found from stage 3 to 4. Based on our analysis of MVD prevalence according to the eGFR level, diabetic patients with an eGFR less than 46 ml/min/1.73 m^2 had a significantly higher risk than those with an eGFR over 46 ml/min/1.73 m^2. For early finding of MVD, including cardiovascular disease, the eGFR range of ≥30 to <46 ml/min/1.73 m^2 is an important level in the diabetic CKD stage. CKD stage 3 should be divided into two substages for management of type 2 diabetic CKD from the perspective of cardiorenal association.

An association between cardiovascular risk and eGFR level was found in some reports.

Yamamoto et al. [9] observed a significantly higher prevalence of cardiovascular disease and stroke when the eGFR was lower than 75 ml/min in diabetic patients, including those without CKD. In the study by Nakagawa et al. [16], a comparison of the eGFR with pulse wave velocity (PWV) showed that an eGFR lower than 45 ml/min/1.73 m^2 predicted arterial stiffness and progression to end-stage renal failure in nondiabetic CKD patients. Go et al. [3] reported that the risks of various outcomes, including hospitalization, death and cardiovascular disease, were increased with an eGFR below 60 ml/min/1.73 m^2; however, these risks were not the same in stage 3 CKD patients. Furthermore, they divided CKD stage 3 patients (with or without diabetes) into two substages and examined the rates of death, cardiovascular events, and hospitalization. Their data showed an increased risk with an eGFR below 45 ml/min/1.73 m^2 (hazard ratio for cardiovascular disease: 2.0 with an eGFR of 30–44 ml/min/1.73 m^2 vs. 1.4 with an eGFR of 45–59 ml/min/1.73 m^2). Go et al. did not consider underlying kidney disease. Our data imply that an eGFR of 46 ml/min/1.73 m^2 is the cut-off for the presence or development of MVD, including CHD, stroke and ASO, in diabetic CKD patients. This eGFR level in diabetic CKD patients was very similar to Go et al.

Hypertension, hyperlipidemia, diabetes duration and CKD are known risk factors for cardiovascular disease. Our present study identified diabetes duration, proteinuria and hyperuricemia as independent risk factors for MVD. Of these factors, hyperuricemia (odds ratio: 1.69) and proteinuria (odds ratio: 1.93) showed the highest odds ratios and were considered to be the independent risk factors for MVD. Proteinuria is the conventional risk factor for cardiovascular disease in diabetic patients [13].

Hypertension was not a risk factor in our study because good control of blood pressure (130/73 mmHg) was being practiced by these patients. Our study showed that hyperuricemia was a risk factor. Recently, the association between uric acid and cardiovascular disease in type 2 diabetic patients was reported. Zoppini et al. [17] reported that elevated uric acid in type 2 diabetic patients is an independent risk factor for cardiovascular disease. Additionally, uric acid has been reported to be associated with the onset of type 2 diabetes [18] and progression of renal impairment in type 2 diabetic nephropathy [19, 20]. Madero et al. [21] reported that in patients with stages 3 to 4 CKD, hyperuricemia is associated with cardiovascular mortality, but not progression to kidney failure. The PIUMA study that followed patients with untreated essential hypertension for 4 years found that when the uric acid level is elevated, the risks of cardiovascular event, cardiovascular death and all-cause death were increased [22].

Uric acid level is conventionally considered to be a marker of renal disease because of the lowered uric acid excretion as CKD progresses; however, in a rat model of hyperuricemia, hypertension and renal arteriolopathy are also observed, which can be suppressed by antihyperuricemic agents [23–25]. These reports suggest that uric acid is a true mediator of progression and aggravation of renal impairment. The importance of uric acid management in diabetic patients so as to retard the progression of diabetic CKD as well as prevent cardiovascular disease development should be clarified.

Our study concluded that the prevalence of MVD, including CHD, stroke and ASO, increases with the progression of CKD stage in type 2 diabetic CKD patients, and this trend is especially seen as CKD progresses from stage 3 to 4. In stage 3 CKD patients, an eGFR of 46 ml/min/1.73 m^2 predicted MVD prevalence. CKD stage 3 should be divided into two substages (eGFR of ≥30 to <46 and ≥46 to < 60 ml/min/1.73 $m^{2)}$. From the present study, an eGFR of 46 ml/min/1.73 m^2 is a useful critical level marker for the prevention and management of type 2 diabetic CKD from the perspective of cardiorenal association. Hyperuricemia is an independent risk factor for MVD, suggesting that uric acid management is important to prevent cardiorenal association in type 2 diabetic CKD.

Conflict of interest The authors have no conflict of interest to disclose.

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慢性腎臓病および糖尿病性腎症における硫化水素の関与

湯沢由紀夫

要約：硫化水素（H_2S）は，有毒で時には致死的なガスであるが，一酸化窒素（NO）および一酸化炭素（CO）に加えて第3のガストランスミッターとして，その生物学的機能が近年大きく注目されている．特に神経系，心血管系，消化器系では，神経伝達物質，血圧のコントロール，インスリン分泌などに深く関与していることが確認され，さらに炎症やアポトーシス，酸化ストレスの誘導にも重要な働きをしている．さらにH_2Sの発現不全状態は，高血圧，動脈硬化，心筋梗塞，糖尿病など生活習慣病の主要な病態を引き起こす．急性腎傷害（AKI）は，直接生命予後に影響する病態として近年注目されている．虚血腎障害モデルに対して，H_2Sドナーの投与により組織傷害および腎機能が改善されたことから，H_2Sは虚血性AKIに対する重要な治療標的として期待される．また，H_2Sは内皮細胞上のアンジオテンシン変換酵素（ACE）の活性の抑制効果がある．さらに，ホモシステインは，Ⅰ型アンジオテンシンⅡ受容体（AT1R）の発現を誘導する．このため，高ホモシステイン血症に伴うH_2S産生低下は，ACE活性の上昇と血管のAT1R発現増加を介して，高血圧や腎の線維化を誘導する．一方，高ホモシステイン血症は慢性腎臓病の主要な危険因子であるが，ホモシステインはH_2Sの基質であることから，H_2S合成酵素（CBS，CSE）の発現不全に由来する内因性H_2S産生低下状態を反映した病態として理解されるようになってきた．糖尿病および糖尿病性腎症に関しては，H_2Sは直接膵でのインスリン分泌に関与し，糖尿病発症に深くかかわることが示された．高血糖に由来する糸球体でのNO発現低下は，内皮細胞障害により糖尿病性腎症における糸球体病変の発症・進展に深く関与し，CSE発現低下に伴うH_2S産生低下は，尿細管・間質での虚血障害を進行させ，腎機能低下が進行すると考えられる．

はじめに

硫化水素（H_2S）は，有毒で時には致死的なガスであるが，一酸化窒素（NO）および一酸化炭素（CO）に加えて第3のガストランスミッターとして，その生物学的機能が近年大きく注目されている．特に神経系，心血管系，消化器系では，神経伝達物質，血圧のコントロール，インスリン分泌などに深く関与していることが確認され，さらに炎症やアポトーシス，酸化ストレスの誘導にも重要な働きをしている(1, 2)．さらにH_2Sの発現不全状態は，高血圧，動脈硬化，心筋梗塞，糖尿病など生活習慣病の主要な病態を引き起こすこともわかり，これらの疾患に対する新たな治療の標的としても注目を集めている(3)．

NO産生に関与する3種類の合成酵素（nNOS, eNOS, iNOS），CO合成に関与する合成酵素（HO1/2/3）と同じく，H_2Sの合成においても3種類の重要な合成酵素（cystathionine-β-synthase（CBS），cystathionine γ-lyase（CSE），3-mercaptopyruvate sulfurtransferase（MST））が存在する．CBSは主に脳や神経系で発現が認められるのに対し，CSEは血管平滑筋，内皮細胞，肝臓，腎臓などで主に発現する．特に腎臓においては，これらの3種類の酵素発現が強く認められることから，H_2Sは腎機能維持に極めて重要な働きをしていると考えられる．

1. 慢性腎臓病と硫化水素

1）腎におけるH_2S合成酵素の発現

すでに腎臓でのCBSとCSEの発現については確認されているが，発現部位の詳細な検討免疫組織学的検討から，両者はともに腎臓の近位尿細管上皮細胞に強

キーワード：CBS，CSE，虚血，慢性腎臓病，糖尿病性腎症
藤田保健衛生大学 腎内科（〒470-1192 愛知県豊明市沓掛町田楽ヶ窪1-98）
E-mail: yukio-y@fujita-hu.ac.jp 原稿受領日：2011年9月24日，依頼原稿
Title: Role of hydrogen sulfide in chronic kidney disease and diabetic nephropathy. Author: Yukio Yuzawa

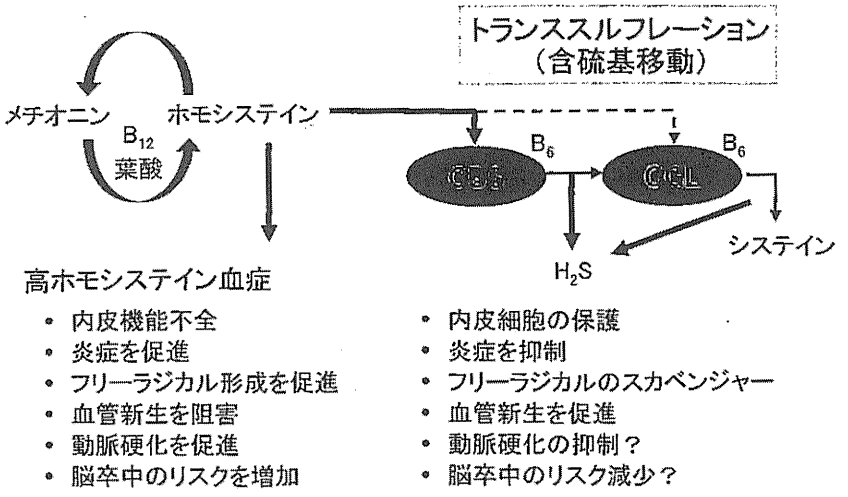

図4 高ホモシステイン血症と血管障害
ホモシステインは H_2S の基質であることから，CBS，CSE の機能不全に由来する内因性 H_2S 産生低下状態を誘導し，血管障害の原因となる．
(Beard RS, et al. Am J Physiol Heart Circ Physiol. 2011;300:H13-H26. 改変)

