DNA Hypermethylation and Inflammatory Markers in Incident Japanese Dialysis Patients

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Key Words
Chronic kidney disease · DNA methylation · Ferritin · Infection · Inflammation · Procalcitonin

Abstract
Background/Aims: Inflammation is an established mortality risk factor in chronic kidney disease (CKD) patients. Although a previous report showed that uremic Caucasian patients with inflammation had signs of global DNA hypermethylation, it is still unknown whether DNA hypermethylation is linked to inflammatory markers including a marker of bacterial infections in Japanese CKD patients. Methods: In 44 consecutive incident dialysis patients (26 males, mean age 59 ± 12 years) without clinical signs of infection, global DNA methylation was evaluated in peripheral blood DNA using the \textit{Hpall/MspI} ratio by the luminometric methylation assay method. A lower ratio of \textit{Hpall/MspI} indicates global DNA hypermethylation. Procalcitonin (PCT), a marker of inflammation due to bacterial infections, was measured using an immunochromatographic assay. Results: The patients were divided into hyper- and hypomethylation groups based on the median value of the \textit{Hpall/MspI} ratio 0.31 (range 0.29–0.37). Whereas patients in the hypermethylation group had higher ferritin levels [133.0 (51.5–247.3) vs. 59.5 (40.0–119.0) ng/ml; \textit{p} = 0.046], there were no significant differences in age, gender, diabetes, smoking, anemia...
or serum albumin levels. However, the \( HpaII/MspI \) ratio showed significant negative correlations with PCT \((p = -0.32, p = 0.035)\) and ferritin \((p = -0.33, p = 0.027)\) in Spearman’s rank test. In a multiple linear regression analysis, PCT and ferritin were associated with a lower \( HpaII/MspI \) ratio \((R^2 = 0.24, p = 0.013)\). \textbf{Conclusion:} In this study, global DNA hypermethylation was associated with ferritin and, most likely, PCT, suggesting that inflammation induced by subclinical bacterial infection promoted DNA methylation.

\section*{Conclusion}

Infectious complications contribute significantly to the increased hospitalization rate in CKD patients who progress to end-stage renal disease and to the high mortality rate among dialysis patients [7, 8]. Various factors including immune dysfunction, protein-energy wasting and comorbid conditions, such as diabetes, dental illness, vascular access devices and immunosuppression drugs, lead to an increased risk of infections in this patient group [9]. The risk of cardiovascular events increase after hospitalization related to infection [10], and cardiac complications worsen the outcomes of pneumonia in CKD patients [11]. Thus, there may be links between infection, inflammation and an increased risk of cardiovascular morbidity and mortality [12]. Indeed, it has been reported that during the 30 days following an infection-related hospitalization, the risk of cardiovascular events increases by 25% in dialysis patients [10].

It is established that inflammatory biomarkers, such as C-reactive protein (CRP), are strong predictors of poor outcome in CKD patients [13]. Ferritin levels have also shown to reflect the inflammatory status in dialysis patients [14]. Procalcitonin (PCT), a precursor of calcitonin and a polypeptide of 116 amino acids (with a molecular weight of 13 kDa), is a biomarker of inflammation induced by bacterial infection [15]. Serum PCT (sPCT) has been reported to increase during bacterial infections in CKD patients [16]. Practically, sources of persistent low-grade inflammation in CKD patients have often been vague. Central catheters [17], periodontal disease [18] and bacterial translocation from the gastrointestinal tract [19] are often verified or suspected as causes of chronic inflammation, but it is likely that many unrecognized cases of subclinical infections with opportunistic pathogens also contribute [20]. The aim of this study is to clarify if inflammation evaluated by CRP and ferritin has an impact on the DNA methylation status and if subclinical bacterial infections detected by PCT levels are involved with this mechanism in Japanese incident dialysis patients.
Subjects and Methods

Subjects
We enrolled 44 Japanese CKD stage 5 patients (26 males and 18 females, mean age 59 ± 12 years) at the initiation of maintenance hemodialysis (HD) or peritoneal dialysis (PD) from June 2007 to August 2009 at Masuko Memorial Hospital and Meiyo Clinic in Aichi prefecture, Japan. This study is an observational study approved by the Ethics Committee of Nagoya University Graduate School of Medicine; informed consent to participate in this study was obtained from all patients. Exclusion criteria were age older than 75 years, signs of acute infectious complications, severe liver dysfunction and unwillingness to participate.

