ELISA Measurement for Urinary 3-Hydroxyproline-containing Peptides and its Preliminary Application to Healthy Persons and Cancer Patients

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Abstract. *Background/Aim: We reported that endogenous urinary 3-hydroxyproline (3-Hyp) is useful for cancer screening because cancer invasion involves the destruction of basement membrane. A simple and sensitive assay is desired. Patients and Methods: An ELISA method using a specific antibody against a synthetic peptide of 10 amino acids including 3-Hyp corresponding to the amino acid sequences of collagen type IV alpha chain was applied to urine samples from 180 healthy controls and 22 cancer patients. Results: The values in controls were 2.44±1.90 (SD) mg peptide/g creatinine for 52 men and 2.87±2.01 for 128 women, while the values in 22 cancer patients were very low at 0.110±0.137 ($p<0.001$). Discussion: The discrepancy in the data between our previous and present studies is based on the difference of targets measured. 3-Hyp-containing peptides in cancer patients might be destroyed by the elevated peptidase levels found in these patients. Conclusion: This ELISA assay may be useful for cancer screening.*

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3-Hydroxyproline (3-Hyp) is found in most types of collagen (I, II, III, IV, V, VIII, X, XI and XVIII) (1-6), but not in other animal proteins except the cystein proteinases of the trematode *Fasciola hepatica* (6). Type I, II, and III collagens each contain one amino acid of 3-Hyp per 1,000 amino acids (2), whereas type IV collagen contains 8 to 25 times as much 3-Hyp (2, 3, 7), although placental type IV collagen contains one amino acid per 1,000 amino acids (8). Therefore, since cancer invasion may involve the destruction of basement membrane (9-12), urinary endogenous 3-Hyp is expected to be increased in cancer patients (13, 14). 3-Hyp would be a relatively specific marker for turnover of type IV collagen (15, 16). In fact, we demonstrated that endogenous urinary 3-Hyp increased in cancer patients and its level may be a useful marker for cancer screening (13).

The original assay method reported previously (13, 14) was based on the measurement by amino acid analysis after urine sample hydrolyzed with strong hydrochloride was concentrated. A simple assay method without any procedures such as hydrolysis and concentration for cancer screening is desirable for the measurement of 3-Hyp. In the previous report (13), urinary 3-Hyp was not determined in most

healthy persons even though their urinary concentration of creatinine (CRNN) was over 60 mg/dl. We utilized a synthetic antigen of 10 amino acids corresponding to the sequence in alpha chain of type IV collagen to produce a polyclonal antibody specific for the detection of 3-hydroxyproline-containing peptides in the urine.

Here we report the development of the assay method for endogenous 3-Hyp-containing peptide in urine with preliminary results of 180 healthy controls and 22 cancer patients.

Patients and Methods

Study participants. Healthy controls: One hundred and eighty healthy controls, 52 males (age 66.6±8.1 years) and 128 females (age 60.5±11.1 years), inhabitants of Ebina City, Kanagawa Prefecture, were included. Under the co-operation of the Ebina City Medical Association and the Ebina City Government, volunteer residents who visited a cancer screening program in the community gave their informed consent to this preliminary test for cancer screening. The Ethical Committee in Tokai University School of Medicine agreed this study on the condition that a counseling service was provided for the volunteers. IO, one of the authors, delivered this service at the City Government as a counselor during 2001 to 2006. All volunteers were all free of complaints and underwent physical examinations (body mass index, blood pressure, *etc*.), conventional laboratory tests including urinalysis (protein, sugar, urobilinogen, and microscopic examination if protein was positive), peripheral blood examination (red and white blood cell counts, hemoglobin, and microscopic examination), blood chemistry (total cholesterol, triglyceride, total protein, fasting blood sugar level, aspartate aminotransferase, alanine aminotransferase, lactic dehydrogenase, gamma-glutamyl transferase, and alkaline phosphatase), chest X-ray, electrocardiogram and gastrofluoroscopy. All urine samples were collected as the first urine of the day without breakfast on condition that voluntary residents did not eat after 8 o'clock on the previous night and excreted their last urine of the previous night before 12 o'clock in order to avoid the effect of exogenous dietary urinary 3-Hyp as described previously (13). Samples were stored without preservatives in a freezer at -20˚C within 3 hours on the same day when volunteers visited the clinics of Ebina City Medical Association members.