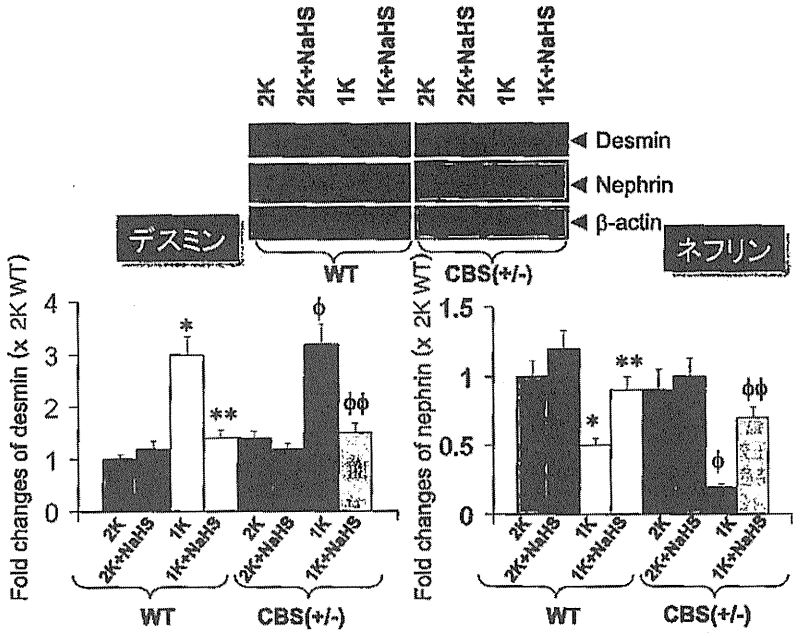

図5 慢性腎臓病における硫化水素の腎保護作用
野生型（WT）と CBS（+/−）マウスの両腎群（2K），片腎摘施行群（1K）にそれぞれ，硫化水素ドナー（NaHS）を投与し，糸球体上皮細胞（ポドサイト）の傷害マーカーであるデスミンおよび，タンパク尿のバリアーとして重要なスリット膜の主要構成タンパク質であるネフリンの発現の変化を観察した．ポドサイト傷害マーカーのデスミンは，野生型，CBS（+/−）ともに片腎群（1K）で有意に上昇したが，NaHS の投与により元のレベルに戻った．また，タンパク尿のバリアーであるネフリンの発現は，片腎群（1K）で低下し，その発現低下は CBS（+/−）群でより大きかった．NaHS の投与によりネフリンの発現の回復が確認された．（文献 19 より改変転載）

ないが，透析患者の白血球の CSE の mRNA 発現は健常人と比べて低下しており，CKD あるいは尿毒症による H_2S の産生酵素の遺伝子の転写抑制により H_2S 発現低下が生ずると考えられている(18)．

高ホモシステイン血症＋H_2S 産生不全状態が CKD の病態に与える影響については，現在解明されつつある．CBS のヘテロマウスに片腎摘を施し，CKD の状態にすると，タンパク尿の出現，糸球体固有細胞のアポトーシス，糸球体での MMP2, 9 の発現増強により，腎障害が進行する．この状態に NaHS を投与し血中の H_2S 濃度を上昇させると，血中ホモシステイン濃度は低下しないにもかかわらず，タンパク尿は減少し，アポトーシスは抑制され，ネフリンの発現回復に示されるような糸球体上皮細胞の保護効果を示したことから，上記の仮説が証明された(19)（図5）．

2. 糖尿病性腎症と硫化水素

1) 糖尿病と H_2S

Kaneko, Niki らは，単離マウス膵島および膵 B 細胞株を用いた実験から，L-システインおよび NaHS 投与によるインスリン分泌の抑制を確認した(20)．さらに，血糖刺激によりマウスの膵 B 細胞で CSE 発現が亢進し，産生誘導された H_2S は，抗アポトーシス作用を介して慢性の糖毒性による膵 B 細胞障害に対して，膵保護的に働くことが示された(21)．これらから，H_2S は直接膵でのインスリン分泌に関与し，糖尿病発症に深くかかわることが示された．

Zucker 糖尿病マウスでも，膵臓での CSE の発現増加および H_2S 産生上昇と血中インスリンの低下・高血糖を認めた(22)．

一方，nonobese 糖尿病ラットでは，糖尿病の進行とともに血中の H_2S レベルと大動脈での H_2S 産生は低下する．筆者らも，1 型糖尿病モデルである CaMTg マウス(23) の腎臓の近位尿細管での CSE の発現低下を認め，尿細管・間質を中心とした腎局所で H_2S の産生が低下する可能性が考えられる．また，糖尿病患者の血中 H_2S レベルの低下も報告されている(24)．

このように種の違いや臓器の違いにより，H_2S 合成酵素の発現および H_2S 産生状態は異なっており，その詳細なメカニズムはまだ十分解明されていない．

2) 糖尿病性腎症と H_2S

すでに述べたように，マウス腎での CBS，CSE の発現は，ともに近位尿細管に限局して強く発現している．これらの糖尿病性腎症における発現の変化を，CaMTg マウス腎を用いて検討すると，CBS はタンパク質レベルでは変化が見られないが，brush border 中心の発現パターンから細胞質全体に瀰漫性の発現に変化した．一方，CSE は，有意な発現量の減少を認めた．PTC の血流も正常マウスに比べ有意に減少しており，この血流低下は，NaHS 投与により回復することから，CSE 発現低下に伴う尿細管局所での H_2S 産生低下が原因と考えられた．

糖尿病性腎症の主体は，高度タンパク尿を特徴とする糸球体障害である．特に，筆者らは，糸球体内での NO-VEGF シグナルのアンバランスによる内皮細胞障害が重要であることを報告してきた(25)．一方，進行性腎障害における腎機能低下に関しては，糖尿病性腎症を含めその原疾患の違いに関係なく，糸球体障害の程度より尿細管・間質障害の程度がより腎機能低下に影響を与える重要な因子となっている．

高血糖に由来する糸球体での NO 発現低下は，内皮細胞障害により糖尿病性腎症における糸球体病変の発症・進展に深く関与し，CSE 発現低下に伴う H_2S 産生低下は，尿細管・間質での虚血障害を進行させ，腎機能低下が進行すると考えられる．

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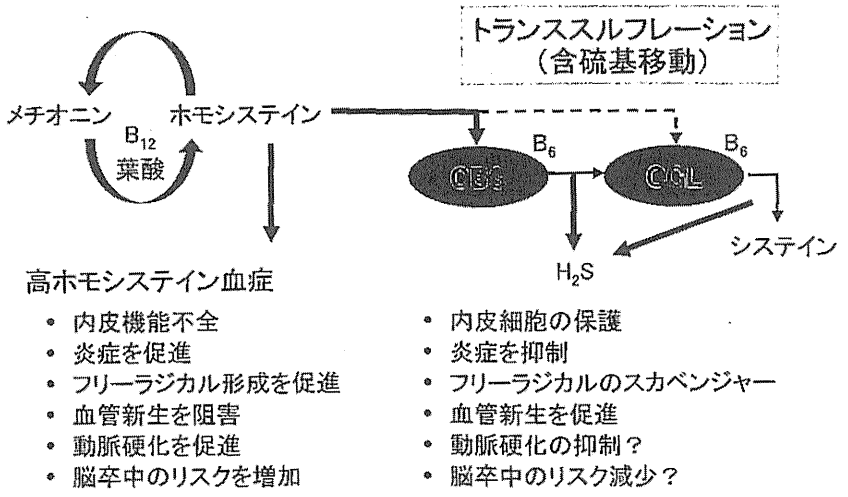

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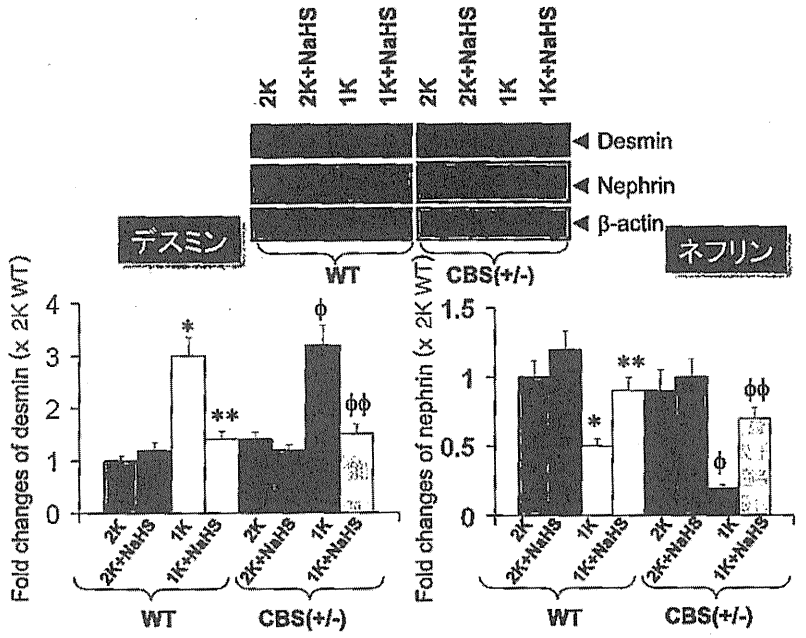

図5 慢性腎臓病における硫化水素の腎保護作用
野生型（WT）とCBS（+/−）マウスの両腎群（2K），片腎摘施行群（1K）にそれぞれ，硫化水素ドナー（NaHS）を投与し，糸球体上皮細胞（ポドサイト）の傷害マーカーであるデスミンおよび，タンパク尿のバリアーとして重要なスリット膜の主要構成タンパク質であるネフリンの発現の変化を観察した．ポドサイト傷害マーカーのデスミンは，野生型，CBS（+/−）ともに片腎群（1K）で有意に上昇したが，NaHSの投与により元のレベルに戻った．また，タンパク尿のバリアーであるネフリンの発現は，片腎群（1K）で低下し，その発現低下はCBS（+/−）群でより大きかった．NaHSの投与によりネフリンの発現の回復が確認された．（文献19より改変転載）

ないが，透析患者の白血球のCSEのmRNA発現は健常人と比べて低下しており，CKDあるいは尿毒症によるH_2Sの産生酵素の遺伝子の転写抑制によりH_2S発現低下が生ずると考えられている(18)．

