Title
CDH13 genotype-dependent association of high-molecular-weight adiponectin with all-cause mortality: The J-SHIPP Study

Short title
Adiponectin and mortality

Authors
Eri Uetani MD, PhD;1) Yasuharu Tabara PhD;1(2) Ryuichi Kawamoto MD, PhD;3) Hiroshi Onuma MD, PhD;4) Katsuhiko Kohara MD, PhD;1) Haruhiko Osawa MD, PhD;4) and Tetsuro Miki MD, PhD1)

Affiliation
1) Department of Geriatric Medicine, Ehime University Graduate School of Medicine, Toon, Japan
2) Center for Genomic Medicine, Kyoto University Graduate School of Medicine, Kyoto, Japan
3) Department of Community Medicine, Ehime University Graduate School of Medicine, Toon, Japan
4) Department of Molecular and Genetic Medicine, Ehime University Graduate School of Medicine, Toon, Japan

Correspondence
Yasuharu Tabara,
Center for Genomic Medicine,
Kyoto University Graduate School of Medicine,
Shogoin-kawaramachi,
Sakyo-ku, Kyoto, 606-8507, Japan
Tel: +81-75-366-7407
Fax: +81-75-751-4167
E-mail: tabara@genome.med.kyoto-u.ac.jp

Word count
Abstract 250 words, body 2,136 words (introduction to discussion)
Two tables, two figures, 30 references
ABSTRACT

Objective: Despite its anti-inflammatory and anti-atherogenic effects, adiponectin is potentially associated with adverse clinical outcomes, such as all-cause mortality. As plasma adiponectin levels are strongly influenced by single nucleotide polymorphisms in the gene encoding T-cadherin (CDH13), we conducted a longitudinal study to investigate the possible link between the CDH13 genotype, plasma adiponectin levels, and all-cause mortality.

Research Design and Methods: This longitudinal study evaluated 2,020 Japanese subjects. Baseline clinical parameters were obtained from subjects’ personal health records as evaluated at annual medical check-ups. Plasma high-molecular-weight adiponectin (HMWA) levels were measured by an ELISA assay, and genotyping was performed by a TaqMan probe assay.

Results: Mean follow-up duration was 6.5 years. Kaplan-Meier analysis showed that HMWA levels were positively associated with mortality (p<0.001). HMWA levels were associated with older age, lower body weight, lower plasma triglyceride and glucose levels, and higher plasma HDL cholesterol. However, the Cox regression analysis showed that the positive association between HMWA and all-cause mortality was independent of these covariates (hazard ratio=1.92, p=0.006). The CDH13 rs4783244 genotype was strongly associated with baseline HMWA levels (per-allele effect size =1.65 µg/ml, p<0.001). In a separate analysis by the CDH13 genotype, the hazard ratio for all-cause mortality was linearly increased with the number of G-alleles (p-value for HMWA*CDH13 genotype interaction=0.023).

Conclusions: Higher plasma HMWA level was an independent prognostic factor for all-cause mortality in a general population. The CDH13 genotype may be a factor that affects not only the plasma level of HMWA but also the prognostic significance of HMWA.

KEYWORDS

Adiponectin, all-cause mortality, CDH13 genotype
Adiponectin is an adipocyte-derived secretory protein that has peripheral insulin-sensitizing activity (1). Adiponectin also exerts systemic anti-inflammatory effects via suppression of the growth and proliferation of macrophage progenitors, as well as the inflammation caused by fully differentiated macrophages (1). Further, cross-sectional studies in various populations have reported that a higher plasma adiponectin level is associated with a lower frequency of type 2 diabetes (2), metabolic syndrome (3), and thinner carotid arterial wall thickness (4). The results of these experimental studies and population-based cross-sectional association studies indicate that adiponectin is a cardio-protective factor in humans.