Cancer patients: Twenty-two cancer patients (5 with gastric cancer, 14 with colon cancer, and 3 with pancreatic cancer; 10 males and 12 females; age 68.6±7.5 years) were enrolled in this study. They were all in-patients at the Department of General Surgery, Inagi Municipal Hospital in Tokyo. Informed consent was obtained from each patient and the study was approved by the Institutional Review Committee. The patients were requested to excrete the last urine of the previous night before midnight. All urine samples were collected in the early morning as the first urine of the day before breakfast at the next day of admission before any treatment such as operation, anticancer therapy, *etc*. All urine samples were stored at –20˚C until measurements were made.

Cancer stage classification: Stage classification was carried out using information on each case based on TNM classification (UICC: http://www.cancerstaging.org/index.html). In general, the stage I group comprised those patients with very early cancer with neither invasion to the surrounding tissue nor metastasis. The stage II group patients had no distinct metastasis including bone metastasis. The stage III group comprised those patients with advanced cancer, and stage IV patients had end-stage disease as described previously (13).

Synthesis of peptide containing 3-Hyp. For the development of the ELISA assay for 3-Hyp-containing peptide, Cys-(3-Hyp-4-Hyp-Gly)$_3$ and Cys-(Pro-4-Hyp-Gly)$_3$ were synthesized as an ELISA antigen, based on the analysis of type IV collagen reported in the literature (3-5, 7, 8). Cys-(Pro-4-Hyp-Gly)$_3$ was used to remove the antibodies that recognize only 4-Hyp but not 3-Hyp from the antibodies raised against Cys-(3-Hyp-4-Hyp-Gly)$_3$ in order to detect only 3-Hyp-containing peptides in the urine. These antigens were prepared by Peptide Institution Co. (Osaka, Japan).

Preparation of polyclonal antibodies against Cys-(3-Hyp-4-Hyp-Gly)$_3$. Five white rabbits were injected with an aliquot of emulsion containing 100 μg to 200 μg of Cys-(3-Hyp-4-Hyp-Gly)3 mixed with Freund complete adjuvant, 4 times every 2 weeks, subcutaneously. *N*-Terminal of Cys was conjugated with keyhole limited hemosianine (KLH) to increase the production of antibody. Several days after the last injection, blood was taken from each rabbit (No. 1-5). Each blood sample was left at 4˚C overnight, followed by centrifugation (3000 rpm, 20 minutes). The obtained sera (No. 1-5) were purified with protein G affinity chromatography. The polyclonal antibodies (No. 1-5) were prepared by the Laboratory for Bio-Immunological Sciences (Gunma, Japan).

Determination of antibody titer against 3-Hyp-containing peptides. Antibodies (No. 1-5) obtained from 5 rabbits were tested to determine the antibody titer against the antigen by inhibition ELISA as follows. The antigen (10 μg/ml, 100 μl/well) was coated on wells of 96 microplates at room temperature for 2 hours. Wells were washed with 10 mM phosphate-buffered saline (PBS). Bovine serum albumin (BSA) at 1% in PBS was used as a blocking agent. An aliquot (100 μl) of each antibody from No. 1 to No. 5 (1000 to 128000 dilution in 10 mM PBS containing 0.1% Tween 20) was placed in each well and subjected to the reaction with the coated antigen at room temperature for 2 hours. After removing the reaction mixture and washing with 10 mM PBS containing 0.1% Tween 20, an appropriate concentration of rabbit IgG-horseradish peroxidase (HRPO, ICN Biomedicals, Inc; 1/5000 vol. % diluted with 10 mM PBS) was added to each well at room temperature for 2 hours. After removing the reaction mixture and washing, the reaction mixture containing peroxide (5 mM/well)/*o*-phenylene diamine (OPD; 100 μl/well) was added to each well and allowed to react at room temperature for 20 minutes. The reaction was stopped by the addition of 100 μl of 1 M H_2SO_4 solution at room temperature for 20 minutes. The color developed was then read at 492 nm with a photometer.