高ホモシステイン血症＋H_2S産生不全状態がCKDの病態に与える影響については，現在解明されつつある．CBSのヘテロマウスに片腎摘を施し，CKDの状態にすると，タンパク尿の出現，糸球体固有細胞のアポトーシス，糸球体でのMMP2, 9の発現増強により，腎障害が進行する．この状態にNaHSを投与し血中のH_2S濃度を上昇させると，血中ホモシステイン濃度は低下しないにもかかわらず，タンパク尿は減少し，アポトーシスは抑制され，ネフリンの発現回復に示されるような糸球体上皮細胞の保護効果を示したことから，上記の仮説が証明された(19)（図5）．

2. 糖尿病性腎症と硫化水素

1) 糖尿病と H_2S

Kaneko, Nikiらは，単離マウス膵島および膵B細胞株を用いた実験から，L-システインおよびNaHS投与によるインスリン分泌の抑制を確認した(20)．さらに，血糖刺激によりマウスの膵B細胞でCSE発現が亢進し，産生誘導されたH_2Sは，抗アポトーシス作用を介して慢性の糖毒性による膵B細胞障害に対して，膵保護的に働くことが示された(21)．これらから，H_2Sは直接膵でのインスリン分泌に関与し，糖尿病発症に深くかかわることが示された．

Zucker糖尿病マウスでも，膵臓でのCSEの発現増加およびH_2S産生上昇と血中インスリンの低下・高血糖を認めた(22)．

一方，nonobese糖尿病ラットでは，糖尿病の進行とともに血中のH_2Sレベルと大動脈でのH_2S産生は低下する．筆者らも，1型糖尿病モデルであるCaMTgマウス(23)の腎臓の近位尿細管でのCSEの発現低下を認め，尿細管・間質を中心とした腎局所でH_2Sの産生が低下する可能性が考えられる．また，糖尿病患者の血中H_2Sレベルの低下も報告されている(24)．

このように種の違いや臓器の違いにより，H_2S合成酵素の発現およびH_2S産生状態は異なっており，その詳細なメカニズムはまだ十分解明されていない．

2) 糖尿病性腎症と H_2S

すでに述べたように，マウス腎でのCBS，CSEの発現は，ともに近位尿細管に限局して強く発現している．これらの糖尿病性腎症における発現の変化を，CaMTgマウス腎を用いて検討すると，CBSはタンパク質レベルでは変化が見られないが，brush border中心の発現パターンから細胞質全体に瀰漫性の発現に変化した．一方，CSEは，有意な発現量の減少を認めた．PTCの血流も正常マウスに比べ有意に減少しており，この血流低下は，NaHS投与により回復することから，CSE発現低下に伴う尿細管局所でのH_2S産生低下が原因と考えられた．

糖尿病性腎症の主体は，高度タンパク尿を特徴とする糸球体障害である．特に，筆者らは，糸球体内でのNO-VEGFシグナルのアンバランスによる内皮細胞障害が重要であることを報告してきた(25)．一方，進行性腎障害における腎機能低下に関しては，糖尿病性腎症を含めその原疾患の違いに関係なく，糸球体障害の程度より尿細管・間質障害の程度がより腎機能低下に影響を与える重要な因子となっている．

高血糖に由来する糸球体でのNO発現低下は，内皮細胞障害により糖尿病性腎症における糸球体病変の発症・進展に深く関与し，CSE発現低下に伴うH_2S産生低下は，尿細管・間質での虚血障害を進行させ，腎機能低下が進行すると考えられる．

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Identification of blood biomarkers of aging by transcript profiling of whole blood

Seiji Nakamura [a,*], Kozo Kawai [b], Yumie Takeshita [c], Masao Honda [c], Toshinari Takamura [c], Shuichi Kaneko [c], Ryo Matoba [a], Kenichi Matsubara [a]

[a] DNA Chip Research Inc., Suehiro-cho, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan
[b] Department of Internal Medicine, Central Hospital of Matto-Ishikawa, Kuramitsu, Hakusan, Ishikawa 924-8588, Japan
[c] Department of Disease Control and Homeostasis, Kanazawa University Graduate School of Medical Science, Takara-machi, Kanazawa, Ishikawa 920-8641, Japan

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ABSTRACT

Immunological changes that inevitably occur with aging are related to the onset of various diseases including autoimmune diseases, immunodeficiency, as well as other age-reflecting (AR) diseases. They are becoming serious problems in the global trend of longevity. To understand the AR changes, we searched for genes whose expression profiles in the whole peripheral blood change dramatically as a function of age using the Agilent whole human genome 44K microarray. After examining two cohorts consisting of 154 healthy people between age 23 and 77, we discovered 16 transcripts strongly and reproducibly correlated with age. Analysis using a publicly available gene expression dataset for a variety of human immune cells revealed that some of these transcripts were highly expressed in specific cell types whose number and function are known to change with age. This analysis shed light on the molecular mechanism of AR immunological system changes. Because of its simplicity, the assay system is expected to be useful for understanding individual health conditions.

1. Introduction

Senescent immune imbalance has been well known and is becoming a serious problem in the global trend of longevity. In addition to the typical disease-associated problems such as autoimmune diseases and immunodeficiency, the immune system of our body is steadily following paths of transition from the young to elderly. Causes, consequences, as well as mechanism of the immune system transition have been studied intensively, but these works have not reached systematic studies because of enormous variety of individuality [1–3].

One approach to this problem is to focus on age-reflecting (AR) shift of transcript balance in the peripheral blood mononuclear cells regardless of gender. Although factors that can affect the profile may be countless, such as those organ activities wherein immune cells are produced, nursed, educated, or mobilized to cope with changes of our physiological condition such as infection, damage of the body parts, hormone balance and drug-intake: These factors should be eliminated when we attempt to compare "normal" transcriptome profiles of man.

We undertook transcriptome studies with whole peripheral blood of normal, healthy people. Because isolation of peripheral blood mononuclear cells (PBMC) for analyses introduces technology-associated uncertainties, such as loss of significant fraction of white blood cells, loss of fragile transcriptome during the purification process, etc., we took whole peripheral blood as the starting material for this study.

Using simple correlation analyses, we compared blood from young, mature, and senior people for AR transcripts. We used two cohorts consisting of 154 healthy people, male and female, between age 23 through 77. Excluded were those regularly taking drugs, having been treated by doctors, or having biased biochemical data. We discovered 16 transcripts that behaved in AR fashion: Some were more abundant in older individuals (AR-increase), whereas others were less abundant in older individuals (AR-decrease). Here, we report results of the analyses, and describe some properties of these AR transcripts.

2. Materials and methods

2.1. Participants

Approval for the study was obtained from the Ethics Committee for Human Genome/Gene Analysis Research at Kanazawa University Graduate School of Medical Science. Blood samples were obtained from healthy volunteers at two related health examination sites in Ishikawa Prefecture: Hokkoku Clinic (site H), Public Central Hospital of Matto Ishikawa (site M). Of these volunteers, none needed routine doctor's visit nor were treated with any drugs. Informed consent was obtained from all subjects, and clinical data were collected.

* Corresponding author.
E-mail address: nakamura@dna-chip.co.jp (S. Nakamura).

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2.2. RNA extraction from blood

Blood samples were collected in PAXgene Blood RNA tubes (BD, NJ, USA), incubated and stored according to the manufacturer's protocol. Total RNA was extracted using the PAXgene Blood RNA Kit (QIAGEN, CA, USA) following the manufacturer's instructions. RNA quantity and quality were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, DE, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA).

2.3. Microarray and data preprocessing

Cy3-labeled cRNA was synthesized from 250 ng of total RNA using Agilent Low RNA Input Linear Amplification Kit PLUS, One-Color (Agilent Technologies). After checking the quality and quantity of the cRNA using a NanoDrop ND-1000 spectrophotometer and an Agilent Bioanalyzer, the cRNA was hybridized at 65 °C for 17 hours to an Agilent Whole Human Genome Microarray 4 × 44K (Design ID: 014850). After washing, microarrays were scanned using an Agilent DNA microarray scanner (Agilent Technologies). All procedures from labeling to scanning were performed according to the manufacturer's instructions. Intensity values of each scanned feature were quantified using Agilent Feature Extraction Software, which performs background subtractions. Normalization was performed using Agilent GeneSpring GX version 11.0.2. (per chip normalization: 75 percentile shift; per gene normalization: none).

2.4. Extraction of the age-reflecting transcripts

Analysis was performed with probes annotated with Entrez Gene ID. Only those probes whose expression data were available at least 1 sample were taken for further analyses. Because the analyses were done on different occasions on samples from sites H and M, the batch cluster effect could give rise to bias to the microarray data. Therefore, we handled the data from the two sites independently to identify the AR transcripts. With this precaution, we selected transcripts whose expression levels correlated with age irrespectively of the two cohort sites. To further clarify the issue, Pearson's correlation and its significance between normalized gene expression levels and age were calculated under the R environment (http://cran.at.r-project.org), and transcripts with false discovery rate (FDR) of 0.25 or less in both sites were selected as the AR transcripts. We further confirmed the findings by taking GSE23515 in NCBI Gene Expression Omnibus (GEO) database [4] that included peripheral blood microarray datasets with age information.

2.5. Hierarchical clustering analysis

Hierarchical clustering analyses based on per gene normalization (divided by the median value of all samples for each transcripts) were performed by Spearman rank correlation similarity metric and complete linkage clustering algorithm using TIGR MultiExperiment Viewer software (http://www.tm4.org/). Distance threshold for dividing the cluster was set to 0.65.

2.6. Assignment of the AR transcripts to blood immune cell types

Identification of the source cells expressing the AR transcripts was done with GSE22886, the sets of Affymetrix microarray data from a variety of resting and activated human immune cells in NCBI GEO [4,5]. Probe information was combined with Entrez Gene ID. There were no Affymetrix probes corresponding to AMZ1 (Gene ID: 155185). Replicated data with the same cell type were averaged. Cell types that exceeded 1.5 IQR of the upper quartile were defined as "Expected cell types".

2.7. Real-time quantitative reverse transcription PCR

cDNA was synthesized from 500 ng total RNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA). Gene expression was analyzed by real-time quantitative PCR using Applied Biosystems 7900HT real-time PCR system. Specific sets of primers and TaqMan probes were obtained from Applied Biosystems. Gene expression level of the target transcript (CD248) was normalized with the values of an endogenous control gene (GAPDH).

Table 1
Summary of demographics of the subjects enrolled in this study.

