カルシフィラキシス

Calciphylaxis

Key words：カルシフィラキシス，ワルファリン，透析，高カルシウム血症

林　松彦

1. 概念・定義

カルシフィラキシスは，1961年に米国のSelyeにより動物実験を基として提唱された概念である．Selyeはanaphylaxisの研究の延長において，感作因子として副甲状腺ホルモン，ビタミンD関連物質などをラットにあらかじめ投与し，刺激因子として金属塩などを投与した際に皮膚，筋症状を呈することを見いだし，anaphylaxisと対比してcalciphylaxisと名づけたものである[1]．その後，腎不全患者を中心として，皮膚の炎症から壊死に至る，動物実験で生じた皮膚症状と類似した病像を呈する臨床例が報告され，二次性の副甲状腺機能亢進症を伴っていたことから，動物実験におけるカルシフィラキシスと同様の病因によることが推測され，カルシフィラキシスと呼ばれるようになった[2-5]．しかし，病理的な所見は全く異なり，ラットでは皮膚間質の異所性石灰化とそれに基づく病変であるのに対し，臨床例では，小動脈中膜の石灰化，内膜の浮腫状肥厚，更に血栓形成を典型的所見としていることが明らかとなってきた[6]．そこで，カルシフィラキシスという病名は適当ではないとの考えから，calcific uremic arteriolopathyという疾患名が提唱され，米国を中心に広まってきている[7]．一方，一部ではあるが腎不全を伴わない症例も確実に存在しており，uremicと断定することはできないことから，この疾患名が最適ともいえない．

明確な疾患の定義はないが，慢性腎不全患者を中心としてみられる紫斑と有痛性の皮膚潰瘍を臨床的特徴として，病理組織的には患部の小動脈中膜の石灰化，内膜の浮腫状肥厚，血栓形成を特徴とする皮膚疾患をカルシフィラキシスと呼んでいる．

2. 疫　　学

我が国での発症率はこれまで統計がなく不明であったが，平成21年度に，著者らが厚生労働省難治性疾患克服研究事業として行った調査では，透析患者において年間2人/1万人以下と推定された．ただし，疾患に対して極めて認知度が低く，実際にはより多く発症している可能性が考えられる．欧米では1-4％の発症率が報告されている．また，全国調査の際，男女の発症率には差がなかったが，一般透析患者に比べてカルシフィラキシスを発症した症例は年齢層が有意に低かった．

3. 病　　因

1960年代の症例報告では二次性副甲状腺機能亢進症が基礎にあることが示唆された．その後の症例対照研究などの結果では，発症危険因子として高カルシウム血症，高リン血症，肥満，肝疾患，低アルブミン血症，原疾患としての糖尿病腎症，などが同定されている[8-11]．著者らの研究班の調査結果では，低アルブミン血症，高カルシウム血症，高血糖が発症危険因子として同定されたが，最も影響を与えていたのはワルファリンの内服であった．これらの危険因子からカルシフィラキシス発症に至る過程は推測の域を出ていないが，カルシウム・リン代謝の異

Matsuhiko Hayashi: Apheresis and Dialysis Center, School of Medicine, Keio University　慶應義塾大学医学部血液浄化・透析センター

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表1　カルシフィラキシス診断基準案(抜粋)

以下の臨床症状2項目と皮膚病理所見を満たす場合，または臨床症状3項目を満たす場合，カルシフィラキシスと診断される．

【臨床症状】

1　慢性腎臓病で透析中，または糸球体濾過率 15 mL/min 以下の症例
2　周囲に有痛性紫斑を伴う，2カ所以上の皮膚の有痛性難治性潰瘍
3　体幹部，上腕，前腕，大腿，下腿，陰茎に発症する，周囲に有痛性紫斑を伴う皮膚の有痛性難治性潰瘍

【皮膚病理所見】

皮膚の壊死，潰瘍形成とともに，皮下脂肪組織ないし真皮の小～中動脈における，中膜，内弾性板側を中心とした石灰化，および，浮腫性内膜肥厚による内腔の同心円状狭窄所見を認める
(皮膚生検は，症状を増悪させたという報告があり，可能な場合に実施する)

(http://www.dialysis.med.keio.ac.jp/kouroushou/index.html に詳細を掲載)