The primary causes of renal disease were glomerulonephritis (n = 10), nephrosclerosis (n = 4), diabetic nephropathy (n = 25) due to diabetes mellitus type 1 (n = 3) and type 2 (n = 22), polycystic kidney disease (n = 1) and other (n = 2) or unknown causes (n = 2). Among 39 patients starting HD, blood access was in most cases obtained by an arteriovenous fistula (n = 36); 3 patients had a graft, and only 1 patient received a double-lumen catheter into his jugular vein but showed no signs of infection. The remaining 5 patients started on PD, and all received a peritoneal catheter in advance when initiation of PD was decided. As mentioned above, patients with apparent current infection were not included in the study; however, 1 patient had hepatitis B and 5 patients had hepatitis C.

Blood Sampling and Laboratory Analysis
Blood samples were collected from all subjects before the start of maintenance dialysis therapy. Hemoglobin, leukocyte counts, platelet counts, serum albumin, total cholesterol, high-density lipid cholesterol, ferritin, creatinine, CRP and intact parathyroid hormone were measured by routine procedures at the clinical laboratory in each facility. sPCT levels were measured using an immunochromatographic assay (BRAHMS Corp, Hennigsdorf, Germany) using the samples kept frozen in −30°C. Estimated glomerular filtration rate (eGFR) was calculated from creatinine values according to the result of a Japanese study: eGFR (ml/min/1.73 m²) = 194 × SCr⁻¹.⁰⁹⁴ × Age⁻⁰.²⁸⁷ × 0.739 (if female) [21]. The Subjective Global Assessment (SGA) was used to evaluate the nutritional status [22]. In brief, each patient was given a score from medical history focused on weight loss, gastrointestinal symptoms and functional capacity and from physical examination focused on loss of subcutaneous fat and muscle, and presence of edema. We classified the patients into three groups based on their SGA score: A = well nourished, B = mild/moderately malnourished and C = severely malnourished.

Measurements of DNA Methylation by LUMA
From a 5-ml EDTA sample of peripheral blood, DNA was extracted using QIAamp® DNA kit. Restriction enzymes (HpaII, MspI and EcoRI) were purchased from New England Biolabs (Beverly, Mass., USA). PSQ™ 96 SNP reagents for pyrosequencing were purchased from Biotage AB (Uppsala, Sweden). DNA quantification was performed using the RediPlate™ 96 PicoGreen® kit from Molecular Probes (Eugene, Oreg., USA). LUMA was run as described elsewhere in detail [23]. Briefly, genomic DNA (200–500 ng) was cleaved with HpaII + EcoRI or MspI + EcoRI in two separate reactions and was run in a 96-well format. Each reaction was performed in duplicates. The digestion reactions were run in a PSQ96™ MA system (Biotage AB). Peak heights were calculated using the PSQ96™ MA software. The HpaII/EcoRI and MspI/EcoRI ratios were calculated as dCTP/(dATP + dTTP) for the respective reactions. The HpaII/MspI ratio was defined as (HpaII/EcoRI)/(MspI/EcoRI).
Statistical Analysis

Data are presented as mean ± SD and/or median and interquartile range (25th–75th percentiles). A p value <0.05 was considered statistically significant. For comparisons between groups, the Wilcoxon rank sum test was used. Nominal variables were tested using the χ² test. Spearman’s rank correlation analysis was used to determine association with HpaII/MspI ratio and selected laboratory biomarkers. Multivariate linear regression analysis was used to assess independent predictors of the HpaII/MspI ratio. All statistical analyses were performed using statistical software JMP version 8.0.1 (SAS Campus Drive, Cary, N.C., USA).