Determination of antibody titer against 4-Hyp-containing antigen. The antigen of peptides containing 4-Hyp, Cys-(Pro-4-Hyp-Gly)$_3$ (10 μg/ml, 100 μl/well) was coated on wells of microplates at room temperature for 2 hours. Wells were washed with 10 mM PBS in order to make the antigen insoluble. BSA at 1% in PBS was used as a blocking agent. An aliquot (100 μl) of antibody solution from No. 2 to No. 5 at a concentration range obtained by dilution of 1000 to 128000 (in 10 mM PBS containing 0.1%

Tween 20) was placed in each well and allowed to react with insoluble coated antigen at room temperature for 2 hours. Subsequent procedures were as described above.

Establishment of 3-Hyp-containing peptide standard curve. Wells of microplates were coated with 3-Hyp- containing peptide antigen (10 μg/ml, 100 μl/well), washed with 10 mM PBS in order to make the antigen insoluble, and BSA solution as a blocking agent was added. In wells of another microplate, an aliquot (50 μl/well) of variable concentration of antigen (3-Hyp-containing peptide) was reacted with 50 μl/well of antibody No. 3 at a constant concentration, 1/1000 diluted with 10 mM PBS/0.1% Tween 20 at 4˚C for 24 hours. The reaction mixture of the latter microplate wells was transferred to each well of the first microplate and the microplates were incubated at room temperature for a further 2 hours. The reaction mixture was removed and the microplates washed with 10 mM PBS containing 0.1% Tween 20, then an optimal concentration of rabbit IgG-HRPO was added. The color reaction mixture containing peroxide/OPD was added and after 20 minutes the reaction stopped by the addition of 100 μl of 1 M H_2SO_4 solution. The color was read at 492 nm as described above. For the measurements of lower concentration (less than 0.2 μg/ml), the color reaction mixture was replaced with 3,3',5,5'-tetramethylbenzidine (TMBZ)/H_2O_2.

ELISA procedures for measuring 3-Hyp-containing peptide in urine. Urine samples were kept below –20˚C in a freezer. Just before measuring, the samples were defrosted and centrifuged at 2000 rpm for 15 minutes. A mixture of urine (50 μl) and specific antibody (50 μl of diluted 1/1000 solution) was placed in the antigen-coated wells of microplates at 4˚C for 24 hours. The reaction mixture was transferred to each well and subjected to the reaction with insoluble coated antigen at room temperature for 2 hours. The color development procedure was the same as described above. The concentration of 3-Hyp-containing peptides in the urine was determined from the absorbance at 492 nm and with the standard curve. When the concentration of 3-Hyp-containing peptide in the urine sample was below the detection limit in sample urine, the value was arbitrarily recorded as the minimum measurable level (0.01 μg/ml) of the peptide using the ELISA method. The level of 3-Hyp-containing peptide in the urine was expressed relative to the concentration of CRNN in the urine.

Original assay method for measuring 3-Hyp in urine. As we reported previously (13, 14), 2 ml of centrifuged supernatant urine and the same volume of concentrated HCl were placed in tight-capped tubes in a block-type hydrolyzer at 150˚C for 1 hour. After hydrolysis, each sample was lyophilized by Speed-Vac Concentrator, and the precipitate was dissolved in 0.02 N HCl and assayed for 3-Hyp and 4-Hyp with an amino acid autoanalyzer (Hitachi 835-50; Hitachi Ltd., Tokyo, Japan) described elsewhere (13). A standard sample of 3-Hyp was prepared from the seeds of *Delonex regia* and purified according to the method of Szymanovicz *et al*. (17). The levels of 3-Hyp in urine were corrected by the urine concentration of CRNN. When the levels of 3-Hyp in urine were below the detectable limit, the level was arbitrarily recorded as 60% (0.060 mg/g creatinine) of the minimum measurable level for 3-Hyp with an amino acid autoanalyzer (0.1 nmol/l) for statistical purposes as described previously (13).

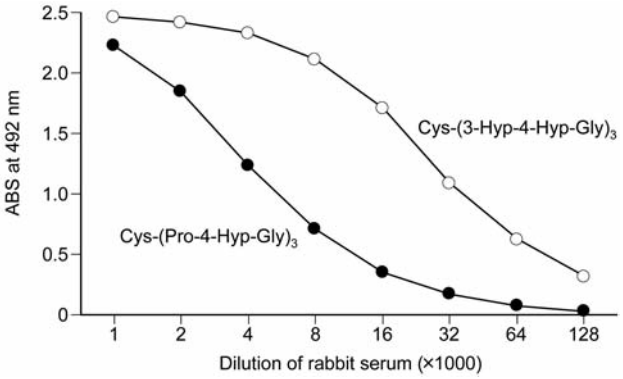

Figure 1. *The antibody titers against the antigens Cys-(3-Hyp-4-Hyp-Gly)$_3$ and Cys-(Pro-4-Hyp-Gly)$_3$ for No. 5 serum used in the present study.*

Statistical analysis. Data were analyzed by EXCEL (Windows Office 2007), and expressed as mean±SD. The F-test and *t*-test were delivered in order to compare differences between groups. A *p*-value of less than 0.05 was considered statistically significant.