常が小動脈の石灰化を生じ，小さな外傷などの外的刺激が加わると局所の炎症，そして血栓形成から皮膚壊死に至るという過程が考えられている．また，ワルファリン内服は，凝固因子の局所での変化と，ビタミンK作用拮抗が血管平滑筋細胞を石灰化させるという基礎実験結果もあり，直接発症に関与している可能性が示唆されている．更に，様々な炎症関連タンパク誘導にかかわる情報伝達物質であるNFκBの活性化が中心的役割を果たす可能性も考慮されている[12]．現在では少なくとも，動物実験でSelyeらが提唱した，副甲状腺ホルモンにより感作された状態で，何らかの刺激により皮膚病変が生じるという機序は臨床的なカルシフィラキシスでは否定的である．

4. 病　　態

近位型と遠位型に大別される．近位型は一般に重症であり，下腿，大腿，臀部，躯幹に有痛性の紫斑で始まる皮膚症状が出現し，次第に潰瘍を形成し，拡大，あるいは病変部が増加していく．痛みが極めて強いのが特徴であり，時に硬膜外麻酔が必要となる．潰瘍への感染，更に皮膚病変が広範な場合低栄養も伴い，敗血症などに至って，死亡率は50％前後である．一方，遠位型は，四肢末梢に生じ，手指，足趾に同様の病変が出現する．近位型に比べて予後が良いとされるが，糖尿病性壊疽，あるいは外傷への感染との鑑別は極めて困難である．

5. 診断と鑑別診断

特徴的な皮膚所見を呈する近位型は，カルシフィラキシスという疾患を知っていれば，それほど困難ではない．確定診断は皮膚生検あるいは病巣掻破の際に採取した組織における特徴的病理所見による．問題は，生検を行う場合，生検による創傷が悪化要因になる可能性が指摘されている点である．**表1**に著者らの研究班が提唱している診断基準案を提示するが，この診断基準により少なくとも，全国調査で集めた臨床例はすべて診断可能であった．鑑別診断としては**表2**に挙げた疾患がある．生化学検査所見，血清反応検査などは非特異的炎症所見のみである．

6. 治療と予後

治療の中心は感染対策と，局所の処置である．過去において副甲状腺摘出術が有効であった症例，あるいはシナカルセトの投与が有効であった症例が報告されており[13]，二次性副甲状腺機能亢進症を伴う症例では有用である可能性がある．一方，副腎皮質ステロイド剤の局所，全身投与は同剤自体が危険因子となる可能性が指摘されており，使用すべきではない．最近，チオ硫酸ナトリウムが有効であったとする症例報告がしばしばみられるが[14]，我が国では適応症は

表2 カルシフィラキシスとの鑑別を要する疾患

- ヘパリン起因性血小板減少症(heparin-induced thrombocytopenia: HIT)に伴う皮膚壊死
- ワルファリン潰瘍
- 全身性皮膚硬化症
- nephrogenic systemic fibrosisの初期病変
- コレステロール塞栓
- 蜂窩織炎
- クリオグロブリン血症
- ハイドレアによる皮膚潰瘍
- 抗リン脂質抗体症候群
- 低温熱傷
- 壊死性筋膜炎
- 下肢静脈瘤に伴う潰瘍病変
- 異所性石灰化に伴う皮膚症状

シアン中毒とヒ素中毒となっている点で使用に抵抗がある．副作用としては重篤な代謝性アシドーシスをきたすことがあるが，その際は恐らく透析により改善が期待され，疾患の予後を考えると，十分なinformed consentの後に使用を考慮してもよいであろう．いずれの治療も，疾患の発症頻度が極めて低いことから，前向き無作為試験は実施が事実上不可能である．恐らく，多くの症例を前向きに集めて統計的解析を行うことが最善と考えられ，著者らの研究班ではレジストリーを公開して情報収集を行っている．過去の報告では外科的な患部のdebridment，集中的内科治療，副甲状腺摘出が有効な治療法として同定されている[15]．

疾患の予後は極めて不良で，50％以上が1年以内に死亡したという報告がある．また，著者らの調査でも死亡率は50％を超えている．今後，この疾患への認知度が高まり，早期に集約的な治療を行うことで予後が改善するかを検討していく必要がある．