In contrast, several longitudinal epidemiological studies have reported that a higher plasma adiponectin level was associated with an increased incidence of cardiovascular disease (5, 6) and mortality (7-12). The underlying mechanism of the inverse association between these cross-sectional association studies and longitudinal prospective studies, the “adiponectin paradox”, is unclear and prevents a complete understanding of the biological role of adiponectin. A number of possibilities warrant investigation including reverse causality stemming from a compensatory increase of adiponectin to subclinical cardiovascular disease risks and adiponectin resistance (13).

Recent advances in DNA array technology have enabled the high-throughput analysis of single-nucleotide polymorphisms (SNPs). Genome-wide association studies (GWAS) have successfully identified SNPs of the CDH13 gene that have a strong association with plasma adiponectin levels (14, 15). The CDH13 gene encodes T-cadherin, a receptor for hexameric and high-molecular-weight forms of adiponectin (HMWA) (16). Although earlier studies (17, 18) and recent GWAS in European subjects (19, 20) reported a strong susceptibility of SNPs in the ADIPOQ gene that encodes adiponectin, GWAS of adiponectin in East-Asian subjects, namely Korean (15) and Japanese (21), identified SNPs in the CDH13 locus, such as rs4783244. Because plasma adiponectin levels in subjects homozygous for the allele that with an
association with higher adiponectin levels (affected allele) were approximately 1.5 times higher than those homozygous for the allele without (unaffected allele) (21), genetic variation might be partially involved in the adiponectin paradox.

To clarify our hypothesis, we analyzed the possible interaction of the CDH13 genotype on the association of plasma levels of HMWA and all-cause mortality in Japanese middle-aged to elderly community residents.

RESEARCH DESIGN AND METHODS

Study subjects
This longitudinal study enrolled community-dwelling Japanese subjects living in Nomura Town, Ehime Prefecture, a mostly rural town with 11,000 inhabitants. Study subjects were recruited through a community-based annual medical check-up process in 2002 as part of the Shimanami Health Promoting Program (J-SHIPP) (3, 22, 23), which was a longitudinal epidemiological study evaluating factors relating to cardiovascular disease (CVD), dementia, and death. Among a total of 2,721 residents who agreed to participate in the J-SHIPP study and completed baseline clinical measurements, 2,020 persons for whom an overnight fasting plasma sample was provided (> 11 h) for HMWA measurement (n=2,084) and CDH13 genotype was determined (n=2,042), and whom mortality during this study period could be followed-up were enrolled in this analysis. All study procedures were approved by the Ethics Committee of Ehime University Graduate School of Medicine, and all subjects provided signed informed consent prior to participation.

Baseline measurements
Baseline clinical characteristics evaluated at medical check-ups, including anthropometric measurements, blood pressure (BP) and basic plasma markers, were obtained from personal
health records. Additional characteristics, including medication and history of CVD, were obtained by individual interviews using a structured questionnaire. Hypertension was defined as any or all of the following: systolic blood pressure (SBP) equal to or greater than 140 mmHg, diastolic blood pressure (DBP) equal to or greater than 90 mmHg, and current use of antihypertensive medication. Subjects with a fasting blood glucose level of more than 126 mg/dl or currently using antihyperglycemic medication were defined as having type 2 diabetes mellitus. Renal function was evaluated based on the estimated glomerular filtration rate (eGFR), which was calculated from the plasma creatinine values using the following formula specifically developed by the Japanese Society of Nephrology for the estimation of the GFR in Japanese: 194 x creatinine\(^{-1.094}\) x age\(^{-0.287}\) x 0.739 (if female).

**Follow-up of all-cause mortality**

Vital status was ascertained until the 30th of November 2009 by checking a residential registration certification, which is under local government control. Since the registration of a person’s death is obligatory under the Family Registration Law in Japan, the local government’s vital statistics database enabled the follow-up of all-cause mortality. Further, as the notification of a change of address is obligatory under the residential registration certificate system, we were informed of subjects who moved outside of the cohort area and treated them as a censored case.