Results

Characteristics and Laboratory Biomarkers

The clinical characteristics are reported in table 1. The patients comprised 26 males (59%) with an average age of 59 ± 12 years (interquartile range 57–67). No patient was on steroids or any other immunosuppressive drugs. The median HpaII/MspI ratio was 0.31 (0.29–0.37). The patients were divided into hyper- and hypomethylation groups based on their median HpaII/MspI ratio. A lower ratio of HpaII/MspI indicates global DNA hypermethylation. There was no significant difference in age, gender, diabetes, smoking habit, nutritional status or medications between the two methylation groups (table 1).
**Table 2. Laboratory biomarkers**

| Laboratory biomarkers                  | Total                  | Global DNA methylation status | p value |
|----------------------------------------|------------------------|------------------------------|---------|
|                                        |                        | HpaII/MspI >median          | HpaII/MspI <median |
| Hemoglobin, g/dl                       | 8.70 ± 1.3             | 8.86 ± 1.4                   | 8.56 ± 1.2 | 0.66   |
| White blood cells, × 10³/mm³           | 5.70 ± 1.7             | 5.40 ± 1.5                   | 6.00 ± 1.9 | 0.39   |
| Thrombocytes, × 10³/mm³                | 20.4 ± 5.8             | 20.4 ± 6.2                   | 20.5 ± 5.5 | 0.72   |
| Albumin, g/dl                          | 3.41 ± 0.6             | 3.40 ± 0.7                   | 3.42 ± 0.5 | 0.82   |
| Total cholesterol, mg/dl               | 172.3 ± 38.8           | 178.4 ± 33.6                 | 166.1 ± 43.3 | 0.10   |
| HDL cholesterol, mg/dl                 | 43.5 ± 12.4            | 44.6 ± 8.8                   | 42.5 ± 15.3 | 0.16   |
| UA, mg/dl                              | 8.43 ± 2.2             | 8.57 ± 2.7                   | 8.29 ± 1.6 | 0.75   |
| eGFR, ml/min                           | 4.86 ± 1.8             | 4.89 ± 1.5                   | 4.83 ± 2.1 | 0.51   |
| Intact PTH, pg/ml                      | 419 ± 248              | 415 ± 251                    | 405 ± 252 | 0.97   |
| Ferritin, ng/ml                        | 147.3 ± 178.8          | 92.9 ± 83.3                  | 201.6 ± 228.8 | 0.046* |
| Median (range)                         | 81.5 (43.3–189.5)      | 59.5 (40.0–119.0)            | 133.0 (51.5–247.3) |
| CRP, mg/dl                             | 0.349 ± 0.718          | 0.211 ± 0.301                | 0.487 ± 0.962 | 0.83   |
| Median (range)                         | 0.06 (0.02–0.33)       | 0.078 (0.028–0.311)          | 0.060 (0.023–0.635) |
| sPCT, ng/ml                            | 0.134 ± 0.166          | 0.107 ± 0.09                 | 0.161 ± 0.215 | 0.95   |
| Median (range)                         | 0.080 (0.030–0.188)    | 0.075 (0.0357–0.153)         | 0.080 (0.028–0.196) |

Data presented as mean ± SD, unless otherwise indicated.

HDL = High-density lipoprotein; UA = uric acid; PTH = parathyroid hormone.

* p < 0.05.

Table 2 shows laboratory biomarkers. The median sPCT was 0.080 ng/ml (0.030–0.188). Although patients with apparent current infections had not been enrolled according to the exclusion criteria, sPCT levels were slightly increased in 3 patients up to the upper limit of normal level (0.50 ng/ml). Whereas patients in the hypermethylation group had higher ferritin levels [133.0 (51.5–247.3) vs. 59.5 (40.0–119.0) ng/ml; p = 0.046], there was no significant difference in anemia, serum albumin levels and other inflammatory markers between the two groups.