Results

Preparation of antibodies against 3-Hyp-containing peptide. Antibodies (No. 1 to No. 5) obtained from 5 rabbits were tested to determine the antibody titer against the antigen after purification. Antibody No. 2 showed the highest titer, and No. 1 the lowest titer; the other three (No. 3, 4 and 5) showed an intermediate titer of almost the same value. Antibody titer against 4-Hyp-containing antigen was determined for the same antibodies (No. 2 to 5). Four-fold higher titer of antibody against 3-Hyp-containing peptide compared with antibody titer against 4-Hyp-containing peptide was found for the antibodies of No. 2, 3, and 5 in the region of ABS>1.0 at 492 nm. The antibody titers against Cys-(3-Hyp-4-Hyp-Gly)3 and Cys-(Pro-4-Hyp-Gly)3 were measured for No. 2 to 5, and the difference was sufficient for the assay for 3-Hyp-containing peptide. Figure 1 shows the curve for antibody titers of No. 5 serum used in the present study.

3-Hyp-containing peptide standard curve. The standard curve showed that 3-Hyp-containing peptide was detectable in the concentration range of 0.1 to 80 μg/ml as shown in Figure 2. The results suggested that ELISA assay could be applied to urine without the need for any additional procedures such as concentration. The color reaction mixture using TMBZ/H_2O_2 improved the sensitivity to a lower concentration of 0.01 μg/ml.

Effect of urinary component interference on the ELISA assay. Urine has many kinds of chemicals which may interfere with the ELISA assay. To examine the effect of urine components on the ELISA assay, two urine samples, one (urine A) with a

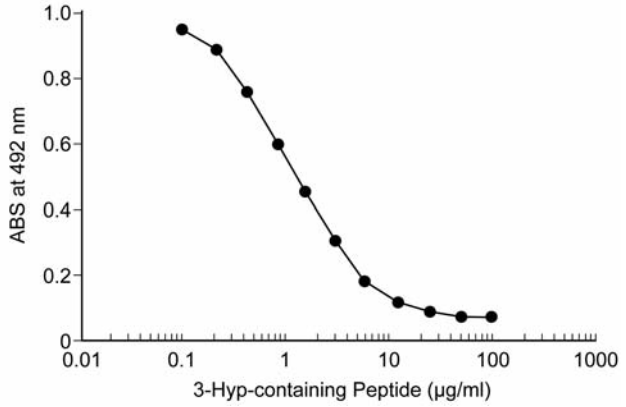

Figure 2. *Standard curve for 3-Hyp-containing peptide using the ELISA assay. 3-Hyp-containing peptide was measured in the concentration range of 0.2 to 80 µg/ml. Repeated improved assay for cancer patients enabled the lower concentration of 0.01 µg/ml to be measured.*

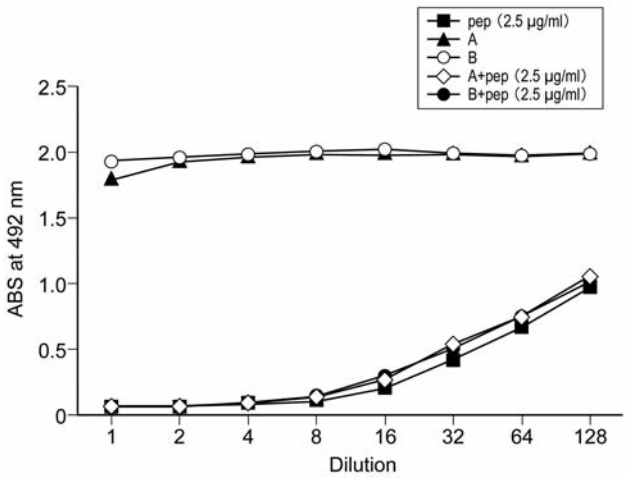

Figure 3. *Effect of urine (sample A and sample B) on the ELISA assay. Two urine samples (A, B) were spiked with a known quantity of 3-Hyp-containing peptide (2.5 µg/ml peptide per assay tube) and analyzed in parallel with those of both urine samples alone. There was no effect of urine chemicals on ELISA assay for 3-Hyp-containing peptide.*