**Measurement of plasma HMWA levels**

Plasma samples were obtained from each subject after a period of overnight fasting of at least 11 h. Samples were immediately frozen and stored at -80 °C until analysis. Plasma concentrations of HMWA were determined using an ELISA assay (Fujirebio Inc., Shinjuku-ku, Tokyo, Japan) (3, 24), which consisted of HMWA-specific capture antibody (IH7) and
HMWA-specific horseradish peroxidase-conjugated detection antibody (POD-IH7). The specificity of this monoclonal antibody has been described elsewhere (25). Standardized HMWA concentrations were determined by human HMWA purified by gelatin-cellulofine column chromatography. Intra- and inter-assay coefficient variations of the adiponectin assay were 4.4% and 9.7%, respectively.

**Genotyping**

DNA was extracted from peripheral blood using a QIAamp DNA blood kit (Qiagen GmbH, Hilden, North Rhine-Westphalia, Germany). The SNP rs4783244 in the CDH13 gene was genotyped by a TaqMan probe assay using commercially available primers and probes purchased from the Assay-on-Demand system (Life Technologies Corporation, Carlsbad, California, USA). Fluorescence of PCR products was measured using an ABI PRISM 7900HT sequence detector (Life Technologies, Minato-ku, Tokyo, Japan).

**Statistical analysis**

Differences in the numeric variables of baseline data were assessed by analysis of variance, and differences in frequency were assessed by a chi-square test. Overall survival ratio was calculated by Kaplan-Meier analysis. Covariate-adjusted hazard ratio (HR) for total mortality was analyzed by Cox proportional hazards regression analysis. Combined HR was calculated under a random effects model. A $p$-value less than 0.05 was considered statistically significant.

**RESULTS**

Baseline clinical characteristics of study subjects are shown in Table 1. Mean HMWA level in female subjects (7.62 ± 4.56 µg/ml) was nearly double that in male subjects (4.12 ± 2.93 µg/ml, $p<0.001$). Subjects were therefore divided by HMWA quartile within each sex and then
combined to avoid potential sex differences (Table 1). Table 1 also summarizes the differences in clinical parameters among HMWA quartiles. Age was strongly and positively associated with HMWA quartile, while BMI showed a significant inverse association. Plasma levels of HDL cholesterol, triglyceride, and glucose were also significantly different among HMWA quartiles. In a simple comparison, systolic blood pressure and eGFR were significantly associated with HMWA quartile; however, these associations disappeared after adjustment for age and BMI.

Mean follow-up duration was 6.5 years, during which time 84 subjects died. Figure 1A shows the Kaplan-Meier plot for all-cause mortality by HMWA quartile, with the highest quartile being strongly associated with all-cause mortality. To clarify whether the prognostic significance of HMWA was independent of possible covariates, Cox regression analysis was performed by a forward covariate selection method. Table 2 summarizes the hazard ratio (HR) of the 4th quartile of HMWA, as well as 10 μg/ml increases in HMWA, on total mortality. In any model adjusted for relevant clinical parameters (Table 2, Model 1 to 3), higher plasma HMWA levels were independently associated with total mortality.

The G-allele of the CDH13 rs4783244 genotype was strongly associated with baseline plasma HMWA level (Figure 2). Although obese subjects, defined as having a body mass index greater than 25 kg/m², showed significantly lower HMWA levels in both sexes (male: obese 3.25±2.22, non-obese 4.49±3.12, p<0.001; female: 6.37±3.68, 8.18±4.86, p<0.001), the association between CDH13 genotype and HMWA was independent of obese status (Figure 2). Per-allele effect size of the genotype calculated by a regression analysis adjusted for age, sex and BMI was 1.65 μg/ml (p<0.001).

Nevertheless, the prognostic significance of HMWA on total mortality was independent of the CDH13 genotype (Table 2, Model 4). In a separate analysis by the genotype, however, HR for all-cause mortality was linearly increased with the number of G-alleles.
(Figure 1B), and the association between HMWA and poor prognosis was seen only in the GG genotype carries. The term of interaction between the CHD13 genotype and plasma HMWA level was identified as a significant factor for all-cause mortality (p=0.023) in the Cox regression analysis adjusted for the same covariates as adjusted in Model 3 of Table 2. These results indicate that the poor prognostic significance of HMWA was genotype-dependent.

