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New Mass-screening Methods for Wilson Disease and Fabry Disease

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Introduction

Wilson disease(WD) often shows liver and brain disease in children aged around 10-12 years, but an early treatment with copper chelating agents prevents the development of these symptoms.

In Fabry disease(FD) an early introduction of the enzyme replacement therapy may prevent an irreversible kidney or cardiac disease. There have been reports on the detection of presymptomatic WD and FD among their family members of the proband by measuring blood holoceruloplasmin (HCP) and α-galactosidase A(α-gal A) or ceramide trihexoside(CTH). Since young children are usually afraid of having blood taken, mass-screening by blood examination is difficult and is unlikely to become widely used. So we conducted non-invasive mass-screening methods to detect presymptomatic WD and FD by measuring urinary HCP and α-gal A, since urinary examinations are performed every year in kindergarten and primary as well as junior high school children as part of routine health care in Japan.

This paper reports new non-invasive mass-screening methods for Wilson and Fabry diseases by measuring urinary HCP and α-gal A proteins.

Wilson disease

In some patients with Wilson disease(WD), the diagnosis is not made for several years after the onset of symptoms. The long-term prognosis of patients detected in the presymptomatic stage is much better than in patients diagnosed by symptoms. A positive cost-benefit balance from WD screening bas been claimed. Because more than 93% of patients have symptoms after the age of 6 years, mass screening for WD in young children has been suggested to improve the prognosis. Two reports describe the successful detection of preschool children with asymptomatic WD by measuring serum ceruloplasmin.

However, we have found that the quantity of holoceruloplasmin(HCP) protein in the urine of patients with WD is significantly lower than in healthy subjects, and that the urinary HCP is relatively stable if the urine samples were kept in small plastic bottles with chlorhexidine gluconate (CHG) at 4℃. Therefore, we hypothesized that early detection of WD is possible by measuring urinary HCP protein.

1. Materials and Methods

The urine and serum or blood samples, were diluted 5 and 10,000 times, respectively, with 50 mM phosphate buffered saline solution. The HCP level of 100 μL of the diluted sample was measured using an ELISA kit with an anti-human HCP monoclonal antibody(Nissho Inc. Osaka). The value was expressed as ng HCP per ml or per mg creatinine of urine and mg HCP per 100

ml of serum or blood. Serum and urinary copper were measured by atomic absorption spectrometry. Informed consent was obtained from all patients with WD and healthy controls or from the parents in the case of minors.

2. Results

The mean urinary HCP level in the control samples at room temperature with or without 5 mg of CHG in 10 ml urine decreased after 3 and 7 days storage to 80.8% and 67.1% or 70.9% and 31.9%, respectively. On the other hand, the mean HCP level did not change significantly in the samples at 4℃ with or without CHG after 3 days storage, while at 4℃ after 7 days storage it decreased to only 95.3% and 93.6% in the samples with and without CHG, respectively.

Therefore, the samples were stored at 4℃ and measurements performed the same day or the next day whenever practicable, but where this was not possible the sample was kept in a small plastic bottle with CHG at 4℃ and tested within 3 days.

Mean urinary HCP levels in young control children tended to show lower values than HCP levels in older children and adults, but there was no significant difference between the levels in male and female subjects. Therefore, the results were evaluated according to age but not sex. The HCP value in patients with WD was significantly lower than in the control subjects(Table 1).

Urinary HCP levels in 2 of 41 patients(5%) were high(42.0 and 43.5 ng/mg creatinine). It has been reported that 5% of patients have a relatively high HCP level in the serum, as well as in the urine, and these patients may be misdiagnosed as normal if patients are screened by HCP levels. The mean HCP concentrations in the blood and urine of 8 children with glomerular disease were 23.8(6.0 mg/dL and 106±8.1(708.3 ng/mg creatinine, respectively, whereas the mean HCP concentrations of 40 healthy adults were 30.3±3.6 mg/dL and 141.5±40.9 ng/mg creatinine, respectively. The values in 19 patients with WD were 4.4±3.4 mg/dL and 11.2±9.2 ng/mg creatinine, respectively, indicating that in WD, significantly lower HCP levels occur in both blood and urine than in controls, whereas children with glomerular disease show higher HCP levels in urine but not in blood than healthy adults.