	Site H	Site M
Subject number	90	64
Gender		
Male	46	35
Female	44	29
Age	40 ± 10 (min: 23, max: 66)	53 ± 11 (min: 27, max: 77)
BMI (kg/m^2)	21.1 ± 2.3 (min: 16.3, max: 27.5)	22.2 ± 2.7 (min: 16.6, max: 28.5)
sBP (mmHg)	110 ± 11 (min: 90, max: 144)	121 ± 16 (min: 92, max: 156)
dBP (mmHg)	66 ± 9 (min: 50, max: 90)	76 ± 9 (min: 57, max: 97)
AST (IU/L)	20 ± 5 (min: 10, max: 36)	21 ± 5 (min: 13, max: 30)
ALT (IU/L)	18 ± 8 (min: 6, max: 39)	19 ± 6 (min: 10, max: 32)
Glucose (mg/dL)	88 ± 9 (min: 76, max: 127)	94 ± 9 (min: 79, max: 120)
HbA1c (%)	5.0 ± 0.2 (min: 4.5, max: 5.6)	5.2 ± 0.3 (min: 4.2, max: 6.1)
Cholesterol (mg/dL)	188 ± 27 (min: 123, max: 245)	196 ± 29 (min: 134, max: 248)
HDL (mg/dL)	67 ± 14 (min: 40, max: 108)	62 ± 15 (min: 30, max: 94)
Triglycerides (mg/dL)	75 ± 33 (min: 30, max: 192)	89 ± 38 (min: 40, max: 194)
RBC (10^4/μL)	463 ± 46 (min: 345, max: 577)	463 ± 35 (min: 404, max: 569)
Platelet (10^4/μL)	22.8 ± 6.0 (min: 11.3, max: 38.0)	23.9 ± 5.8 (min: 10.6, max: 47.6)
WBC (/μL)	5688 ± 1388 (min: 2900, max: 9300)	4998 ± 1235 (min: 2910, max: 7940)
Neutrophil (/μL)	3455 ± 1095 (min: 1372, max: 6125)	2927 ± 940 (min: 1118, max: 5057)
Eosinophil (/μL)	147 ± 109 (min: 9, max: 539)	151 ± 104 (min: 12, max: 572)
Basophil (/μL)	35 ± 21 (min: 7, max: 112)	21 ± 12 (min: 0, max: 52)
Monocyte (/μL)	296 ± 104 (min: 129, max: 644)	261 ± 75 (min: 90, max: 449)
Lymphocyte (/μL)	1755 ± 505 (min: 896, max: 3572)	1638 ± 499 (min: 698, max: 3008)

Data are expressed as mean ± SD.

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Table 2
List of 11 transcripts whose expression levels are positively correlated with age (AR-increase).

Agilent Probe ID	Gene name	Gene symbol	Gene ID	Site H		Site M		Correlation average	Expected cell types
				Correlation coefficient	FDR	Correlation coefficient	FDR		
A_24_P18137	Neurofilament, light polypeptide	NEFL	4747	0.505	2.17E-03	0.480	1.06E-01	0.492	Memory T-resting (naïve)[a]
A_23_P44674	Cysteine-rich protein 1 (intestinal)	CRIP1	1396	0.419	3.36E-02	0.468	1.07E-01	0.443	NA
A_32_P186731	Isthmin 1 homolog (zebrafish)	ISM1	140862	0.492	2.35E-03	0.391	2.13E-01	0.442	Memory T-resting (naïve)[a], CD4 T cell (naïve and resting)[a]
A_23_P62959	Pleckstrin homology-like domain, family A, member 3	PHLDA3	23612	0.498	2.17E-03	0.385	2.13E-01	0.441	Macrophage-7 day (of differentiatior
A_23_P215048	KIAA0408	KIAA0408	9729	0.419	3.36E-02	0.424	1.99E-01	0.422	B cell (naïve), Helper Th1-12 h (of differentiation)
A_23_P52610	Damage-specific DNA binding protein 2, 48 kDa	DDB2	1643	0.447	1.56E-02	0.396	2.13E-01	0.421	Helper Th2-48 h (of differentiation)
A_23_P92073	Poly(ADP-ribose) polymerase family, member 3	PARP3	10039	0.426	3.08E-02	0.405	2.07E-01	0.415	NA
A_23_P79482	Chimerin (chimaerin) 1	CHN1	1123	0.403	6.26E-02	0.369	2.44E-01	0.386	Memory T-resting (naïve)[a], Memory T-activated[a]
A_23_P391228	Mannosidase, endo-alpha-like	MANEAL	149175	0.381	1.10E-01	0.385	2.13E-01	0.383	NA
A_23_P23924	Calpain 2, (m/II) large subunit	CAPN2	824	0.347	2.12E-01	0.416	2.03E-01	0.382	NA
A_24_P383649	Archaelysin family metallopeptidase 1	AMZ1	155185	0.373	1.30E-01	0.364	2.49E-01	0.368	NA

Correlation coefficients were independently obtained with the site H dataset and site M datasets. Transcripts are arranged according to the "Correlation Average" from the two sites. Assignment of source cells expressing these transcripts are noted in the Materials and methods section.
[a] Over 3 IQR of the upper quartile.

Table 3
List of five transcripts whose expression levels are negatively correlated with age (AR-decrease).

Agilent Probe ID	Gene name	Gene symbol	Gene ID	Site H		Site M		Correlation average	Expected cell types
				Correlation coefficient	FDR	Correlation coefficient	FDR		
A_23_P52697	CD248 molecule, endosialin	CD248	57124	−0.604	3.80E-06	−0.467	1.07E-01	−0.535	CD8 T cell (naïve and resting)[a]
A_24_P314786	Solute carrier family 4, sodium bicarbonate transporter, member 10	SLC4A10	57282	−0.494	2.35E-03	−0.518	4.28E-02	−0.506	B cell (naïve), Memory B-IgM
A_24_P930111	Solute carrier family 4, sodium bicarbonate transporter, member 10	SLC4A10	57282	−0.485	2.90E-03	−0.496	8.62E-02	−0.491	B cell (naïve), Memory B-IgM
A_24_P348806	Pleckstrin homology domain containing, family A member 7	PLEKHA7	144100	−0.350	2.08E-01	−0.479	1.06E-01	−0.415	Plasma B-PBMC [a], Plasma B-bone marrow
A_23_P32444	Matrix-remodelling associated 8	MXRA8	54587	−0.369	1.38E-01	−0.364	2.49E-01	−0.366	Plasma B-bone marrow

Correlation coefficients were independently obtained with the site H dataset and site M dataset. Transcripts are arranged according to the "Correlation Average" from the two sites. Assignment of source cells expressing these transcripts are noted in the Section 2.
[a] Over 3 IQR of the upper quartile.

3. Results

3.1. Clinical characteristic of the two cohorts

Demography of participants in two cohorts of healthy individuals is shown in Table 1. Site H consisted of 46 males and 44 females. Site M consisted of 35 males and 29 females. The balance of gender is deemed reasonable. At site H, the average age of subjects was 40 ± 10, the age range from 23 to 66. At site M, the average age was 53 ± 11, and the age range from 27 to 77. The slight difference in average and distribution of age did not affect the AR studies. As noted, none of these volunteers were suffering from a disease, or had clinical data suggesting the need of medical attention. Bloods were collected using PAXgene Blood RNA tubes, RNA extracted and analyzed using Agilent 44K microarray.

3.2. Identifying the age-reflecting (AR) transcripts

We performed Pearson correlation analyses for the AR transcripts, independently with the H and M datasets. Although members in these two cohorts were not overlapping, we were able to obtain 16 common transcripts that show AR behavior (FDR $\leqslant$ 0.25). The results are shown in Tables 2 and 3, in which transcripts are ordered by the average of the correlation coefficient between the two cohort sites. In Table 2 are listed 11 transcripts whose expression levels are positively correlated with age (AR-increase). The correlation coefficients are 0.505 through 0.347 (FDR: 2.17E-3–2.12E-1) for site H, 0.480 through 0.364 (FDR: 1.06E-1–2.49E-1) for site M. Table 3, on the other hand, lists five transcripts that behave inversely, i.e. their expression levels are negatively correlated with age (AR-decrease). The correlation coefficients were −0.604 through −0.350 (FDR: 3.80E-6–2.08E-1) for site H, −0.518 through −0.364 (FDR: 4.28E-1–2.49E-1) for site M.

Scatter plots of the representative genes, NEFL and CRIP1 (the AR-increase transcripts), and CD248 and SLC4A10 (the AR-decrease transcripts) are shown in Fig. 1. The profiles from sites H and M are nearly superimposable.

To further confirm these findings, we searched the NCBI GEO databases [4] for similar datasets. We found that the GSE23515 data fitted well. Relevant parts of the data are copied in Supplementary

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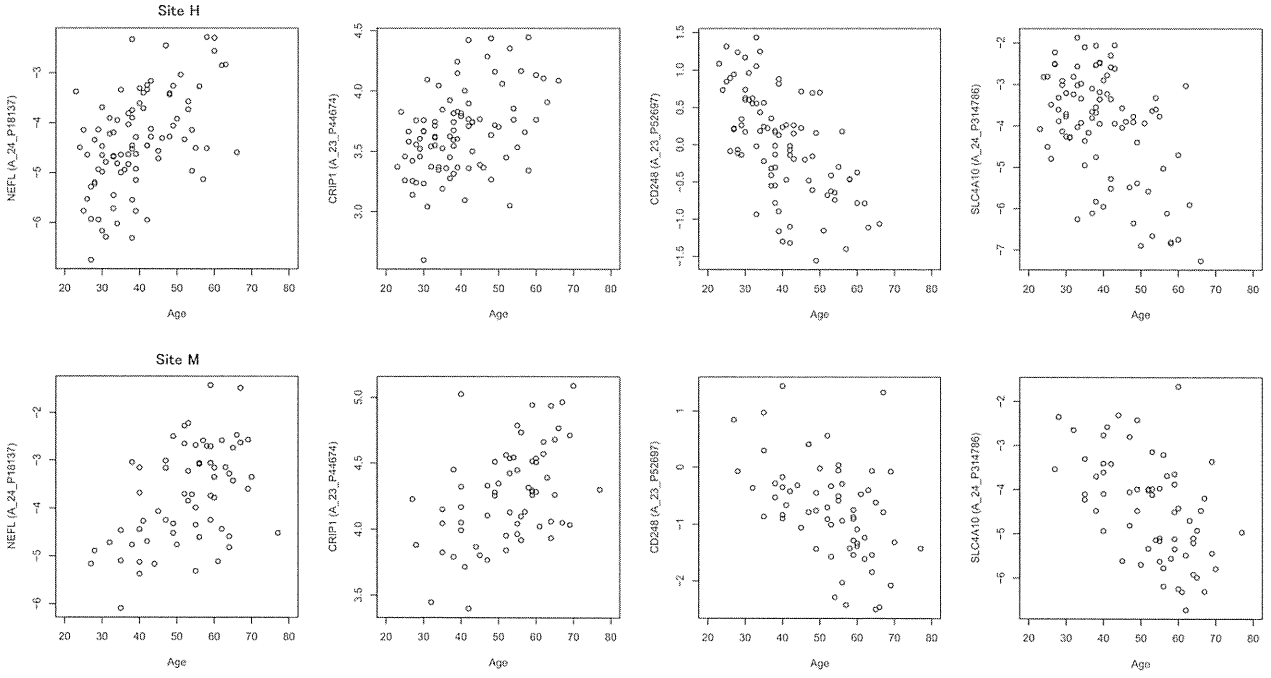

Fig. 1. Representative scatter plots showing correlations between age and some of the AR transcripts. Upper panel shows data from site H, and lower panel data from site M.