CONCLUSIONS
In this longitudinal study in a general population, we observed that higher plasma HMWA levels were significantly associated with all-cause mortality. Further, the association between HMWA and mortality showed a high degree of variation among CDH13 rs4783244 genotypes. To our knowledge, this is the first study showing a genotype-dependent association of HMWA with all-cause mortality.

Our results confirmed the increased risk of all-cause mortality in subjects with a higher baseline HMWA level. Several previous studies reported a similar relationship in general populations (5, 8-10), as well as in patients with type 2 diabetes (7), coronary artery disease (6, 11), and chronic heart failure (12). Higher plasma HMWA in a general population is a complex phenotype which may represent a clustering of sub-clinical risk statuses, namely reduced renal and cardiac function, obesity, insulin resistance and metabolic disorders, atherosclerosis, systemic inflammation, weight loss in elderly, and anemia. However, these studies were conducted mainly in European subjects (7, 9-12) or in European and African American subjects (5, 6, 8), with no positive results being reported from East-Asian subjects. In contrast, it has been reported that the Japanese population have distinct anthropometric characteristics, including a lower frequency of obesity compared with developed Western countries (26), and considerably lower plasma adiponectin levels (27), even in comparison with Japanese Americans living in Hawaii (28). In populations with lower HMWA levels, our results
demonstrate the cross-validity of the prognostic significance of HMWA.

We also found an inverse association between HMWA and all-cause mortality between CDH13 TT and CDH13 GG genotype carriers, with the HR of the CDH13 TT genotype failing to reach statistical significance, presumably due to the smaller sub-sample size. Although the CDH13 gene is known to encode T-cadherin, a receptor for HMWA, the functionality of each CDH13 rs4783244 genotype or other unidentified responsible SNPs in linkage disequilibrium have not been clarified. However, T-cadherin knockout mice were reported to show dramatically increased plasma adiponectin levels due to impaired linkage between adiponectin and target cells (29). It is therefore possible that the G-allele of the CDH13 genotype causes secondary hyper-adiponectinemia by altering the amount or functionality of T-cadherin. This genetically dependent adiponectin resistance and consequent reduction in adiponectin’s anti-inflammatory and anti-atherogenic effects may explain the increased mortality ratio in subjects with higher HMWA levels. Because differences in HMWA levels for each CDH13 rs4783244 genotype were similar between non-obese and obese subjects (Figure 2), the production of adiponectin and formation of high-molecular-weight isoforms might not be a factor in the genotype-specific mortality ratio.

According to the HapMap database (http://hapmap.ncbi.nlm.nih.gov/), frequency of the CDH13 rs4783244 genotype is similar across all ethnic groups. However, GWAS in European subjects (19-20) identified only the ADIPOQ gene as susceptible loci for plasma adiponectin levels. Although more large-scale GWAS might identify the CDH13 gene as another susceptible loci, it is probable that genetic polymorphisms in the ADIPOQ gene have a larger effect than those in the CDH13 genotype in European populations regardless of the type of adiponectin complex (30). Because the ADIPOQ gene encodes adiponectin, our “genetic adiponectin resistance” hypothesis involving the CDH13 gene as a means of explanation might not be easily extrapolated to other populations. However, our results provide novel supporting
evidence that adiponectin resistance is a key mechanism of the adiponectin paradox, which is lacking in other proposed mechanisms such as renal dysfunction and decreased adiponectin clearance, weight loss or sarcopenia in the elderly, and compensatory rise in adiponectin due to subclinical risks (reverse causality) (13). Our present results also excluded the possibility of confounding effects of decreased renal function.