With the collaboration of 122 primary schools in Tokyo in 1998 and 1999, the urinary HCP was measured in 22,639 and 26,180 children, respectively. The mean HCP levels in the screened samples were 103.3±72.2 ng/mL in 1998 and 87.5±58.1 ng/mL in 1999, but 148 children in 1998 and 275 children in 1999 showed levels below the 3rd centile, with mean values of 8.2±4.4 ng/mL in 1998 and 9.4±7.4 ng/mL in 1999. We asked these child to bring another sample for reexamination, and we obtained 138 samples in 1998 and 261 samples in 1999. In 12 of the 138 samples and in 29 of the 261 samples, the HCP levels at this second measurement were likewise below the 3rd centile; these children were invited to our outpatient clinic for further investigation. Of these, 10 children in 1998 and 26 in 1999 were investigated and one child in each year(Cases A and B, respectively) were found to have very low urinary and serum HCP levels, together with low serum copper and high basal urinary copper concentrations(Table 2). However, physical examination revealed no hepatosplenomegaly and the Kayser-Fleischer ring was not detected in either case. Hematologic examination and liver function tests were all within the normal ranges, except for low levels on the hepaplastin test(Case A, 52.0%; Case B, 61.7% [normal range, 70-130%]).

After oral loading in Case A and B with 800 mg penicillamine, urinary copper excretion in both cases was increased to 718.9 and 1696.9 ng/mg

creatinine, respectively. These results were not as high as reported in other patients with WD.

Analysis of the ATP 7B gene revealed the presence of mutations of R778L at Exon 8 and A803T at Exon 9 in Case A, and D1,267A at Exon 18 and D1,296N at Exon 18 in Case B - abnormalities already identified in Japanese patients with WD.

Therefore, these cases were diagnosed as compound heterozygotes of WD.

3. Discussion and Conclusion

These results suggest that noninvasive mass screening for WD is possible by measuring urinary HCP levels where there is no apparent kidney disease. But approximately 5% of patients with WD have a relatively high HCP level in the serum as well as in the urine, and these patients may be misdiagnosed as normal in our screening method. We wish to report, however, that 2 cases of Wilson disease were found by testing urine samples from 48,819 children. The diagnosis was confirmed by clinical laboratory tests and the detection of a mutated ATP7B gene.

Fabry disease

Early trials demonstrated the feasibility of enzyme replacement to correct the metabolic defect in Fabry disease(FD). These data suggest that clinical outcome of the patient will become favorable. In some patients, however, the diagnosis is not made for several years after onset of symptoms when irreversible kidney or cardiac damage has occurred. It may be worthwhile to make an early detection and treatment in young children with FD in order to improve the prognosis. We have found that the quantity of α-galactosidase A(α-gal A) protein as well as enzyme activity in the urine or patients with FD is significantly lower than in healthy subjects. Therefore, we hypothesized that non-invasive early detection of FD is possible by measuring urinary α-gal A protein.

1. Material and Methods

The α-gal A protein of 100 μL of non-diluted serum and 4 times diluted urine sample with phosphate buffered saline solution was measured by using an enzyme-linked immunosorbent assay (ELISA) with an antihuman α-gal A monoclonal antibody. Fluorometric assay of α-gal A activity in serum and urine samples was performed as previously described with minor modification.

The mean levels of urinary α-gal A protein and activity in the control samples did not change significantly at 4℃ after 3 days' storage, whereas after 7 days' storage, it decreased to 70-95%. Conversely, the levels of the samples at room temperature decreased after 3 days storage to 85-96%. Therefore, the samples were stored at 4℃ and measurements were performed the same day or the next day whenever practicable.

2. Results

Urinary α-gal A protein and the activity in 40 control subjects were measured and a positive correlation(γ=0.911) was demonstrated between two levels. We examined the levels of α-gal A protein in serum and urine of 9 control subjects, 8 patients with FD and 3 heterozygotes. A positive correlation(γ=0.758) between two levels was observed(Fig. 1).

Frequency of the levels of α-gal A protein in urine of 1447 adult controls and 8 patients with FD is shown in Fig 2. The mean α-gal A concentrations in the urine of 1447 control subjects and 8 patients with FD were 18.53±14.62 and 0.58 ±0.23 ng/mg creatinine respectively. There was a significant difference between the two levels. 53 out of 1447 control subjects and all 9 patients showed below the 3rd centile level of the urinary

α-gal A protein in the first test, which corresponding to 3 ng/mg creatinine. We asked these control subjects and patients, whose α-gal A levels were below the 3rd centile, to give us the second urine sample for reexamination. The α-gal A levels of controls at this measurement were all above the 3rd centile value but levels of the patients were low.

3. Discussion and Conclusion

These results suggest that noninvasive mass screening for FD is possible by measuring urinary α-gal A protein. The diagnosis is confirmed by the measurement of CHT in urine and plasma. The concentration of CTH in the plasma and urine was determined quantitatively by tandem mass spectrometry using an internal standard, C-17 CTH, which was kindly given by Dr. Kevin Mills.