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腎臓内科学

わが国におけるcacliphylaxisについての全国調査結果と診断規準案策定

National surveillance and diagnostic criteria of calciphyalxis in Japan

Calciphylaxisとは

Calciphylaxis(カルシフィラキシス)は，1960年にSelyeらが動物実験から提唱した概念である．感作因子として副甲状腺ホルモン，ビタミンD関連物質などをラットにあらかじめ投与し，刺激因子として金属塩などを投与した際に皮膚への石灰沈着などの症状を呈する現象を，anaphylaxisと対比してcalciphylaxisと命名したものである[1]．

その後，慢性透析患者を中心として発症する，皮膚の有痛性で多発性・難治性潰瘍を主病変とする疾患が報告され，その発症原因として細小血管の石灰化が主因として考えられたことからcalciphylaxisとよばれるようになった．したがって，動物実験の概念とは異なる病態であり，calcific uraemic atreriolopathyという名称を提唱する研究者もいるが[2]，現在も一般的にはcalciphylaxisとよばれている．

Calciphylaxisは皮膚潰瘍への細菌感染から敗血症などを併発して死亡率が50%を超えることが報告され，欧米での発症率は調査対象などにより異なるが，透析患者の数%に上る可能性も示されている[2]．正確な発症状況については現在，アメリカ，ヨーロッパ，それぞれにおいてレジストリーが作成されており，近い将来，さらに詳細な検討結果が発表されることが期待される．

わが国における全国調査と診断規準案

一方，わが国では本疾患の発症状況についての系統的な調査・研究はなされたことはなく，実態はまったく不明であった．そこでcalciphylaxisの臨床上の重要性から，同疾患の実態把握，病態解明，診断基準作成を目的に著者を研究代表者とした平成21年度厚生労働省難治性疾患克服研究事業「Calciphylaxisの診断・治療に関わる調査・研究」により，はじめて全国調査が実施された．

2009年8月に日本透析医学会所属3,760施設に対して調査票を配布し，151施設から計249例の症例を経験したことが報告された．その結果，症例の性別・年齢分布はわが国の透析患者全体と比べ，男女比はほぼ同等であるが，年齢分布は30代，40代，50代に多い傾向を示した．また，calciphylaxisについて“よく知っている”，“だいたい知っている”と答えた施設は40%にとどまっていた．さらに，67例について病理所見などを含む詳細な臨床情報が集められたが，臨床症状，病理所見よりcalciphylaxisと確定診断できた症例はこのなかの35例であった．これら35例に基づいて表1のような診断規準案を作成し，現在その妥当性を検証中である．

おわりに

2010年度以降，レジストリーを作成するとともに，対照研究，前向き観察研究を実施中であり，さらに本症の実態が明らかとなることが期待される．なお，表1の診断規準は抜粋であり，本研究の詳細とともに，以下のホームページを参照されたい(ホームページアドレス http://www.dialysis.med.keio.ac.jp/kouroushou/index.html)．

表1 Calciphylaxis診断規準案抜粋

以下の臨床症状2項目と皮膚病理所見を満たす場合，または臨床症状3項目を満たす場合，calciphylaxisと診断される．

臨床症状

1．慢性腎臓病で透析中，または糸球体濾過率15 ml/min以下の症例
2．周囲に有痛性紫斑をともなう，2カ所以上の皮膚の有痛性難治性潰瘍
3．体幹部，上腕，前腕，大腿，下腿，陰茎に発症する，周囲に有痛性紫斑をともなう皮膚の有痛性難治性潰瘍

皮膚病理所見

皮膚の壊死，潰瘍形成とともに，皮下脂肪組織ないし真皮の小～中動脈における，中膜，内弾性板側を中心とした石灰化，および，浮腫性内膜肥厚による内腔の同心円状狭窄所見を認める．

(皮膚生検は，症状を増悪させたという報告があり，可能な場合に実施する)

(http://www.dialysis.med.keio.ac.jp/kouroushou/index.html に詳細を掲載)

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林 松彦／Matsuhiko HAYASHI
慶應義塾大学医学部血液浄化・透析センター

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NEPHROLOGY – ORIGINAL PAPER

Association of peripheral artery disease and long-term mortality in hemodialysis patients

Shigeru Otsubo · Mariko Kitamura · Takako Wakaume · Aiji Yajima · Miwa Ishihara · Masayo Takasaki · Syuitsu Ueda · Hisayuki Sugimoto · Kimiko Otsubo · Naoki Kimata · Takashi Akiba · Kosaku Nitta