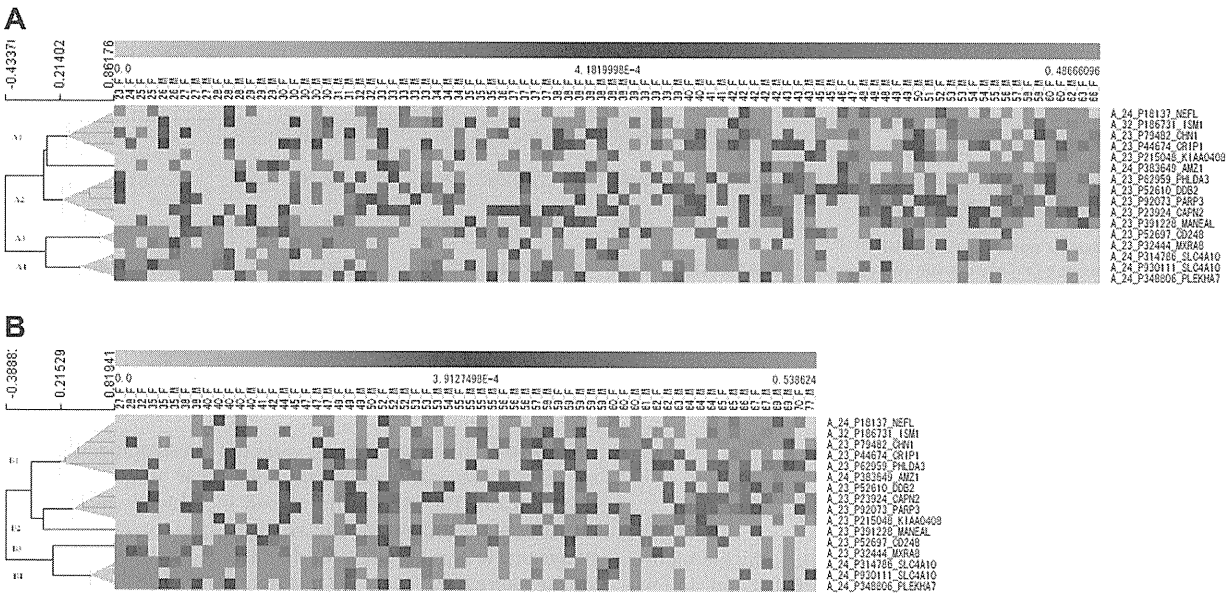

Fig. 2. Hierarchical clustering analysis of 16 AR transcripts based on the expression levels of (A) site H with 90 samples and (B) site M with 64 samples. Details of clustering analyses are described in Materials and methods section. Sample labels represent age and gender.

Tables 1 through 3. Although the subject number is only 24, and the age range is from 21 to 64, the dataset can serve as a perfect source of AR transcripts, and the results showed excellent agreement with ours. For comparison, scattergrams with the four of the transcripts (NEFL, CRIP1, CD248, SLC4A10) are displayed (Supplementary Fig. S1).

3.3. Hierarchical clustering analysis of the AR transcripts

To examine the uniformity/heterogeneity of these and other AR transcripts, we performed hierarchical cluster analysis as shown in Figs. 2A (site H) and 2B (site M). The AR-increase transcripts were grouped into four clusters in Figs. 2A and 2B. Members in cluster A1 show excellent agreement with those in cluster B1. Similarly, members in cluster A2 almost perfectly match with members in clusters B2. As with AR-decrease transcripts, members in A3 and A4 clusters almost perfectly have their counterparts in clusters B3 and B4, respectively. Having these transcripts been extracted independently from the sites H and M, it is highly likely that the AR-increase and AR-decrease transcripts are robust markers, consisting of at least two groups each.

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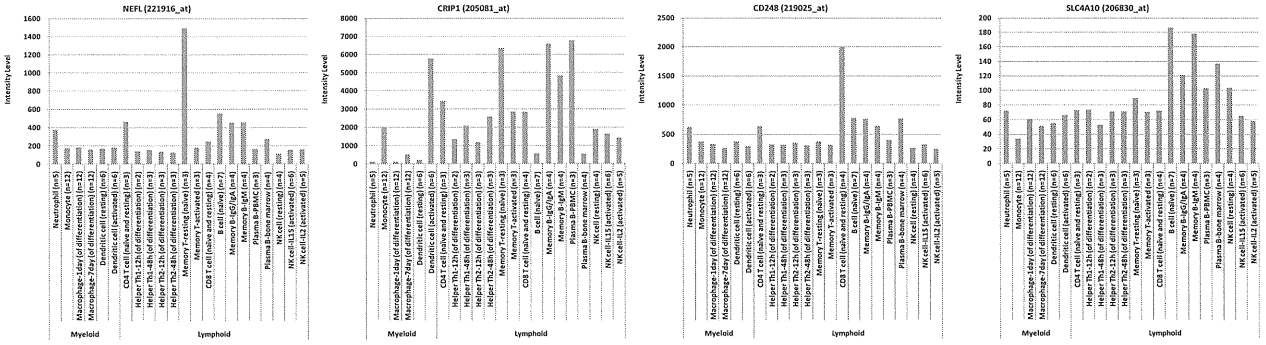

Fig. 3. Expression profiles of the top AR transcripts, NEFL and CRIP1 (AR-increase), and CD248 and SLC4A10 (AR-decrease) in various immune cells under variety of conditions. Texts in parentheses next to the gene symbols indicate Affymetrix probe ID. Data were taken from [4,5].

3.4. Assignment of the AR transcripts to blood immune cell types

Abbas et al. [5] examined gene expression profiles of immune cells under variety of conditions. Although their data were obtained with cultured cells, we took it liberty to refer to these data for assigning our AR transcripts. The selected four AR transcripts in Fig. 1 are displayed in Fig. 3. Other transcripts in Tables 2 and 3 are collectively displayed in Supplementary Fig. S2 in a similar way. The names of referred cells thus obtained are displayed in Tables 2 and 3. Regardless of AR-increase or AR-decrease, significant fractions of the AR transcripts are highly associated with T lymphocytes and B lymphocytes. For example, the top AR-increase transcript, neurofilament light polypeptide (NEFL), is specifically expressed in the memory T resting (naïve) cell, and the top AR-decrease transcript, endosialin (CD248), is highly expressed in the CD8 T (naïve and resting) cell.

3.5. Real-time RT-PCR verification of the AR transcripts

We took CD248, the transcript that showed the strongest correlation with age in microarray expression, and examined it in real-time RT-PCR for verification. We were able to show the expected correlation between this gene expression and age (Fig. 4).

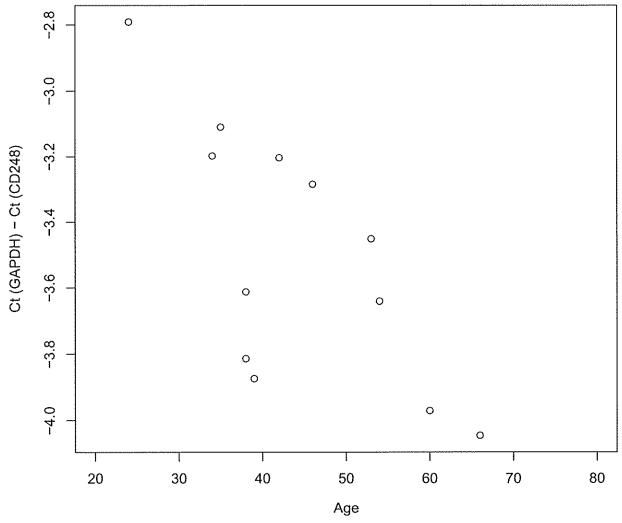

Fig. 4. Real-time RT-PCR verification of the correlation between age and the gene expression level of CD248. Data are shown in scatter plot. The gene expression level of CD248 was normalized to the expression of GAPDH (endogenous control gene).

4. Discussion

Senescence of man and dynamic shift of immune activity have attracted researchers. Studies have been conducted to search for an age-dependent correlation with immune cell differentiation, production, cell population shift in organs or in blood stream [6]. Microarray technologies, which allow transcriptome analyses, have opened the door to a new era in this research field.

We attempted to extract age-reflecting (AR) transcripts in human peripheral blood. The profile of transcripts in the peripheral blood has been believed to be very complex, as population of cells will be unique from a person to person, and will reflect the host's physiological condition, such as disease, infection, injury, fatigue, hormone unbalance and so on, under bewilderingly complex transcriptional controls of immune cells. Nevertheless, we were able to extract AR marker transcripts through cohort studies, independently ran at two sites (90 and 64 participants, age 23–77) using simple correlation analysis between transcripts and age. We found 16 AR transcripts, 11 of which are AR-increase and 5 AR-decrease. Analyses of an another dataset obtained from NCBI GEO have yielded essentially the same results, indicating that these AR transcripts represent a set independent of ethnicity, and can be taken as AR biomarkers of man. Whitney et al. [1] and Eady et al. [7] have attempted similar approaches. Unfortunately, the former authors failed to present a gene list, and the latter authors handled too few samples for qualified data. Furthermore, the latter authors employed isolated peripheral blood mononuclear cells (PBMC), that are liable to errors because of some cell loss, degradation of some mRNA and changes in active genes during purification. As noted, the peripheral blood transcriptome profile is highly affected by donor's physiological condition, such as health condition, exercise, food intake, day rhythm, and many more. Despite these possible factors for complication, our AR transcripts gave rise to reproducible results after taking appropriate precautions.

