Japan), according to the manufacturer's instructions (Cardenas *et al.*, 2008; Suzuki *et al.*, 2002). The detection limit of this kit is 18.8 ng ml^{-1} of protein concentration (equivalent to 10^4–10^5 bacterial cells ml^{-1}). Bacterial DNA was extracted from stool specimens that tested positive for the *H. pylori* antigen using the QIAmp DNA stool mini kit (Qiagen) according to the manufacturer's instructions with the following minor modifications. Approximately 1 g of each stool specimen was suspended in 3.0 ml ASL buffer (supplied in the kit). After mixing the suspension, approximately 1.2 ml of the supernatant was used for DNA extraction. The extracted DNA was dissolved in 200 µl AE buffer (supplied in the kit) and stored at −20 °C until further use.

PCR analysis. All PCR analyses were performed according to methods described in a previous report (Hirai *et al.*, 2009). The sequences of the primers and the probe used in this study are shown in Table 1. For the template, we used a solution containing 50 ng DNA µl^{-1} or a 10-fold dilution of the DNA sample (approx. 5–10 ng µl^{-1}), which minimized the effect of the inhibitors possibly present in the solution. We performed real-time PCR to detect the 16S rRNA gene of *H. pylori*. In order to detect the East Asian *cagA* genotype, nested PCR was performed using genotype-specific primers. We performed two rounds of PCR. The first round was performed using a common forward primer (F1) and either of the two reverse primers (R1 or R2) (Fig. 1b, c). The PCR cycling conditions for the first round were as follows: 95 °C for 10 min, then 40 cycles at 94 °C for 15 s, 55 °C for 30 s and 68 °C for 30 s. The second round was performed using 1 µl of the PCR products obtained in the first round as the template. In the second round, primers specific to these two types were used in separate reactions. The cycling conditions of the second round of PCR were as follows: 94 °C for 2 min, then 30 cycles at 98 °C for 10 s and 63 °C for 30 s. The PCR products were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining.

Data analysis. All results were analysed by χ^2 analysis. The level of statistical significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

Prevalence of *H. pylori*

In Japan, there has been a considerable increase in the incidence of gastric cancer in middle-aged individuals (Yoshida *et al.*, 2006); hence, for this study, middle-aged and older individuals who did not exhibit any subjective symptoms and had not received medical treatment in the previous 3 months were enrolled. The age distribution of the 186 participants enrolled in this study is shown in Table 2. Of the 186 participants, 75 (40.3%) tested positive for the *H. pylori* antigen (Table 3). The incidence of *H. pylori* infection in each age group ranged from 33.3 to 51.2%; no significant difference was observed among the age groups in this regard (Table 2).

The study revealed that a certain number of healthy asymptomatic Japanese individuals who were older than 39 years were infected with *H. pylori*; this was directly determined using the individuals' stool specimens. However, previous studies that employed the serological method reported higher incidences (70–80%) of *H. pylori* infection among individuals who were ⩾40 years old (Asaka *et al.*, 1992; Fujisawa *et al.*, 1999; Yamagata *et al.*, 2000). It is likely that the difference between the present results and those reported by previous studies may be attributable to the different detection systems employed in these studies. The serological method may tend to yield a relatively higher infection rate than the antigen detection method using stool specimens because the serological method also detects past and cured infections. It has been shown that the sensitivity and specificity of the antigen detection method used in this study are comparable to those of the breath test, which is widely used as a standard method for the detection of *H. pylori* infection (Cardenas *et al.*, 2008).

Detection of the *H. pylori* 16S rRNA gene

Total bacterial genomic DNA was extracted from stool specimens that tested positive for the *H. pylori* antigen. In order to confirm the existence of *H. pylori* genomic DNA in the extracted DNA, real-time PCR was performed using