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Abstract
Background Peripheral artery disease (PAD) is a common complication in hemodialysis patients. The ankle-brachial blood pressure index (ABI) has been widely used to screen for subclinical PAD. In the present study, we investigated the association between ABI and long-term (up to 8.8 years) mortality among hemodialysis patients.
Methods A total of 86 consecutive patients receiving maintenance hemodialysis who underwent an ABI examination between 2001 and 2003 were retrospectively enrolled in this study. Patients with an ABI of less than 0.9 were considered as having PAD; those with an ABI of more than 0.9 in both legs were considered as being free from PAD. We examined the relationship between mortality and several risk factors.
Results During the follow-up period, 43 deaths were recorded. In the univariate regression analysis, the mortality hazard ratio (HR) of patients with PAD was 1.67 (95% confidence interval [CI], 1.18–2.28). Other predictive variables for mortality included male gender, age, and diabetes mellitus ($P = 0.006$, $P = 0.024$, and $P = 0.023$, respectively). A multivariate Cox analysis identified PAD and male gender as independent predictors of mortality ($P = 0.033$ and $P = 0.028$, respectively). The impact of age and diabetes mellitus on mortality was no longer significant in the multivariate analysis.
Conclusion After a relatively long-term observation period, a multivariate analysis indicated that PAD acted independently of other risk factors, including advanced age and the presence of diabetes mellitus. ABI measurements can be used to identify high-risk hemodialysis patients requiring intensive follow-up care.

Keywords Ankle-brachial index · Chronic kidney disease · Long-term outcome · Peripheral artery disease

S. Otsubo (✉) · M. Kitamura · T. Wakaume · M. Ishihara · M. Takasaki · S. Ueda · H. Sugimoto · K. Otsubo
Sangenjaya Hospital, 1-21-5 Sangenjaya, Setagaya-ku, Tokyo 154-0024, Japan
e-mail: sotsubo@kc.twmu.ac.jp

S. Otsubo
Faculty of Human Care, Department of Nursing, Tohto College of Health Sciences, Saitama, Japan

A. Yajima
Towa Hospital, Tokyo, Japan

N. Kimata · T. Akiba
Department of Blood Purification, Kidney Center, Tokyo Women's Medical University, Tokyo, Japan

K. Nitta
Department of Medicine, Kidney Center, Tokyo Women's Medical University, Tokyo, Japan

Introduction

Peripheral artery disease (PAD) is a common complication in hemodialysis patients. Epidemiological and clinical studies in the general population have demonstrated that the presence of PAD is associated with an increased risk of myocardial infarction and stroke [1, 2]. Limited available data also suggest that PAD is prevalent among hemodialysis patients [3–5] and is associated with poor outcomes [3, 6, 7]. The ankle-brachial blood pressure index (ABI) has been widely used to screen for subclinical PAD and is believed to be strongly correlated with PAD of the lower limbs [8]. A recent study revealed that ABI is an independent and strong predictor of all-cause and cardiovascular mortality in hemodialysis patients [6]. However, the average follow-up period of this previous study was only 2 years [6], and no information is available about ABI and long-term mortality in hemodialysis patients. In the present study, we investigated the association between ABI and long-term (up to 8.8 years) mortality among hemodialysis patients.

Materials and methods

A total of 86 consecutive patients receiving maintenance hemodialysis who underwent an ABI examination between 2001 and 2003 were retrospectively enrolled in this study. Clinical data, including age, gender, duration of hemodialysis therapy, diabetes mellitus complications, and smoking habits, were collected. The clinical follow-up data were obtained from the hospital records. The clinical endpoint was defined as death from any cause. Patients with an ABI of less than 0.9 were considered as having PAD (PAD group); those with an ABI of more than 0.9 in both legs were considered as being free from PAD (non-PAD group) [9]. We compared the clinical data among the PAD group and the non-PAD group and examined the relationship between mortality and several risk factors. Among 41 patients who were more than 60 years old, we also compared the survival rate between the PAD group and the non-PAD group.