Most of the genes for AR transcripts are well known, although their roles in AR phenotypes have not been well established. The 16 transcripts do not match the hypothetical longevity genes deduced from SNPs by Sebastiani [8], nor are included in the reference-driven pathway members around sirtuin [9], mTOR [10], Klotho [11]. Though not proved, the 16 AR transcripts may be biomarkers some of which reflect the population change of cells in the blood in the course of aging, and some other reflect gene expression control change within cells. When we examine the 16 transcripts in the GSE22886 dataset in conjunction with the cognate cells, many of them are expressed highly and preferentially in T or B lymphocytes.

Although the roles of the 16 AR marker transcripts in the process of senescence wait further elucidation, we can make some

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inference based on the four clusters of transcripts shown in Fig. 2A and B. Members in Group 1 (A1 and B1 clusters) show AR-increase. At least three of these genes (NEFL, CHN1, ISM1) are uniquely expressed (Over 3 IQR of the upper quartile) in memory T cells (Fig. 3 and Supplementary Fig. S2). Notice that the number of these cells shows increase with aging [12]. Thus, genes in this group may reflect changes in memory T cell number in the aging process.

Group 2 consisted of genes in A2 and B2 clusters. These genes also showed AR-increase in expression. DDB2 and PARP3 included in this group are known for their DNA-repair activities [13,14] or for involvement in cellular accumulation of reactive oxygen species [15], deeply associated with premature senescence. We may be able to infer that the Group 2 genes reflect cellular activity change, rather than cell population shift, caused by senescence.

Group 3 consisted of genes in A3 and B3 clusters. These genes showed AR-decrease expression. No definitive AR functions have been reported for the genes in this group, but CD248 may need special attention: This gene is specifically-expressed in CD8 T cell (Fig. 3), and is active [16]. This gene is also expressed in endothelial progenitor cells [17]. Both cells decreased in number with aging [18,19].

Genes in Group 4, consisting of A4 and B4 clusters, also showed AR-decrease in expression. In cell type assignment analysis, SLC4A10 (Fig. 3) and PLEKHA7 (Supplementary Fig. S2) were highly expressed in B cells. These findings suggest that the decline of gene expression in Group 4 with aging may reflect age-related changes in B cell function.

Because of the paucity of data on gene expression in different cell types in peripheral blood, our argument based on known gene functions in known cell, as above, is incomplete. Nevertheless, we argue that the cluster structures of the 16 transcripts assigned to the four groups (Fig. 2) are stable and relatively homogeneous, demonstrating that a significant number of genes in each group show similar clustering profile. Thus, it may be allowed to take one or several well-characterized genes in each group, such as NEFL (Group 1), DDB2 (Group 2), CD248 (Group 3), and SLC4A10 (Group 4), as representing majority of the genes in each of the four groups. We will be able to discover more AR genes as we extend this line of works. As discussed, some of them may reflect dynamic change of immune cell population, and others may represent emerging/recessing function of immune cells. Further studies on the function of these AR genes and their assignment to cells will provide insights into the roles played by immune system in conjunction with aging of man. In addition, this easy assay system could be used for monitoring the health condition of individuals: For example, the stable balance of the marker set may be taken as reflecting a stable health condition, whereas their distorted proportion could be taken as an alarm of physiological change of the examinee. Notice that fatigue, dementia, unnoticed cancer, drug or supplement intake, and many other temporary or long-lasting physiological changes may induce a balance shift.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2012.01.018.

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INTERNAL MEDICINE

□ CASE REPORT □

Slowly Progressive Insulin-Dependent Diabetes in a Patient with Primary Biliary Cirrhosis with Portal Hypertension-Type Progression

Yumie Takeshita[1], Toshinari Takamura[1], Oto Inoue[1], Miki Okumura[1], Kenichiro Kato[1], Hajime Sunagozaka[1], Kuniaki Arai[1], Hirofumi Misu[1], Minoru Nakamura[3], Yasuni Nakanuma[2] and Shuichi Kaneko[1]

Abstract

A 73-year-old woman had previously been diagnosed with CREST syndrome, PBC and diabetes. Hepatic fibrosis was not evident, in spite of the transudative ascites and active esophageal varices. ACA were positive, whereas AMA and anti-gp210 antibodies were negative. She showed low urinary excretion of C-peptide and was weakly positive for anti-GAD antibody. She was diagnosed with a form of PBC that progresses via portal hypertension rather than liver failure and with SPIDDM. Her HLA type did not contain risk allele for IDDM or PBC. SPIDDM should be considered when patients with PBC with portal hypertension-type progression develop diabetes.

Key words: SPIDDM, primary biliary cirrhosis, portal hypertension, anti-centromere antibodies (ACA), CREST syndrome

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Case Report

A 73-year-old woman was admitted to our hospital in April 2009 for control of diabetes and ascites. She had no family history of diabetes or liver disease. She had developed Raynaud's phenomenon at 40 years of age and sclerodactyly when she was 44. She had been diagnosed with calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia (CREST) syndrome based on her clinical features and the presence of anti-centromere antibodies (ACA). At 65 years of age, a routine screening revealed abnormal serum alkaline phosphatase (ALP) and γ-glutamyl transpeptidase (γ-GTP) levels, postprandial hyperglycemia, and elevated levels of hemoglobin A1c (HbA1c). She did not have a history of alcohol or drug abuse, and was negative for anti-mitochondrial antibodies (AMA), the M2 fraction of AMA, and viral markers for hepatitis B and C. A liver biopsy showed chronic non-suppurative destructive cholangitis (CNSDC)-like bile duct injuries with granulomatous reactions, and intraepithelial lymphocytic infiltration (Fig. 1A). Based on these findings, the patient was diagnosed with primary biliary cirrhosis (PBC) and diabetes. After ursodeoxycholic acid (UDCA) and sulfonylurea (glimepiride 1 mg) were administered, her ALP level remained high, but her diabetes was well controlled.

In June 2009, at 73 years of age, the patient was referred to our hospital because of ascites and worsening diabetes. She presented with anemia, skin thickening, sclerodactyly, palmar erythema without vascular spider, hepatomegaly, an abdominal fluctuation suggestive of ascites, and edema of the leg. Her laboratory data (Table 1) showed elevations in ductal enzyme levels and a preserved hepatic reserve. Her diabetes was poorly controlled (FPG, 341 mg/dL; HbA1c,

[1]Department of Disease Control and Homeostasis, Kanazawa University Graduate School of Medical Science, Japan, [2]Department of Human Pathology, Kanazawa University Graduate School of Medical Science, Japan and [3]National Hospital Organization (NHO) Nagasaki Medical Center and Department of Hepatology, Nagasaki University Graduate School of Biochemical Sciences, Japan
Received for publication August 31, 2011; Accepted for publication September 27, 2011
Correspondence to Dr. Toshinari Takamura, ttakamura@m-kanazawa.jp

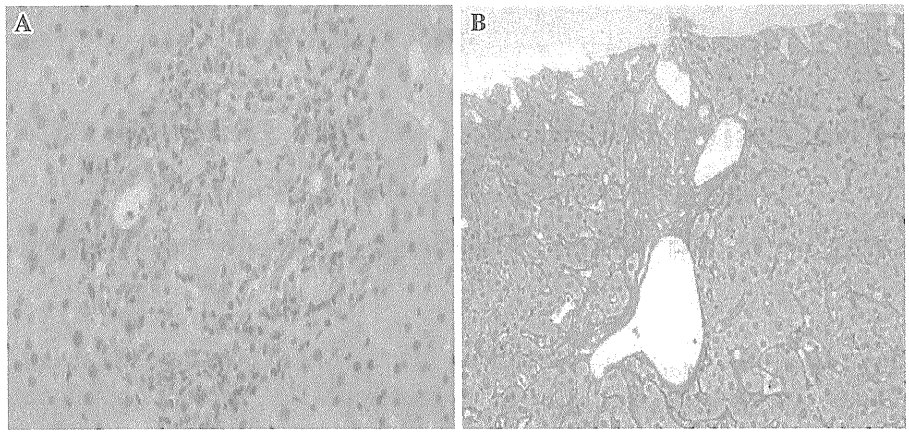

Figure 1. Histological findings from liver needle biopsy specimens. A. Damaged interlobular bile duct associated with a granulomatous reaction. B. The portal tract is sclerotic, and an abnormal blood vessel herniation in the parenchyma is present.

Table 1. Laboratory Data on Admission

WBC	6320	/μL	BUN	28	mg/dL	IgG	893	mg/dL
RBC	2.86×10^6	/μL	Cr	0.39	mg/dL	IgA	389	mg/dL
Hb	9.9	g/dL	ALP	1725	IU/L	IgM	726	mg/dL
Ht	23.3	%	GTP	142	IU/L	Anti-TPO Ab	2.7	IU/mL
Plts	261×10^3	/μL	AST	19	IUL	Anti-Tg Ab	2880	IU/mL
FPG	341	mg/dL	ALT	29	IU/L	HBsAg	Negative	
HbA1c (JDS value)	9.1	%	Amy	28	IU/L	HCVAb	Negative	
			T-Bil	0.4	mg/dL	ANA	640	
			TP	5.7	g/dL	AMA	Negative	
			Alb	3.9	g/dL	AMA-M2	Negative	
			PT	92	%	Anti-GAD Ab	3.2	IU/mL
			CPR	0.3	ng/mL	IA-2 Ab	<0.4	IU/mL
						ACA	120.2	
						Anti-gp210 Ab	0.7	

JDS, Japanese Diabetes Society; PT, prothrombin time; Anti-TPO Ab, thyroperoxidase antibody; Anti-Tg Ab, thyroglobulin antibody; HBsAg, hepatitis B virus surface antigen; HCV, antibody to hepatitis C virus; ANA, anti-nuclear antibodies; AMA, anti-mitocondrial autoantibodies ; Anti-GAD Ab, glutamic acid decarboxylase antibody; IA-2 Ab, insulinoma-associated antigen-2; ACA, anti-centromere antibodies

9.1%). She was positive for various autoantibodies, including anti-glutamic acid decarboxylase antibody (GAD Ab), Tg antibodies, and anti-centromere antibodies, but she was negative for AMA. Her human leukocyte antigen (HLA) type was DRB1*010101, DQB1*050101, DPB1*020102/0501, DQA1*0101.