Table 1. Oligonucleotide primers and a probe used for PCR analysis

Gene	Primer/probe	Sequence	Reference
16S rRNA	Forward	5′-TGC GAA GTG GAG CCA ATC TT-3′	Yamazaki *et al.* (2005a)
	Reverse	5′-GGA ACG TAT TCA CCG CAA CA-3′	Yamazaki *et al.* (2005a)
	Probe	5′-(FAM) CCT CTC AGT TCG GAT TGT AGG CTG CAA C (TAMRA)-3′	Yamazaki *et al.* (2005a)
cagA	F1	5′-GGA ACC CTA GTC AGT AAT GGG TT-3′	Hirai *et al.* (2009)
	F2	5′-CCA ATA ACA ATA ATA ATG GAC TCA A-3′	This study
	R1	5′-GCT TTA GCT TCT GAT ACC GCT TGA-3′	Hirai *et al.* (2009)
	R2	5′-AAT TCT TGT TCC CTT GAA AGC CC-3′	Hirai *et al.* (2009)
	EA-F	5′-AAA GGA GTG GGC GGT TTC A-3′	Yamazaki *et al.* (2005a)
	EA-R	5′-CCT GCT TGA TTT GCC TCA TCA-3′	Yamazaki *et al.* (2005a)

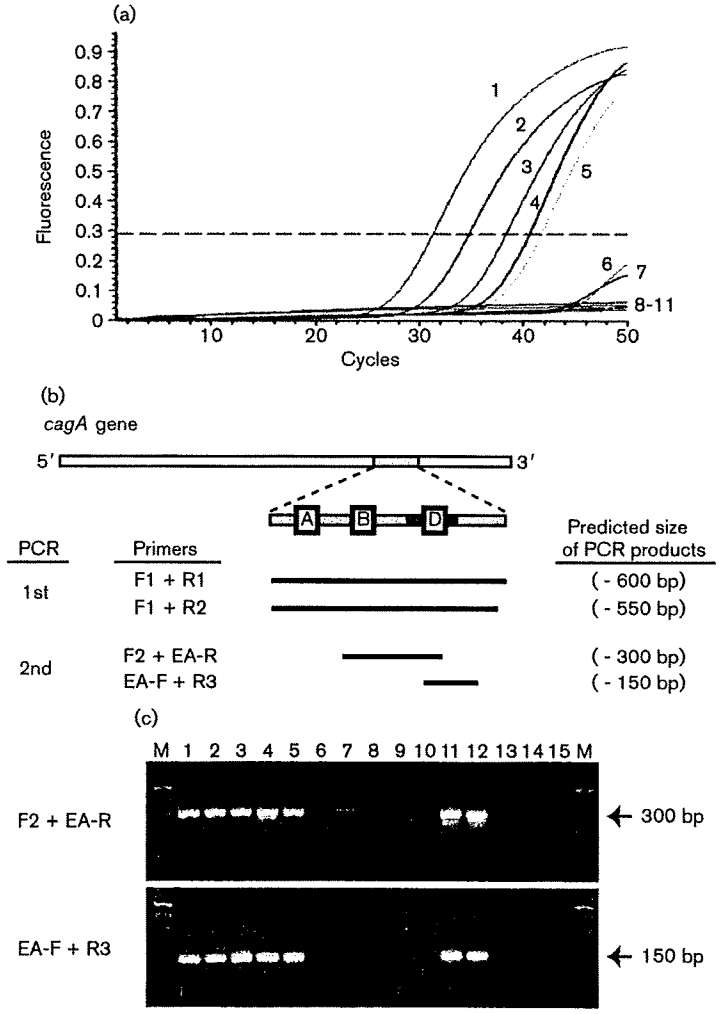

Fig. 1. Real-time PCR analysis of the 16S rRNA gene and genotyping of *cagA* encoding the virulence factor. (a) Representative amplification plots of real-time PCR targeting the 16S rRNA gene. Curves: 1–5, *H. pylori* DNA-positive specimens; 6–10, *H. pylori* DNA-negative specimens; 11, negative control (water); broken line, threshold cycle line. (b) Diagram showing the positions of the PCR products on the *cagA* gene. Alignment of the primer pairs used for genotyping and the sizes of PCR products are shown. (c) Representative detection of PCR products by agarose gel electrophoresis. Arrows indicate the expected sizes of the PCR products. Lanes: 1–5, East Asian *cagA* specimens; 6 and 7, Western *cagA* specimens; 8 and 9, unclassified *cagA* specimens; 10, a *cagA*-negative specimen; 11 and 12, DNA of East Asian *cagA*-positive *H. pylori* strains used as reference; 13 and 14, DNA of Western *cagA*-positive *H. pylori* strains used as reference; 15, negative control (water); M, molecular marker.