Several limitations of this study warrant mention. First, we could not evaluate a cause-specific mortality, particularly cardiovascular mortality. Results of long-term analysis for cardiovascular mortality would strengthen our findings. Second, we did not analyze other molecular isoforms of adiponectin. As T-cadherin is a receptor for HMWA and the HMW isoform is considered an active form, further investigations regarding isoform specificity will be necessary. Third, this study contained approximately 2,000 study subjects. Replication studies, particularly in East-Asian populations, with a larger sample size are therefore needed to confirm our present findings.

In summary, our longitudinal study demonstrated that the prognostic significance of HMWA is genotype-specific. Our findings may provide the insight necessary to help solve the adiponectin paradox, and further highlight the importance of genetic factors in understanding the contribution of adiponectin levels to adverse clinical outcomes.

ACKNOWLEDGEMENTS
The authors have no conflicts of interest to disclose.

Contribution of each author: E.U conducted research data acquisition, statistical analysis, and manuscript writing; Y. T conducted research data acquisition, statistical analysis, and manuscript writing; R.Y and H.On conducted research data acquisition; K.K and H. Os conducted research data acquisition, and contributed to the Conclusions section; and T.M supervised the study. Y.T is the guarantor of this work and, as such, had full access to all the
data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

This study was supported by a Grant-in-Aid for Scientific Research from The Ministry of Education, Culture, Sports, Science and Technology of Japan; a Science and Technology Incubation Program in Advanced Regions from the Japan Science and Technology Agency; a Grant-in-Aid for Scientific Research from the Japan Arteriosclerosis Prevention Fund; and a Research Promotion Award from Ehime University.

We thank Nicholas Crabb and Guy Harris of DMC Corporation for help in the preparation of this manuscript.
REFERENCES