**Correlation between Global DNA Methylation Status and Inflammatory Biomarkers**

We investigated CRP, ferritin and PCT as inflammatory markers, global DNA methylation status, and albumin and SGA scores as nutritional parameters. As shown in table 3, the HpaII/MspI ratio showed significant negative correlations with PCT (ρ = −0.32, p = 0.035) and ferritin (ρ = −0.33, p = 0.027). CRP was positively correlated with PCT (ρ = 0.31, p = 0.049) and ferritin (ρ = 0.37, p = 0.014). Serum albumin and SGA score were not correlated with the HpaII/MspI ratio. Since a lower ratio of HpaII/MspI means global DNA hypermethylation, a more severe inflammatory status was associated with accelerated DNA methylation.

**Multivariate Regression Analysis for Global DNA Methylation Status**

Next, we investigated the contributing factors to the HpaII/MspI ratio in a multivariate linear regression analysis. PCT and ferritin, but not CRP, were associated with a lower HpaII/MspI ratio (R² = 0.24; table 4).
Discussion

Following death due to CVD, infection-related death is the second most common cause of death in CKD patients, accounting for about 20% of the mortality in CKD stage 5 patients [7]. Alterations of the immune system in the uremic milieu are linked to the susceptibility to infections as well as to immune activation, resulting in persistent inflammation that accelerates atherosclerosis and CVD mortality [12]. The possible factors by which a chronic subclinical inflammatory state could be related to increasing CVD risk induced by infections include endothelial dysfunction [24] and an altered coagulation system [25]. It has been reported that infections might precipitate overt CVD through activation of systemic inflammation [26].

In the present study, we investigated global DNA methylation and inflammatory biomarkers, including sPCT as a marker of asymptomatic bacterial infections, in an observational study of Japanese incident dialysis patients. Unlike other inflammatory markers, sPCT

Table 3. Correlations between HpaII/MspI ratio, inflammatory markers and nutritional parameters

|                  | WBC  | Albumin | SGA  | CRP  | Ferritin | PCT  |
|------------------|------|---------|------|------|----------|------|
| HpaII/MspI ratio | ρ    | −0.1826 | NS   | −0.1103 | −0.1273 | −0.3343 | −0.3226 |
| p                |      |         |      |       |          |       |         |
| WBC              | ρ    | −0.2937 | NS   | −0.087 | 0.1128   | 0.2495 | 0.0194  |
| p                |      |         |      |       |          |       |         |
| Albumin          | ρ    | 0.1237  | NS   | −0.1147 | −0.1527 | −0.0854 |
| p                |      |         |      |       |          |       |         |
| SGA              | ρ    | −0.0821 | NS   | −0.1394 | −0.0606 |
| p                |      |         |      |       |          |       |         |
| CRP              | ρ    | 0.3739  | 0.3059 |
| p                |      |         |       |       |          |       |         |
| Ferritin         | ρ    | 0.1299  | NS   |
| p                |      |         |       |       |          |       |         |

NS = Not significant; WBC = white blood cells. * p < 0.05.

Table 4. Multivariate regression model predicting HpaII/MspI ratio in CKD stage 5 patients

| Parameter       | Parameter estimate | Standard error | p value |
|-----------------|--------------------|----------------|---------|
| Intercept       | 0.450              | 0.079          | <0.0001 |
| Age >61 years   | −0.025             | 0.023          | 0.3     |
| Female gender   | 0.010              | 0.012          | 0.4     |
| CRP             | 0.007              | 0.008          | 0.4     |
| PCT             | −0.025             | 0.011          | 0.034*  |
| Ferritin        | −0.030             | 0.013          | 0.029*  |

The adjusted R² of the model was 0.24. Age was dichotomized by the median value. * p < 0.05.
does not increase, or is only slightly elevated, in viral, localized infections, autoimmune diseases and during stress following surgical operations [27]. Thus, sPCT is considered to be a useful marker to distinguish bacterial infections from non-infectious inflammatory disease [28, 29]. In our study, sPCT concentrations showed a mean of 0.13 ± 0.17 ng/ml, and sPCT levels in 3 patients were slightly above the level of 0.5 ng/ml, although none of the patients had signs of current infection. As we found a positive association between sPCT and CRP, patients who showed signs of inflammation may be those who had contracted latent bacterial infections.