primers targeting the 16S rRNA gene (Yamazaki *et al.*, 2005a). The detection limit for the 16S rRNA gene was 10 copies μg^{-1}. As shown in Table 3, the 16S rRNA gene was detected in 65 of 75 (86.7 %) DNA samples obtained from stool samples that tested positive for the *H. pylori* antigen. This high detection rate indicated that the method used for DNA extraction was effective. In addition, *H. pylori* DNA was detected in fewer than 5 % of the DNA samples extracted from randomly selected stool specimens that tested negative for the *H. pylori* antigen. These findings indicate that the results of the present study revealed the prevalence of *H. pylori* infection in all the study participants.

Table 2. Prevalence of *H. pylori* infection and the ratio of the East Asian *cagA* genotype in the age groups

Age group	No. tested	No. antigen positive (%)	No. East Asian genotype positive (%)
40–45	55	20 (36.4)	8 (14.5)
46–50	42	16 (38.1)	11 (26.2)
51–55	42	16 (38.1)	7 (16.7)
56–60	41	21 (51.2)	13 (31.7)
>60	6	2 (33.3)	2 (33.3)

Table 3. Summary of *H. pylori* antigen, DNA and *cagA* detection

	No. tested	No. positive (%)
H. pylori antigen test	186	75 (40.3)
Real-time PCR 16S rRNA	75	65 (86.7)
Genotyping of *cagA* East Asian genotype	65	41 (63.1)

Detection of the East Asian *cagA* genotype

The East Asian *cagA* genotype was detected by performing nested PCR using the DNA samples extracted from the participants' stool specimens (Fig. 1b, c). First, using two pairs of gene-specific primers, we amplified a region at the 3′-end of the *cagA* gene that codes for multiple EPIYA phosphorylation motifs. Next, we confirmed the presence of the East Asian *cagA* genotype by performing two separate rounds of PCR using specific primer pairs (F2+EA-R and FA-F+R3). As shown in Fig. 1(c), the specificity of the detection method was confirmed by using *H. pylori* strains with the East Asian *cagA* genotype as a reference. The East Asian *cagA* genotype was detected in 41 of 65 (63.1 %) genomic DNA samples of *H. pylori* (Table 3). The incidence of *cagA*-positive *H. pylori* ranged from 40.0 to 100.0 % across all age groups (Table 2). There was no significant difference between the age groups, except for the group comprising individuals aged $\geqslant$60 years, because of the small sample number of participants in this age group.

The results of the present study indicated that 22.0 % of the healthy asymptomatic Japanese individuals participating in the study may be infected with the highly virulent *H. pylori* strain. A considerably higher number of healthy individuals were found to have infection with the highly virulent East Asian *cagA*-positive *H. pylori* in Japan than in Thailand [where 2.8 % (5/179) healthy asymptomatic individuals were positive for the highly virulent *H. pylori* infection; unpublished data]. The cause of the highly virulent *H. pylori* infection in a considerably high number of asymptomatic Japanese individuals is unknown. However, in a recent report, it has been suggested that (1) the geographical distribution of *H. pylori* strains harbouring a certain virulence factor genotype and (2) the incidence of cancer are responsible for the high incidence of *H. pylori* infection among asymptomatic Japanese individuals (Yamaoka *et al.*, 2008). The findings of Yamaoka *et al.* (2008) were based on genotype analysis of *H. pylori* strains that were clinically isolated from patients; however, their finding of a high incidence of gastric cancer in countries where the East Asian CagA is predominant is in agreement with the result obtained in our study.

A recent study showed that the eradication of *H. pylori* significantly suppressed the development of metachronous gastric cancer (Fukase *et al.*, 2008). The report does not directly suggest that the eradication of *H. pylori* infection in healthy asymptomatic individuals will suppress the onset of gastric cancer in the future, but it highlights the significance of *H. pylori* infection in gastric cancer development. Therefore, a silent infection with a highly virulent strain of *H. pylori*, such as one with the East Asian *cagA* genotype, in healthy individuals may be a critical public health issue in the prevention of gastric cancer.