16. Hug C, Wang J, Ahmad NS, Bogan JS, Tsao TS, Lodish HF. T-cadherin is a receptor for


Table 1. Clinical parameters of HMWA quartiles

<table>
<thead>
<tr>
<th></th>
<th>Total subjects</th>
<th>Q1 (501)</th>
<th>Q2 (507)</th>
<th>Q3 (507)</th>
<th>Q4 (505)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ANOVA</td>
</tr>
<tr>
<td>HMW adiponectin (µg/ml)</td>
<td>6.09 ± 4.33</td>
<td>2.34±1.07</td>
<td>4.21±1.51</td>
<td>6.36±2.05</td>
<td>11.43±4.60</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>62.4 ± 12.3</td>
<td>57.9 ± 11.9</td>
<td>60.9 ± 12.4</td>
<td>63.3 ± 11.7</td>
<td>67.6 ± 11.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sex (male%)</td>
<td>44.2</td>
<td>44.3</td>
<td>43.8</td>
<td>43.4</td>
<td>44.2</td>
<td>0.998</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.5 ± 3.2</td>
<td>24.6 ± 3.2</td>
<td>23.8 ± 3.2</td>
<td>23.3 ± 2.9</td>
<td>22.2 ± 3.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>139 ± 22</td>
<td>140 ± 22</td>
<td>136 ± 21</td>
<td>139 ± 23</td>
<td>142 ± 22</td>
<td>0.001</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>203 ± 35</td>
<td>205 ± 36</td>
<td>204 ± 35</td>
<td>201 ± 33</td>
<td>203 ± 34</td>
<td>0.302</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dl)</td>
<td>62 ± 16</td>
<td>56 ± 14</td>
<td>60 ± 14</td>
<td>63 ± 15</td>
<td>68 ± 16</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>113 ± 74</td>
<td>139 ± 92</td>
<td>115 ± 69</td>
<td>107 ± 64</td>
<td>91 ± 58</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>eGFR (mL/min/1.73m²)</td>
<td>80 ± 17</td>
<td>82 ± 18</td>
<td>80 ± 17</td>
<td>81 ± 17</td>
<td>78 ± 17</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>98 ± 21</td>
<td>102 ± 24</td>
<td>98 ± 23</td>
<td>98 ± 22</td>
<td>95 ± 15</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Antihyperglycemic medication (%)</td>
<td>3.4</td>
<td>3.8</td>
<td>2.8</td>
<td>4.1</td>
<td>3.0</td>
<td>0.575</td>
</tr>
<tr>
<td>Type 2 diabetes (%)</td>
<td>7.4</td>
<td>10.6</td>
<td>6.9</td>
<td>6.7</td>
<td>5.4</td>
<td>0.011</td>
</tr>
<tr>
<td>CDH13 genotype (G-allele %)</td>
<td>65.8</td>
<td>51.7</td>
<td>65.3</td>
<td>68.3</td>
<td>77.9</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation. Quartile of HMWA was calculated within each sex and then combined to avoid potential sex differences. Statistical significance in numeric variables was assessed by analysis of variance (ANOVA), while differences in frequency were assessed by a chi-squared test. Trends in numeric variables among the HMWA quartile was assessed by regression analysis adjusted for age and BMI. The estimated glomerular filtration rate (eGFR) was calculated from plasma creatinine values using the following formula: 194 x creatinine^{-1.094} x age^{-0.287} x 0.739 (if female). Type 2 diabetes was defined as a fasting plasma glucose level greater than 126 mg/dl, or the current use of medication to lower blood glucose levels. G-allele frequency of the CDH13 rs4783244 genotype is shown in the Table. BMI, body mass index; BP, blood pressure; HMWA, high-molecular-weight adiponectin.
<table>
<thead>
<tr>
<th>Model</th>
<th>Adjusted factors</th>
<th>HMW Adiponectin 10μg/ml</th>
<th>p-value</th>
<th>HMW Adiponectin 4th quartile</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model 1</td>
<td>Age, sex</td>
<td>2.03 (1.34-2.96)</td>
<td>0.001</td>
<td>1.78 (1.19-2.68)</td>
<td>0.006</td>
</tr>
<tr>
<td>Model 2</td>
<td>Age, sex, BMI</td>
<td>1.85 (1.20-2.76)</td>
<td>0.006</td>
<td>1.63 (1.08-2.48)</td>
<td>0.021</td>
</tr>
<tr>
<td>Model 3</td>
<td>Age, sex, BMI, SBP, TC, HDLC, glucose, CVD, eGFR, current smoking, current medication</td>
<td>1.91 (1.20-2.96)</td>
<td>0.007</td>
<td>1.59 (1.03-2.46)</td>
<td>0.035</td>
</tr>
<tr>
<td>Model 4</td>
<td>Age, sex, BMI, SBP, TC, HDLC, glucose, CVD, eGFR, current smoking, current medication, CDH13 genotype</td>
<td>1.83 (1.12-2.91)</td>
<td>0.017</td>
<td>1.51 (0.97-2.37)</td>
<td>0.068</td>
</tr>
</tbody>
</table>

Hazard ratio (HR) was calculated by a cox regression analysis. CVD indicates previous history of cardiovascular diseases (namely stroke and myocardial infarction). Medication includes antihypertensive drugs and antihyperglycemic drugs. BMI, body mass index; SBP, systolic blood pressure; TC, total cholesterol; HDLC, high-density lipoprotein cholesterol; eGFR, estimated glomerular filtration rate.
FIGURE LEGENDS

Figure 1. HMWA and all-cause mortality
A: Kaplan-Meier plot of survival by HMWA quartile. Subjects were divided by HMWA quartile within each sex and then combined to avoid potential sex differences. B: Hazard ratio of HMWA with total mortality and CDH13 rs4783244 genotype. Hazard ratio of HMWA (continuous variable, per 10 µg/ml) for each CDH13 rs4783244 genotype was calculated by a Cox regression analysis adjusted for age and sex. Diamond represents the combined odds ratio calculated by a random effects model.

Figure 2. Mean HMWA level and CDH13 rs4783244 genotype
Values are mean ± standard deviation. Statistical significance was assessed by analysis of variance (ANOVA). Number of subjects for each genotype is shown in the column. Obesity was defined as a body mass index greater than 25 kg/m².
Figure 1
209x148mm (300 x 300 DPI)
Figure 2
209x148mm (300 x 300 DPI)