ABI was determined in all the patients using an ABI-form (Colin, Japan), which simultaneously measures bilateral arm and ankle blood pressures (brachial and posterior tibial arteries, respectively) using the oscillometric method. The blood pressure was measured after a dialysis session and after the patients had rested in a supine position for at least 5 min. The ABI was calculated using the ratio of the ankle systolic pressure divided by the arm systolic pressure. The systolic pressure of the arm without dialysis access and the lower value of the ankle pressure were used for the calculation.

Statistical analysis

All data were expressed as means ± SD. The Student *t* test was used for comparisons between continuous variables. The chi-square or Fisher exact probability test was applied for categorical data. Univariate and multivariate analyses used the Cox proportional hazards model. The survival curves were estimated using the Kaplan–Meier method followed by a log-rank test. All statistical calculations were performed using JMP 5.1 software. P values less than 0.05 were considered to denote statistical significance.

Results

The patient background characteristics are shown in Table 1. Among the 86 patients, 19 patients had PAD (22.1%). The patients in the PAD group were significantly older (65.2 ± 8.3 years) than those in the non-PAD group (58.3 ± 8.6 years, $P = 0.0027$). The percentage of patients with diabetes mellitus was also higher in the PAD group (42.1%) than in the non-PAD group (13.4%, $P = 0.0099$). No differences in the duration of hemodialysis and the smoking habits were observed between the two groups.

During the follow-up period, 43 deaths were recorded. Table 2 shows a Cox proportional hazards analysis of the covariates for predicting mortality. In the univariate regression analysis, the hazard ratio (HR) of patients with PAD was 1.67 (95% confidence interval [CI], 1.18–2.28). Other predictive variables for mortality included male gender, age, and diabetes mellitus ($P = 0.006$, $P = 0.024$, and $P = 0.023$, respectively). The multivariate Cox analysis identified PAD and male gender as independent predictors of mortality ($P = 0.033$ and $P = 0.028$, respectively). The impact of age and diabetes mellitus on mortality was no longer significant in the multivariate analysis.

Table 1 Background characteristics of the study participants

	Total	PAD group	Non-PAD group	*P* value
Number *n* (male, % male)	86 (60, 69.8%)	19 (14, 73.7%)	67 (46, 68.7%)	NS
Age (years)	59.8 ± 9.0	65.2 ± 8.3	58.3 ± 8.6	0.0027
Duration of hemodialysis (years)	15.1 ± 10.1	16.7 ± 9.9	14.6 ± 10.1	NS
Diabetets mellitus *n*, (%)	17 (19.8%)	8 (42.1%)	9 (13.4%)	0.0099
Smoking *n*, (%)	18 (20.9%)	3 (15.8%)	15 (22.4%)	NS

Table 2 Cox proportional hazards regression analysis for mortality

Parameters	Univariate		Multivariate	
	Hazard ratio (95% CI)	*P* value	Hazard ratio (95% CI)	*P* value
PAD (Y)	1.63 (1.18–2.28)	0.004	1.58 (1.04–2.38)	0.033
Gender (male)	1.67 (1.15–2.61)	0.006	1.55 (1.05–2.46)	0.028
Age (per year)	1.04 (1.01–1.08)	0.024	1.02 (0.98–1.06)	0.305
Duration of dialysis (per 1 year)	0.98 (0.95–1.01)	0.208	0.99 (0.95–1.02)	0.446
Diabetets mellitus (Y)	1.48 (1.06–2.02)	0.023	1.15 (0.76–1.71)	0.504
Smoking (Y)	1.19 (0.82–1.65)	0.338	1.25 (0.84–1.80)	0.263

Table 3 Background characteristics of study participants over the age of 60 years

	Total	PAD group	Non-PAD group	*P* value
Number *n* (male, % male)	41 (30, 73.2%)	14 (10, 71.4%)	27 (20, 74.1%)	NS
Age (years)	67.4 ± 5.0	68.7 ± 6.4	66.7 ± 4.0	NS
Duration of hemodialysis (years)	12.3 ± 8.7	13.0 ± 8.9	11.9 ± 8.7	NS
Diabetets mellitus *n*, (%)	10 (24.4%)	8 (57.1%)	2 (7.4%)	0.001
Smoking *n*, (%)	5 (12.2%)	1 (7.1%)	4 (14.8%)	NS

The background data for hemodialysis patients over the age of 60 years at the start of the observation period are shown in Table 3. Among the 41 patients, 14 patients had PAD. The patients' age was not significantly different between the two groups. The rate of diabetes mellitus complications was higher in the PAD group (57.1%) than in the non-PAD group (7.4%, $P = 0.0001$). Figure 1 shows a comparison of survival curves for patients with PAD and those without PAD. Even though the age was similar in the two groups, the curves showed a statistically significant difference between the two groups ($P = 0.0289$). Patient survival rate at 8 years was 28.6% in the PAD group and 61.6% in the non-PAD group.