low concentration of 3-Hyp as determined by the original assay and another (urine B) with a higher concentration, were mixed with a known volume of 3-Hyp-containing peptide, and were assayed by the ELISA method at 492 nm. The results for both samples with the known volume of 3-Hyp-containing peptide (2.5 µg/ml peptide/ assay tube) were parallel to those of each urine alone. A minimal effect of urine components was found in the ELISA assay for 3-Hyp-containing peptide, as shown in Figure 3.

3-Hyp-containing peptide concentration in 180 healthy controls. The concentration of 3-Hyp-containing peptide were determined in all samples by ELISA at 492 nm and found to be in the range from 0.47 to 3.96 (µg/ml) using the standard curve shown in Figure 2. Although 14 samples in 52 healthy males (27%) and 32 samples in 128 healthy females (25%) had less than 60 mg/dl of CRNN, absorbance in the ELISA method at a range from 0.47 to 1.57 (µg/ml) was clearly detectable as shown in Table I. The levels of 3-Hyp-containing peptide were 2.44±1.90 mg/g CRNN (range 0.65-10.52) in 52 healthy males, and 2.87±2.01 mg/g CRNN (range 0.92-17.31) in 128 healthy females (Table II). The highest values of 10.52 and 17.31 were calculated as follows: 0.873 µg/ml peptide divided by 8.3 mg/dl CRNN, and 0.496 µg/ml peptide by 8.1 mg/dl CRNN (see Table I). There was no statistical difference between the values for the 52 healthy males and 128 healthy females (Table II). The levels of the peptide healthy males aged 50-59, 60-69 and 70-81 years were not statistically different, while in healthy females the levels in the 50 to 59-years-old group were statistically higher than those in the 30 to 39-years-old group (p=0.0008) and the 70 to 89-years-old group (p=0.009). The values for females aged 60-69 years were also statistically higher than those aged 70-89 years (p=0.0269), as shown in Table II.

3-Hyp-containing peptide concentration in 22 cancer patients. The concentrations of 3-Hyp-containing peptides were below the detection limit in 7 out of 22 cancer patients. Four of the seven samples had less than 60 mg/dl CRNN (Table III). In urine sample with a low CRNN concentration, however, the peptide was detected in all healthy controls by ELISA. There were marked differences between the 180 healthy controls and 22 cancer patients as shown in Tables I-IV. The concentrations of 3-Hyp-containing peptide were detected in the other 15 cancer patients in the range from 0.005 to 0.28 (µg/ml) by the ELISA method. The values for the patients were very low in comparison with the value of 0.47 µg/ml, the lowest concentration of peptide in 180 healthy persons. The levels of 3-Hyp-containing peptide were 0.110±0.137 mg/g CRNN in 22 cancer patients, 0.127±0.152 mg/g CRNN in 14 colon cancer patients, 0.117±0.107 mg/g CRNN in 5 gastric cancer patients, and 0.016±0.004 mg/g CRNN in 3 pancreatic cancer patients, as shown in Table IV. Nine cases with stage I and stage II colon cancer had 3-Hyp levels of 0.131±0.170 mg/g CRNN, while the levels for 5 patients with stage III and IV colon cancer were lower (0.121±0.113) (Table IV).

Urinary endogenous 3-Hyp by original assay in 180 healthy controls and 22 cancer patients. The concentration of 3-Hyp measured with an amino acid autoanalyzer were below the detection limit in 41 samples of 48 healthy males (85.4%) (in 4 samples, 2 were not assayed for the lack of sample volume, and 2 produced an interfering amino acid peak), and 3-Hyp was not detected in 96 of 107 healthy females (89.7%) (in 21 samples, 7 were not assayed for the lack of sample volume, and 14 produced an interfering amino acid peak). On the

Table I. *The levels of 3-Hyp measured by an amino acid autoanalyzer and those of 3-Hyp-containing peptide by ELISA in 20 out of 180 healthy persons.*