Abdominal CT revealed hepatomegaly and collateral vascularization, in the paraesophageal region. Gastrointestinal endoscopic examinations showed esophageal varices (linear and white varices without red coloring). We performed hepatic venography to further investigate the collateral vascularization and esophageal varices. The patient's wedged hepatic vein pressure (WHVP) was 11 mmHg and her hepatic venous pressure was 4 mmHg. The normal Hepatic Venous Pressure Gradient (the difference between the WHVP and the free hepatic venous pressures) value is between 1 and 5 mmHg (1). However, while transudative ascites were present, there was no evidence of portal hypertension. A needle liver biopsy was performed. The lobular architecture was relatively normal, and there was dense portal fibrosis without bridging. At the edges of the portal areas, abnormal blood vessels, frequently reported in idiopathic portal hypertension (2), were observed (Fig. 1B). Cholangitis, which had been detected in a previous biopsy (Fig. 1A), was not found.

Concerning the etiology of diabetes, the GAD Ab titer was 3.2 U/mL (normal range [NR], <1.5 U/mL). Plasma levels of basal circulating C-peptide immunoreactivity (CPR) and urinary excretion of CPR were as low as 0.3 ng/mL (NR, 0.94-2.8 ng/mL) and 12 μg/day (NR, 20.5-198 μg/day), respectively. We examined the responses of CPR and glucagon to arginine in the arginine challenge test. ΔCPR and Δglucagon were calculated from the difference between peak values and the base values of CPR and glucagon. Arginine challenge yielded a weak CPR response (ΔCPR, 0.1 ng/mL) and an exaggerated glucagon response (Δglucagon, 382 pg/mL), which are characteristic of type 1 diabetes (3). Based on these findings, a diagnosis of slowly progressive insulin-dependent diabetes mellitus (SPIDDM) was made. Basal-bolus insulin therapy (28 U/day of insulin lispro and 6 U/day of insulin glargine) reduced the patient's HbA1c to

6.5% after 6 months.

Discussion

Collectively, the present patient was diagnosed as having PBC, type 1 diabetes and autoimmune thyroid disease (AITD) based on positivity in Tg antibody and a heterogeneous internal echo finding of the thyroid. Therefore, it may be possible that our patient is included in the entity of autoimmune polyglandular syndrome type 3 that is composed of type 1 diabetes and AITD (4). In gerenal, the prevalence of GAD Ab is significantly higher in patients with AITD than in healthy control subjects (5). However, the present patient's HLA type was different from the frequent DRB1*0405-DQB1*0401 haplotype observed in type 1 diabetes and AITD (5). Chronic liver disease has also been implicated as a complication of Hashimoto's thyroiditis, and the term "hepatothyroiditis syndrome" has been proposed to describe this condition (6). Thyroid disease is also found in about 10-15% of patients with PBC, and AITD is the most common (7). The prevalence of GAD Ab is 5.5% in patients with PBC, higher than that in the healthy population (8). However, to the best of our knowledge, this is only the second reported case of SPIDDM complicated by PBC. SPIDDM (9), which is also referred to as latent autoimmune diabetes in adults (10), generally occurs in adults after a clinical course involving the control of type 2 diabetes with oral hypoglycemic agents. Because the level of GAD Ab was relatively low in the present patient, we should rule out insulinopenic type 2 diabetes in our patient. In this regard, we previously reported that arginine-induced CPR and glucagon responses were negatively and positively correlated with each other in patients with type 1 and type 2 diabetes, respectively (3). Autoimmune type 1 diabetes is caused by a targeted immune reaction that destroys β-cells while leaving the α-cell mass relatively unaffected (11). Therefore, intra-islet insulin deficiency determines the exaggerated glucagon response to arginine in type 1 diabetes. Because the present patient showed a diminished insulin response and exaggerated glucagon response to arginine challenge, we came to the conclusion that our patient has type 1 diabetes rather than type 2 diabetes.

There are at least two different types of progression in PBC: hepatic failure-type progression, which is characterized by the presence of anti-gp210 antibodies, and portal hypertension-type progression, which is characterized by the presence of ACA (12). The present patient was positive for ACA and was therefore deemed to be at high risk of portal hypertension-type progression, rather than hepatic failure-type progression (12). Indeed, she presented with portal hypertension with transudate ascites and esophageal varices without advanced liver fibrosis. The reasons for the different types of progression are not known, but one could argue that specific immunological interactions in the presence of an additional autoimmune disorder may influence the clinical picture and favor a better liver disease outcome (13).

It might be possible that the pathology of SPIDDM and CREST-PBC overlap syndrome are associated in the present patient because exacerbation of diabetes and portal hypertension developed in parallel in the clinical course. SPIDDM and PBC are both autoimmune diseases and share common features. For example, infiltration of CD8+T lymphocytes occurs in the exocrine pancreas in SPIDDM (14) and peripheral damage to the bile ducts is seen in PBC (15). Thus, CD8+T lymphocytes may have played a pathological role in the present case. On the other hand, the HLA susceptibilities in type 1 diabetes and CREST-PBC overlap syndrome are different. HLA-DQA1*0301-DQB 1*0401 haplotype is often present in SPIDDM (16), whereas HLA-Cw6 is often present and HLA-DR2 is often absent in CREST-PBC overlap syndrome (17). The HLA type of the present patient was different than other reported cases of IDDM and PBC. Thus, accumulation of the similar cases complicated with SPIDDM and CREST-PBC overlap syndrome will be necessary to shed light on the common pathology and genetic basis of this condition.

Generally, patients with PBC and collagen disease have lower rates of liver transplantation and liver-related death, and a slower rate of increase in bilirubin levels, compared to patients with PBC alone (18). More attention should be paid to the progression of portal hypertension (i.e., varices and ascites) and diabetic complications, which might be determinants of prognosis.

As a lesson from this case, we suggest that SPIDDM should be considered when patients with CREST-PBC overlap syndrome with portal hypertension-type progression develop diabetes.

The authors state that they have no Conflict of Interest (COI).

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Dietary glycemic index and risk of type 2 diabetes mellitus in middle-aged Japanese men

Masaru Sakurai[a,*], *Koshi Nakamura*[a], *Katsuyuki Miura*[b], *Toshinari Takamura*[c], *Katsushi Yoshita*[d], *Yuko Morikawa*[a], *Masao Ishizaki*[e], *Teruhiko Kido*[f], *Yuchi Naruse*[g], *Yasushi Suwazono*[h], *Shuichi Kaneko*[c], *Satoshi Sasaki*[i], *Hideaki Nakagawa*[a]

[a] *Department of Epidemiology and Public Health, Kanazawa Medical University, 1-1 Daigaku, Uchinada, Ishikawa 920-0293, Japan*
[b] *Department of Health Science, Shiga University of Medical Science, Seta Tsukinowa-cho, Otsu 520-2192, Japan*
[c] *Department of Disease Control and Homeostasis, Kanazawa University Graduate School of Medical Science, 13-1 Takara-machi, Kanazawa 920-8641, Japan*
[d] *Department of Food Science and Nutrition, Graduate School of Human Life Science, Osaka City University, 3-3-138 Sugimoto, Sumiyoshi-ku, Osaka 558-8585, Japan*
[e] *Department of Social and Environmental Medicine, Kanazawa Medical University, 1-1 Daigaku, Uchinada, Ishikawa 920-0293, Japan*
[f] *School of Health Sciences, College of Medical, Pharmaceutical and Health Sciences, Kanazawa University,5-11-80, Kodatsuno, Kanazawa 920-0942, Japan*
[g] *Department of Community and Geriatric Nursing, Toyama University, 2630 Sugitani,Toyama 930-0194, Japan*
[h] *Department of Occupation and Environmental Medicine, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chiba 260-8670, Japan*
[i] *Department of Social and Preventive Epidemiology, School of Public Health, the University of Tokyo, Hongo 7-3-1, Tokyo 113-0033, Japan*

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ABSTRACT

This cohort study investigated the association between dietary glycemic index (GI), glycemic load (GL), and the incidence of type 2 diabetes mellitus in middle-aged Japanese men, and the effect of insulin resistance and pancreatic B-cell function on the association. Participants were 1995 male employees of a metal products factory in Japan. Dietary GI and GL were assessed using a self-administered diet history questionnaire. The incidence of diabetes was detected in annual medical examinations over a 6-year period. The association between GI, GL, and the incidence of diabetes was evaluated using Cox proportional hazards models. During the study, 133 participants developed diabetes. Age- and body mass index–adjusted hazard ratios across the GI quintiles were 1.00 (reference), 1.62, 1.50, 1.68, and 1.80; and those of GL were 1.00 (reference), 1.07, 1.48, 0.95, and 0.98. The hazard ratio for the highest GI quintile was significantly greater than that for the lowest quintile. The influence of GI was more pronounced in the lowest insulin resistance subgroups. GI and pancreatic B-cell function were independently associated with the incidence of type 2 diabetes mellitus; participants with low B-cell function and the highest tertile of GI had the highest risk of diabetes. Dietary GI is associated with the incidence of diabetes in middle-aged Japanese men. GI and B-cell function were independently associated with incidence of diabetes.

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* *Corresponding author.* Tel.: +81 76 286 2211; fax: +81 76 286 3728
E-mail address: m-sakura@kanazawa-med.ac.jp (M. Sakurai).

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1. Introduction

The prevalence of type 2 diabetes mellitus is similar in Asian and Western countries even though the prevalence of obesity is lower in Asia [1]. The high incidence of diabetes in the relatively lean Asian population may be explained, in part, by the presence of more abdominal fat in Asians as compared with white people of similar body mass index (BMI) [2,3]. Furthermore, nonobese Asians who have low pancreatic B-cell function are at high risk for diabetes [4-6].

Dietary factors may also play a role in the high incidence of diabetes in the Asian population. An association between dietary glycemic index (GI), glycemic load (GL), and the incidence of type 2 diabetes mellitus has been reported in Western countries [7-9]; however, the association between GI and type 2 diabetes mellitus in the Asian population is not clear because high-GI rice is a significant part of the Asian diet [10-14] and Asian GI values are higher than those in Western countries [15-19]. At present, the only study examining the relationship between GI and type 2 diabetes mellitus in the Asian population was conducted in women [12]; and none have investigated the association in Asian men.