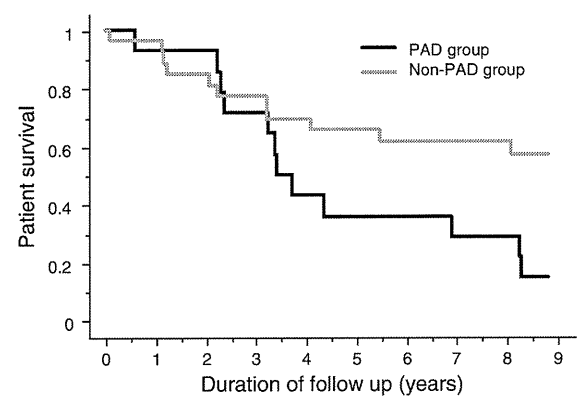

Fig. 1 Survival probabilities of hemodialysis patients with or without PAD who are over the age of 60 years. Log-rank test, $P = 0.0289$

Discussion

The prevalence of PAD in our center was 22.1%. This prevalence was higher than that reported by the United States Renal Data System (USRDS) (15%) and similar to that reported by the HEMO Study (23%) in patients undergoing maintenance hemodialysis [4, 10]. This value is much higher than previously published data in an Asian population of type 2 diabetes patients without chronic kidney disease (10%) and almost the same as that reported for Taiwanese peritoneal dialysis patients (19.6%) [11, 12]. In dialysis patients, both atherosclerosis (mainly affecting the intima of the arteries) and arteriosclerosis (affecting predominantly the media of large- and middle-sized arteries diffusely) are highly prominent. Arteriosclerosis, characterized by reduced arterial compliance (i.e., reduced elasticity of the arteries), is due to increased fibrosis, loss of elastic fibers, and extensive vessel wall calcification [13]. The transformation of vascular smooth muscle cells into chondrocytes or osteoblast-like cells seems to be a key element in vascular calcification pathogenesis, in the context of passive calcium and phosphate deposition due to abnormal bone metabolism and impaired renal excretion [14].

Some studies have reported conventional risk factors for PAD in hemodialysis patients, including advanced age, white race, male gender, diabetes mellitus, coronary artery disease, cerebrovascular disease, smoking, and left ventricular hypertrophy [4, 15]. Dialysis-specific factors included the duration of dialysis, a malnourished status, hypoalbuminemia, disorders of mineral metabolism, and hypoparathyroidism [4, 15, 16]. In our study, PAD was significantly correlated with advanced age and diabetes in hemodialysis patients, similar to the results of previous reports [4, 15]. The duration of hemodialysis tended to be longer in PAD patients than in non-PAD patients but was not statistically significant. This difference may be a result of the rather small number of examined patients. Other associations with PAD could not be evaluated because of the lack of data.

Cardiovascular disease is the leading cause of death in both chronic kidney disease and peritoneal dialysis/hemodialysis patients [13, 16]. Vascular disease prevention in these patients is therefore important to reduce the incidence of cardiovascular events and the high morbidity and mortality [16]. An important issue in the management of patients with PAD is the elevated risk of cardiovascular disease, stroke, and premature death among patients with PAD of the lower limbs [2, 14, 17]. ABI is a simple, reliable tool that can be used to diagnose the existence and severity of PAD. ABI was determined using the ABI-form (Colin, Japan), which simultaneously measures the arm and ankle blood pressures using an oscillometric method. This automatic device allowed us to measure the ABI quickly, non-invasively, and reproducibly. Several recent investigations have also shown an association between an abnormal ABI and cardiovascular morbidity and mortality [18, 19]. As the results of our relatively long-term observation period (maximum of 8.8 years) showed, PAD was an independent risk factor for mortality. The survival of hemodialysis patients older than 60 years of age was 28.6% in the PAD group and 61.6% in the non-PAD group at 8 years. The multivariate analysis indicated that PAD acted independently of the other risk factors, including an advanced age and diabetes mellitus. Thus, the measurement of ABI effectively identified high-risk hemodialysis patients, a target population requiring intensive follow-up. Previously, multiple conventional risk factors for mortality in hemodialysis patients were analyzed [6]. Although diabetes mellitus was a strong predictor in the univariate analysis, it did not have a statistically significant impact on mortality in the multivariate analysis. These results are similar to those obtained in the present study, suggesting that the impact of diabetes mellitus might be mediated by atherosclerotic vascular disease, represented by a low ABI [6]. In our study, a male gender was another independent risk factor for mortality, similar to the results of a previous report [6].