DNA methylation is a key mechanism for control of gene expression. The global DNA methylation level generally decreases with aging and is lower in males than in females [30]. In our study, whereas DNA hypermethylation was consistently associated with elevated ferritin levels in all there statistical methods [comparisons between the two groups divided by DNA methylation status (Wilcoxon rank sum test), Spearman’s rank test and multivariate linear regression analysis], we could find statistically significant differences between DNA hypermethylation and PCT levels by only the latter two methods. We deduce this discrepancy from the findings that PCT levels were more deviated from normal distribution and more centralized in lower layers because we enrolled patients without obvious infections. We could not find any association between DNA hypermethylation and CRP levels. CRP is a common inflammatory marker but is non-specific. We speculate that various kinds of inflammatory and non-inflammatory stimuli could alter DNA methylation [31], while there perhaps might be a certain kind of inflammation that is not related to aberrant DNA methylation. Moreover, CRP is a rapidly moving target. We also speculate that DNA methylation should be altered by chronic inflammation inducing dysfunction of iron metabolism with increasing ferritin levels, and that CRP might be not enough to detect low-grade inflammations, especially in Japanese CKD patients because their CRP levels seem naturally inclined to be much lower than those in Western CKD patients [32, 33].

Few studies on DNA methylation have been published in the context of uremia. Ingrosso et al. [34] reported that global DNA methylation in a selected group of maintenance HD patients was lower than that in healthy controls, and this hypomethylation status was associated with hyperhomocysteinemia. In stage 2–4 CKD patients, the global DNA methylation level was not associated with renal function and atherosclerosis [35]. Another study of unselected incident and prevalent dialysis patients showed that inflammation was associated with global DNA hypermethylation – a feature associated with increased mortality [6]. Although the basic epigenetic status of the DNA is set, change basically refers to a heritable change and influenced maternal factor in utero and is, to a large extent, heritable. DNA methylation patterns may fluctuate in response to changes in inherited genetic polymorphisms, diet and environmental factors, such as uremic toxins, sodium and infections in the uremic milieu [3, 36]. These results imply that environmental factors have the power to alter the epigenetic code and, ultimately, the phenotype. Further indirect support for our finding is a study showing that bacterial endotoxin altered DNA methylation and gene expression in an animal model [37]. Moreover, chronic inflammation by infection of Helicobacter pylori induced cell proliferation via DNA hypermethylation [38], which suggests that inflammation induced by bacterial infection and/or bacterial toxins may have the potential to alter DNA methylation status.

Some limitations of this study should be acknowledged. First, the small sample size makes it difficult to draw firm conclusions. Second, although we analyzed sPCT levels, we could not define the exact infectious cause of inflammation. We checked inflammatory markers including PCT at only one time close to the start of dialysis, while serial measurements would have been more informative. Third, because we did not compare the DNA methylation status to clinical outcomes, we could not evaluate if DNA methylation predicts
outcome. Finally, we did not analyze DNA methylation status in age- and gender-matched healthy controls.

In conclusion, the present study demonstrates that global DNA hypermethylation is associated with elevated inflammatory markers including PCT in Japanese incident dialysis patients. Our results suggest that inflammation may play a role in DNA hypermethylation, and subclinical bacterial infection may be, in part, involved in this mechanism, although further studies are needed to clarify the role of aberrant DNA methylation in the premature mortality of CKD patients.

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Disclosure Statement

Bengt Lindholm is an employee of Baxter Healthcare Corporation. Peter Stenvinkel is a member of the Scientific Advisory Board of Gambro.

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