A high-GI diet is associated with insulin resistance and postprandial hyperglycemia and hyperinsulinemia, which may cause pancreatic B-cell failure and diabetes mellitus [20]. However, no studies evaluating the influence of insulin resistance or B-cell function on the association between GI and the incidence of diabetes have been reported.

In this 6-year prospective study of Japanese men, we investigated the relationship between dietary GI, GL, and the risk of developing type 2 diabetes mellitus. The objectives of the study were to investigate whether dietary GI and GL are associated with the risk of diabetes and to examine the effect of insulin resistance and B-cell function on the relationship.

2. Methods

2.1. Participants

The study participants were male employees of a factory that produces zippers and aluminum sashes in Toyama Prefecture, Japan. Detailed information on the study population has been previously reported [6,13]. The Industrial Safety and Health Law in Japan requires that employers conduct annual health examinations for all employees. A test for diabetes mellitus was conducted during annual medical examinations between 2003 and 2009. In 2003, 2275 (89%) of 2543 male employees aged 35 to 55 years received health examinations and responded to the diet survey. Of these 2275 potential participants, 280 (12%) were excluded: 139 were diabetic or had high fasting plasma glucose (≥126 mg/dL) at the time of the baseline examination, 70 did not have fasting plasma insulin levels measured at the baseline examination, 9 men had a total daily calorie intake less than 500 kcal or greater than 5000 kcal, and 62 did not participate in consecutive follow-up annual health examinations. Thus, 1995 participants were included in the present study.

2.2. Data collection

The annual health examination included a medical history, physical examination, anthropometric measurements, and the measurement of fasting plasma glucose, fasting insulin, glycated hemoglobin (HbA_{1c}), and serum lipid levels. Height was measured without shoes to the nearest 0.1 cm using a stadiometer. Weight was measured with participants wearing only light clothing and no shoes to the nearest 0.1 kg using a standard scale. Body mass index was calculated as weight/height^2 (kilograms/square meter). Blood pressure was measured using a mercury sphygmomanometer after the subject rested for 5 minutes in a seated position. All measurements were taken by trained staff.

Plasma glucose levels were measured enzymatically using an Abbott glucose UV test (Abbott Laboratories, Chicago, IL), and plasma insulin levels were determined using radioimmunoassay (Shionogi, Tokyo, Japan). HbA1c was measured by high-velocity liquid chromatography using a fully automated HbA_{1c} analyzer (Kyoto Daiichi Kagaku, Kyoto, Japan). Total cholesterol and triglycerides were measured using an enzyme assay. High-density lipoprotein (HDL) cholesterol was measured using direct methods. Insulin resistance was calculated by the homeostasis model assessment (HOMA) method using the formula: HOMA-IR = fasting insulin (microunits per milliliter) × fasting plasma glucose (milligrams per deciliter)/405 [21]. The HOMA of β-cell function (HOMA-B) was calculated using the following formula: HOMA-B = 360 × fasting insulin (microunits per milliliter)/[fasting plasma glucose (milligrams per deciliter) − 63] [21].

A questionnaire was used to identify voluntary health-related behaviors such as alcohol consumption, smoking, and habitual exercise. A self-administered questionnaire was also used to collect information about a medical history of hypertension, dyslipidemia, diabetes, the use of antidiabetic medication, and a family history of diabetes. High blood pressure and dyslipidemia were defined using the Japanese criteria for metabolic syndrome [22]: *high blood pressure* was defined as a systolic blood pressure of at least 130 mm Hg or a diastolic blood pressure of at least 85 mm Hg; *dyslipidemia* was defined as serum triglycerides of at least 150 mg/dL or HDL cholesterol less than 40 mg/dL.

2.3. Dietary assessment and calculation of dietary GI and GL

Dietary habits during the preceding month were assessed using a self-administered diet history questionnaire (DHQ) [23]. The DHQ was developed to estimate the dietary intakes of macronutrients and micronutrients for epidemiological studies in Japan. A detailed description of the methods used for calculating dietary intakes and the validity of the DHQ have been reported previously [11,24,25]. Estimates of dietary intake for 147 food and beverage items, energy, and nutrients were calculated in 2007 using an ad hoc computer algorithm developed for the DHQ that was based on the Standard Tables of Food Composition in Japan [26].

Of the 147 food and beverage items included in the DHQ, 6 (4.1%) were alcoholic beverages, 8 (5.4%) contained no available carbohydrate, and 63 (42.9%) contained less than 3.5 g of available carbohydrate per serving. The calculation of

dietary GI and GL was thus based on the remaining 70 items. The GI databases used were an international table of GI [27], several publications concerning the GI of Japanese foods [28-30], recent articles on GI values published after the publication of the international GI table [31,32], and an online database provided by the Sydney University Glycemic Index Research Service [33]. Although concerns have been expressed regarding the utility of GI for mixed meals (overall diet) [34,35], many researchers have shown that the GI of a mixed meal can be consistently predicted as the weighted mean of the GI values of each of the component foods [36,37]. We calculated dietary GI by multiplying the percentage contribution of each food to the daily carbohydrate intake by the GI value of the food and then summed these products. GL was calculated by multiplying the dietary GI by the total daily carbohydrate intake and dividing by 100. We used energy-adjusted values by the density method (per 1000 kcal) for dietary GL [11].

2.4. *Diagnosis of diabetes*

Fasting plasma glucose and HbA_{1c} were measured during the annual medical examinations. Participants with HbA_{1c} greater than 6.0% were given a 75-g oral glucose tolerance test (OGTT). According to the definition of the American Diabetes Association [38] and the Japanese Diabetes Society [39], the diagnosis of diabetes was confirmed by at least one of the following observations: (1) a fasting plasma glucose concentration of at least 126 mg/dL, (2) 2-hour glucose level of at least 200 mg/dL in a 75-g OGTT, or (3) treatment with insulin or an oral hypoglycemic agent.

2.5. *Statistical analysis*

We calculated the incidence rates and hazard ratios (HRs) for diabetes according to the quintile of dietary GI, dietary GL, and total energy intake. The Cox proportional hazard model was used to calculate HRs adjusted for multiple variables, including age (<40, 40-44, 45-49, ≥50 years), BMI (<22, 22-25, ≥25 kg/m^2), family history of diabetes (no, yes), alcohol consumption determined by the DHQ (nondrinker, consumed <20 g/d, consumed ≥20 g/d), smoking status (never, ex-smoker, or current smoker), habitual exercise (no, yes), total energy intake (kilocalories per day, quintile), and dietary total fiber intake (grams per 1000 kcal, quintile). The HR for diabetes was calculated separately for BMI (<22, 22-25, ≥25 kg/m^2), the HOMA-IR or HOMA-B tertile in each GI tertile, and the joint effects of GI and BMI, HOMA-IR, or HOMA-B by cross-classifying participants by both variables. The statistical analyses were conducted using the Statistical Package for the Social Sciences (SPSS version 12.0J, Tokyo, Japan). A P value < .05 was deemed statistically significant.

3. Results

The mean participant age at baseline was 46.0 years, and the mean BMI was 23.4 kg/m^2. The mean dietary GI was 69.2, and the mean dietary GL (/1000 kcal) was 87.9. White rice was the largest contributor to dietary GI (61.2%), followed by noodles (5.4%), bread (5.2%), and confectioneries (4.9%).

The participants' baseline characteristics according to the dietary GI and GL quintile are shown in Table 1 (GI) and Table 2 (GL). No association was observed between dietary GI and age, BMI, serum lipid levels, fasting plasma glucose and insulin, blood pressure, prevalence of high blood pressure, or dyslipidemia. The higher GL quintiles were associated with significantly lower HDL cholesterol, lower fasting plasma glucose, higher fasting insulin, lower systolic/diastolic blood pressure, and a lower prevalence of high blood pressure. Furthermore, high GI and GL were associated with lower dietary energy intake, lower fat intake, lower dietary fiber intake, and higher carbohydrate intake.

During the 6-year follow-up (8988 person-years), we documented 133 cases of diabetes. Among these, 115 diagnoses were based on high fasting plasma glucose levels, 16 were diagnosed according to a 75-g OGTT, and 2 participants had been treated with hypoglycemic medication.

The crude incidence rates (per 1000 person-years) across the GI quintiles from lowest to highest were 10.1, 15.7, 13.6, 16.1, and 18.3, respectively (Table 3). The age- and BMI-adjusted HRs (model 1) across the GI quintiles were 1.00 (reference), 1.62, 1.50, 1.68, and 1.80. The HR of the highest GI quintile was significantly higher than that of the lowest quintile. Further adjustment for family history of diabetes, alcohol intake, smoking, physical activity, the presence of high blood pressure, and dyslipidemia at baseline (model 2) did not affect the HRs. When we used a model adjusted for the variables used in model 2 plus dietary factors (model 3), the HRs across the quintiles were higher than those in models 1 and 2; and the HRs for the fourth and fifth quintiles were significantly higher than that of the first quintile.

The crude incident rates (per 1000 person-years) across the GL quintiles were 13.3, 15.0, 19.5, 12.4, and 14.0 (Table 3). The age- and BMI-adjusted HRs across the BMI quintiles were 1.00 (reference), 1.07, 1.48, 0.95, and 0.98; and no association was found between GL and the incidence of diabetes. The relationships remained nonsignificant even after additional adjustments for potential confounders (models 2 and 3).

Because GI was inversely associated with total energy intake and total fiber intake (Table 1) and positively associated with the incidence of diabetes, we further evaluated the association between total energy intake and total fiber intake and the incidence of diabetes (Table 3). There were no associations between the total energy intake, total fiber intake, and incidence of diabetes.

We analyzed the association between GI and the incidence of diabetes separately in subgroups based on the degree of BMI, insulin resistance, or pancreatic B-cell function at baseline. There were no differences in the associations between GI and baseline characteristics among the different BMI, insulin resistance, and B-cell function subgroups (Supplemental Table 1). High GI was associated with a significantly higher risk of diabetes in participants with a BMI less than 22 kg/m^2, but not in the subgroup with a BMI of 22 to 24.9 kg/m^2 or in participants with a BMI of at least 25 kg/m^2 (Table 4). Similarly, significant positive associations were observed in participants in the lowest HOMA-IR and HOMA-B tertiles, but not in the other tertiles (Table 4). We examined the joint effects of GI and BMI/HOMA-IR/HOMA-B by cross-classifying participants by both variables (Fig. 1). We found a significant