No.	Gender	Age (year)	3-Hyp (μg/ml)	CRNN (mg/ml)	Relative level (mg/g CRNN)+	Pep (μg/ml)	Relative level (mg/g CRNN)+
22	m	65	ND	0.08	0.06	0.87	10.52
23	m	65	ND	0.81	0.06	1.64	2.02
24	m	66	not suffic	0.22		1.03	4.61
25	m	66	ND	1.28	0.06	1.96	1.53
26	m	67	ND	0.29	0.06	1.18	4.01
27	m	67	ND	0.50	0.06	0.70	1.42
28	m	68	ND	1.45	0.06	1.73	1.19
29	m	68	ND	0.69	0.06	1.23	1.78
30	m	69	0.494	2.60	0.19	1.68	0.65
31	m	69	ND	0.52	0.06	1.00	1.91
147	f	67	ND	0.61	0.06	1.41	2.32
148	f	67	ND	0.67	0.06	1.29	1.92
149	f	67	ND	0.26	0.06	1.20	4.60
150	f	67	ND	0.44	0.06	1.37	3.12
151	f	67	ND	0.23	0.06	0.71	3.13
152	f	68	ND	0.49	0.06	1.35	2.76
153	f	68	ND	1.21	0.06	2.46	2.04
154	f	68	ND	0.14	0.06	1.15	7.99
155	f	69	0.738	1.05	0.70	1.73	1.65
156	f	69	ND	0.12	0.06	1.27	10.4

m: Male; f: female; 3-Hyp: 3-Hyp as measured by an amino acid autoanalyzer; +: the level of endogenous urinary 3-Hyp relative to urinary creatinine (CRNN); Pep: the concentration of 3-Hyp-containing peptide by ELISA; ND: not detected, no 3-Hyp peak; not suffic: urine specimen too small to assay.

Table II. *The urinary level of endogenous 3-Hyp-containing peptide measured by ELISA in 180 healthy persons.*

	3-Hyp (amino acid autoanalyzer) mg/g CRNN	3-Hyp-containing-peptide (ELISA) mg/g CRNN
180 Healthy persons	0.123±0.196 (n=155+)	2.746±1.991 (n=180)
52 Healthy males	0.115±0.144 (n=48)	2.44±1.90 (n=52)
40-49 years	0.689, 0.06 (n=2)	2.299, 1.599 (n=2)
50-59 years	0.135±0.151 (n=5)	3.20±1.73 (n=5)
60-69 years	0.065±0.026 (n=24)	2.67±2.46 (n=26)
70-81 years	0.149±0.163 (n=17)	1.99±0.59 (n=19)
128 Healthy females	0.125±0.214 (n=108)	2.87±2.01 (n=128)
30-39 years	0.233±0.386 (n=6)	*1 1.73±0.44 (n=6)
40-49 years	0.060 (n=8)	2.55±1.07 (n=11)
50-59 years	0.136±0.226 (n=31)	*2 3.48±2.74 (n=39)
60-69 years	0.099±0.135 (n=45)	*3 2.90±1.86 (n=49)
70-89 years	0.168±0.287 (n=18)	*4 2.21±0.65 (n=23)

*Statistical difference at p=0.0008 between *1 and *2, p=0.009 between *2 and *4, and p=0.027 between *3 and *4. +A total of 9 samples for amino acid autoanalyzer were too small; 16 samples had a 3-Hyp peak covered by that of another amino acid, and thus 3-Hyp was not assessed by amino acid autoanalyzer.

other hand, in 22 cancer patients 3-Hyp in 13 samples was below the detection limit. This difference between healthy controls and cancer patients for the occurrence of unmeasurable 3-Hyp levels was the same as described previously (13). For the statistical evaluation, the levels in 41 males, 96 females, and 13 cancer patients were replaced with 0.06 mg/g CRNN as described in the Materials and Methods. As shown in Tables I-IV, the endogenous urinary levels of 3-Hyp measured with an amino acid autoanalyzer were significantly lower in 180 healthy persons than those in 22 cancer patients (p=0.037) as described previously (13).