Our study had several limitations. First, the study was a retrospective examination of patients evaluated at a single institution, and there were differences in care, including medications, over the course of the study. Second, the associations of other factors with PAD could not be evaluated because of a lack of data.

Conclusions

PAD is strongly correlated with advanced age and diabetes in hemodialysis patients. After a relatively long-term observation period, a multivariate analysis indicated that PAD acted independently of other risk

factors, including age and diabetes mellitus. ABI is a simple, reliable tool that can be used to diagnose the existence and severity of PAD. Thus, ABI measurements can be used to identify high-risk hemodialysis patients requiring intensive follow-up care.

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Comparison between Whole and Intact Parathyroid Hormone Assays

Masatomo Taniguchi,[1] Motoko Tanaka,[2] Takayuki Hamano,[15] Shohei Nakanishi,[3] Hideki Fujii,[4] Hitoshi Kato,[5] Fumihiko Koiwa,[6] Ryoichi Ando,[7] Naoki Kimata,[8] Takashi Akiba,[8] Takashi Kono,[10] Keitaro Yokoyama,[9] Takashi Shigematsu,[11] Takatoshi Kakuta,[12] Junichiro James Kazama,[13] Yoshihiro Tominaga,[14] and Masafumi Fukagawa[12]

[1]Department of Medicine and Clinical Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka, [2]Department of Nephrology, Akebono Clinic, Kumamoto, [3]Chibune General Hospital, Osaka, [4]Division of Nephrology and Kidney Center, Kobe University Graduate School of Medicine, Hyogo, [5]Department of Nephrology and Hypertension, Saitama Medical University, Saitama Medical Center, Saitama, [6]Division of Nephrology, Department of Internal Medicine, Showa University Fujigaoka Hospital, Kanagawa, [7]Department of Nephrology, Musashino Red Cross Hospital, [8]Department of Blood Purification, Kidney Center, Tokyo Women's Medical University, and [9]Division of Kidney and Hypertension, Department of Internal Medicine, Jikei University School of Medicine, Tokyo, [10]Mihama Narita Clinic, Chiba, [11]Division of Nephrology and Blood Purification Medicine, Department of Medicine, Wakayama Medical University, Wakayama, [12]Division of Nephrology, Endocrinology and Metabolism, Tokai University School of Medicine, Kanagawa, [13]Division of Blood Purification Therapy, Niigata University Medical and Dental Hospital, Niigata, and [14]Division of Endocrine Surgery, Department of Transplant and Endocrine Surgery, Nagoya Second Red Cross Hospital, Aichi, Japan; and [15]Clinical Epidemiology Unit, Center for Clinical Epidemiology and Biostatistics, University of Pennsylvania School of Medicine, Philadelphia, PA, USA.

Abstract: The standard measurement of parathyroid hormone (PTH) is the intact PTH (iPTH) assay, which is used for approximately 90% of Japanese dialysis patients. The iPTH assay reacts not only with 1–84 PTH, but also with large truncated fragments of non-1–84 PTH, including 7–84 PTH. On the other hand, the whole PTH assay is specific for 1–84 PTH. The aim of the current study was to define the validity of both whole and intact PTH assays. A total of 738 hemodialysis patients were enrolled from twelve dialysis services. The serum PTH level was evaluated by both intact and whole PTH assays simultaneously. Non-1–84 PTH was determined by subtracting the whole PTH value from that of the intact PTH assay. The median level of whole PTH was 121 pg/mL, and that of iPTH was 210 pg/mL. The whole PTH assay had a very high correlation with the iPTH assay ($r = 0.870, P < 0.001$). For 43 out of 738 patients (5.8%) the value for intact PTH—whole PTH was <0. Both assays significantly correlated with non-1–84 PTH ($P < 0.001$), while the iPTH assay, particularly, had a very high correlation with non-1–84 PTH ($r = 0.791$). As a whole, 18% of the total population was misclassified into a different Japanese guideline category. Stratified by Japanese guideline classifications, 28% of patients within an iPTH target range were misclassified. Using Bland–Altman plot analysis, as the serum PTH level increased, there was a large difference between two assays. Both PTH assays correlate strongly, although the whole PTH assay may be more useful for precise evaluation of PTH function than the iPTH assay. **Key Words:** Intact parathyroid hormone, non-1–84 parathyroid hormone, N-parathyroid hormone, whole parathyroid hormone.