To our knowledge, this is the first report on the prevalence of *H. pylori* infection among healthy asymptomatic Japanese individuals that describes results that were directly revealed by genetic analyses. We found a relatively high incidence of infection with the highly virulent *H. pylori* strain among asymptomatic adult Japanese individuals. Further investigations with a larger number of participants are required to determine precisely the significance of *H. pylori* with a virulence factor genotype in the development of gastric cancer.

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REFERENCES

Asaka, M., Kimura, T., Kudo, M., Takeda, H., Mitani, S., Miyazaki, T., Miki, K. & Graham, D. Y. (1992). Relationship of *Helicobacter pylori* to serum pepsinogens in an asymptomatic Japanese population. *Gastroenterology* **102**, 760–766.

Azuma, T. (2004). *Helicobacter pylori* CagA protein variation associated with gastric cancer in Asia. *J Gastroenterol* **39**, 97–103.

Cardenas, V. M., Dominguez, D. C., Puentes, F. A., Aragaki, C. C., Goodman, K. J., Graham, D. Y. & Fukuda, Y. (2008). Evaluation of a novel stool native catalase antigen test for *Helicobacter pylori* infection in asymptomatic North American children. *J Pediatr Gastroenterol Nutr* **46**, 399–402.

Chattopadhyay, S., Datta, S., Chowdhury, A., Chowdhury, S., Mukhopadhyay, A. K., Rajendran, K., Bhattacharya, S. K., Berg, D. E. & Nair, G. B. (2002). Virulence genes in *Helicobacter pylori* strains from West Bengal residents with overt *H. pylori*-associated disease and healthy volunteers. *J Clin Microbiol* **40**, 2622–2625.

Covacci, A., Censini, S., Bugnoli, M., Petracca, R., Burroni, D., Macchia, G., Massone, A., Papini, E., Xiang, Z. & other authors (1993). Molecular characterization of the 128-kDa immunodominant antigen of *Helicobacter pylori* associated with cytotoxicity and duodenal ulcer. *Proc Natl Acad Sci U S A* **90**, 5791–5795.

Devi, S. M., Ahmed, I., Khan, A. A., Rahman, S. A., Alvi, A., Sechi, L. A. & Ahmed, N. (2006). Genomes of *Helicobacter pylori* from native Peruvians suggest admixture of ancestral and modern lineages and reveal a western type *cag*-pathogenicity island. *BMC Genomics* **7**, 191.

Fujisawa, T., Kumagai, T., Akamatsu, T., Kiyosawa, K. & Matsunaga, Y. (1999). Changes in seroepidemiological pattern of *Helicobacter pylori* and hepatitis A virus over the last 20 years in Japan. *Am J Gastroenterol* **94**, 2094–2099.

Fukase, K., Kato, M., Kikuchi, S., Inoue, K., Uemura, N., Okamoto, S., Terao, S., Amagai, K., Hayashi, S. & Asaka, M. (2008). Effect of eradication of *Helicobacter pylori* on incidence of metachronous gastric carcinoma after endoscopic resection of early gastric cancer: an open-label, randomised controlled trial. *Lancet* **372**, 392–397.

Higashi, H., Tsutsumi, R., Fujita, A., Yamazaki, S., Asaka, M., Azuma, T. & Hatakeyama, M. (2002a). Biological activity of the *Helicobacter pylori* virulence factor CagA is determined by variation in the tyrosine phosphorylation sites. *Proc Natl Acad Sci U S A* **99**, 14428–14433.

Higashi, H., Tsutsumi, R., Muto, S., Sugiyama, T., Azuma, T., Asaka, M. & Hatakeyama, M. (2002b). SHP-2 tyrosine phosphatase as an intracellular target of *Helicobacter pylori* CagA protein. *Science* **295**, 683–686.

Hirai, I., Sasaki, T., Fujimoto, S., Moriyama, T., Azuma, T. & Yamamoto, Y. (2009). A method for assessment of *Helicobacter pylori*