Discussion

Cancer invasion and metastasis involve the destruction of basement membrane (9-12). Matrix metalloproteinases (MMPs), especially MMP-1, MMP-2, MMP-3, MMP-9 and Membrane-type 2 matrix metalloproteinase (MT2-MMP) participate in the destruction of basement membrane composed of type IV collagen, glycoproteins and proteoglycan (9-12). The Authors have focused on the role of MMP-1 in the invasion and metastasis of gastric cancer (18-21), colon cancer (19), hepatocellular carcinoma (22, 23) and pancreatic cancer (24). We assumed that tissue destruction by MMPs in cancer tissue causes an increase in type IV collagen destruction followed by the increase in excretion of 3-Hyp-containing peptide in urine (13).

We developed an assay for endogenous urinary 3-Hyp using an amino acid autoanalyzer, but detectably high 3-Hyp peaks in amino acid autoanalysis were limited to 118 samples among 211 healthy persons (56%), 49 out of 99 patients with nonmalignant diseases (50%), and 86 out of 96 patients with cancer (90%) in our previous paper (13). The levels of 3-Hyp in cancer patients were significantly higher than those in healthy persons (p<0.001), and those in patients with nonmalignant diseases (p<0.05) (13). The sensitivity of 3-Hyp was 44% although the detection of stage I (early) cancer was low. The specificity of this original assay for 3-Hyp as a cancer screening test was 96% (13).

Since reporting the original assay method for 3-Hyp (13), we have developed a new sensitive and simple ELISA assay, as a cancer screening test in the present study. Although the antigen is not derived directly from type IV collagen, the sequence of 10 amino acids, Cys-(3-Hyp-4-Hyp-Gly)$_3$, has been reported in the literature as relating to type IV collagen structure (2, 3, 6-8). The serum (No. 2, 3 and 5) in the present study showed antibody for Cys-(3-Hyp-4-Hyp-Gly)$_3$, but not for Cys-(Pro-4-Hyp-Gly)$_3$ although the detailed structures of inhibitory peptides in the urine for Cys-(3-Hyp-4-Hyp-Gly)$_3$ had not yet been clarified in the present study.

Table III. *The levels of 3-Hyp measured by an amino acid autoanalyzer and those of 3-Hyp-containing peptide by ELISA in 22 cancer patients.*

Gender	Age (year)	Organ	Stage	T	N	M	3-Hyp (μg/ml)	CRNN (mg/ml)	Relative level (mg/gCRNN)	Pep (μg/ml)	Relative level (mg/gCRNN)
m	65	Colon	I	1	0	0	1.456	1.230	1.200	0.06	0.049
m	65	Colon	I	1	0	0	ND	0.661	0.060	ND*	0.015
f	64	Colon	I	1	0	0	ND	0.169	0.060	ND	0.592
m	75	Colon	II	2	0	0	ND	0.810	0.060	0.01	0.074
m	74	Colon	II	2	0	0	0.367	1.412	0.260	0.03	0.021
f	60	Colon	II	2	0	0	1.580	3.824	0.413	0.28	0.073
f	63	Colon	II	2	0	0	2.770	2.127	1.302	0.12	0.056
f	67	Colon	II	2	0	0	ND	0.526	0.060	ND	0.190
m	76	Colon	II	2	0	0	ND	0.947	0.060	ND	0.106
m	59	Colon	III	3	1	0	1.576	1.506	1.046	0.12	0.080
f	67	Colon	III	3	3	0	1.038	3.248	0.320	0.14	0.043
f	75	Colon	III	3	2	0	ND	0.292	0.060	ND	0.342
m	63	Colon	III	3	2	0	ND	2.263	0.060	0.08	0.035
m	58	Colon	IV	4	2	1	ND	0.931	0.060	0.10	0.107
m	67	Stomach	III	3	1	0	ND	1.028	0.060	0.06	0.058
f	87	Stomach	IV	4	3	1	ND	0.319	0.060	ND	0.313
f	67	Stomach	IV	4	3	1	1.020	1.411	0.723	0.08	0.057
f	58	Stomach	IV	4	2	1	ND	0.690	0.060	ND	0.145
f	78	Stomach	IV	4	3	1	0.758	0.950	0.798	0.01	0.011
f	77	Pancreas	IV	4	3	1	covered	0.280		0.005	0.018
f	76	Pancreas	IV	4	3	1	ND	0.460	0.13	0.005	0.011
m	69	Pancreas	IV	4	3	1	ND	1.042	0.06	0.02	0.019

m: Male; f: female; 3-Hyp: 3-Hyp as measured by an amino acid autoanalyzer; Relative level: 3-Hyp concentration relative to creatinine (CRNN); ND: not detected, no 3-Hyp peak; covered: 3-Hyp peak was covered by another amino acid peak; Pep: the concentration of 3-Hyp-containing peptide by ELISA; ND*: the concentration of 3-Hyp-containing peptide was not determinable by ELISA. As the minimum estimation of 0.10 μg/ml, NDs were replaced by 0.010 for statistical analysis; TNM stage was based on the International Classification of Cancer Staging.

The present results show the very low levels of 3-Hyp-containing peptide relative to CRNN in 22 cancer patients compared with those in 180 healthy controls. The levels in patients with stage I or II colon cancer were significantly lower than those in 180 healthy controls, and those in stage III and IV of colon cancer were the lowest. The discrepancy in the data between our previous study (13) and the present study is based on the difference of assay targets. In our previous study, we measured the level of 3-Hyp amino acid hydrolyzed from urinary peptides (13), but have we assayed the level of high molecular weight 3-Hyp-containing peptides.

3-Hyp-containing peptide as an antigen in the present study has 10 amino acids and its molecular weight is 1128, 7.8 times higher than 3-Hyp alone. In 180 healthy persons, the ratio of 3-Hyp-containing peptide to 3-Hyp by weight observed is 22.3 (2.746/0.123, mean values in Table II). This ratio is higher than the theoretical ratio (7.8). This difference between ratios is assumed to be due to the following two possibilities; one is that the antibody used in the present study may react to the 4-Hyp-containing peptides that might have been present at such high amounts and might have inhibitory activity in the detection system used in the present study for 3-Hyp-containing peptides. Another possibility is that some 3-Hyp-containing peptides in urine may have a higher inhibitory activity than the original antigen. As a third possibility, the determination by amino acid analysis including hydrolysis and concentration of the urine sample may give rise to a lower estimation, since 3-Hyp degrades much faster during the hydrolysis (6).

Table IV. *The urinary levels of endogenous 3-Hyp-containing peptide measured by ELISA in 22 cancer patients.*

	3-Hyp (amino acid autoanalyzer) mg/g CRNN	3-Hyp-containing peptide (ELISA) mg/g CRNN
All samples	0.329±0.409 (n=21*)	0.110±0.137 (n=22)
Colon cancer	0.359±0.447 (n=14)	0.127±0.152 (n=14)
Stage I & II	0.386±0.477 (n=9)	0.131±0.170 (n=9)
Stage III & IV	0.309±0.382 (n=5)	0.121±0.113 (n=5)
Gastric cancer	0.340±0.344 (n=5)	0.117±0.107 (n=5)
Pancreatic cancer	0.094±0.058 (n=2)	0.016±0.004 (n=3)

*n 1 sample, the peak of 3-Hyp was covered by another amino acid peak, and thus was not assessed by the amino acid autoanalyzer. This was a case of pancreatic cancer with jaundice.

On the other hand, in cancer patients, the ratio of 3-Hyp-containing peptide/3-Hyp by weight is 0.33 (0.110/0.329, mean values in Table IV). This ratio is much lower than the theoretical ratio. One possibility is that the destruction of type IV collagen may be increased and, temporally, 3-Hyp-containing peptide increases in the cancer tissue. This high molecular weight peptide may be destroyed by proteolytic enzymes, such as MMPs and/or peptidases such as cathepsin B and D, followed by increased endogenous urinary excretion of 3-Hyp because MMPs and the other enzymes mentioned above are frequently found at abnormally high levels in tumors and have been proposed as agents in the metastatic cascade (9-12, 18-28). Another possibility is that the structure of the basement membrane in cancer tissue is different from that in the normal tissue, and the 3-hydroxylation of type IV collagen in the cancer tissue could be different, thus the metabolic stability of basement membrane collagens is less stable. Clarifying why the patients with colon cancer stage I and II showed significantly low peptide levels in comparison with healthy controls in the present study will give us a further clue to the relationship between early stage of cancer and basement membrane collagen metabolism.

Application of this ELISA assay extending to diseases other than cancer, including liver fibrosis or chronic kidney diseases, may elucidate the relation of basement membrane metabolism with the diseases. It is most important to determine whether the ELISA assay is useful for cancer screening, especially for the detection of early cancer, since the method is simple and very convenient as the samples are of urine. We believe that the antigen detected in the present study may open a new door in oncology.

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