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Address correspondence and reprint requests to Dr Masatomo Taniguchi, Department of Medicine and Clinical Science, Graduate School of Medical Sciences, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812-8582, Japan. Email: masatomo@kcu.med.kyushu-u.ac.jp

Secondary hyperparathyroidism is commonly observed in dialysis patients. Measurement of parathyroid hormone (PTH) values is used to assess the severity of hyperparathyroidism, to decide when to start a vitamin D receptor activator or cinacalcet, and

to monitor the effectiveness of treatment. The standard measurement of PTH is the intact PTH (iPTH) assay, which was developed in late 1980s. In Japan, the iPTH assay was used in approximately 90% of all dialysis patients in the data of Japanese Renal Data Registry 2009, although, the iPTH assay was shown to react not only with 1–84 PTH but also with large truncated fragments of non-1–84 PTH, including 7–84 PTH (1–4). In humans, 20–60% of PTH measured with the iPTH assay corresponds to non-1–84 PTH (1,3–5). In dialysis patients, the percentage of non-1–84 PTH calculated by the iPTH assay is generally greater than normal people (1–6). The whole PTH assay has been shown to be specific for 1–84 PTH. Serum concentrations of large, truncated fragments of non-1–84 PTH can be determined by subtracting the whole PTH value from that measured with the iPTH assay (7,8). In parathyroidectomized rats, a 7–84 PTH infusion, which is a main fragment of non-1–84 PTH, was shown to inhibit a calcemic action of simultaneously infused 1–84 PTH (7). These results suggest that large, truncated PTH fragments similar to 7–84 PTH may be an important cause of skeletal resistance to 1–84 PTH in uremia. In a study of hemodialysis patients, the 1–84 PTH/non-1–84 PTH ratio was less than 1 only in those patients with low bone turnover (8). This result also suggests that large carboxy-terminal fragments may antagonize the skeletal effects of 1–84 PTH. Moreover, the 7–84 PTH fragment, like other carboxy-terminal fragments, binds only to the carboxyl PTH receptor and does not affect the binding of 1–84 PTH to the PTH related peptide (PTHrP) receptor (9–11). Thus, it would appear that the 7–84 PTH fragment may decrease the calcemic action of 1–84 PTH through its interaction with the carboxyl PTH receptor. In order to assess the severity of hyperparathyroidism accurately, it is important to understand the characteristics and the differences of both whole and intact PTH assays.

The aim of the current study was to define the validity of both whole and intact PTH assays by assessing: (i) the comparison of distribution; (ii) the correlation between both assays; (iii) the association of both assays with patient characteristics; and (iv) the comparison between whole PTH and calculated 1–84 PTH levels.

PATIENTS AND METHODS

Patient selection

A total of 738 hemodialysis patients were enrolled in Japan from twelve hemodialysis clinics in five prefectures between June 2000 and May 2006. These associated clinics included: Tokai University School of Medicine, Sekisinkai Kawasaki Clinic, Dai2-Rokushima Clinic, Sekishin Clinic, Japan Red Cross Musashino Hospital, Nakano Clinic, Kyonan Clinic, Suda Clinic, Tokyo Women's Medical University, Mihama Narita Clinic, Matsushita-Kai Akebono Clinic, and Sumiyoshi Clinic Hospital. Entry criteria included having been hemodialysis patients in the preceding three months, the ability to provide informed consent for participation, and being of an age greater than 19 years.

Data collection and PTH assays

Blood used for PTH analyses was collected from the study participants as part of a special lab specimen draw. The serum PTH level was evaluated by both intact and whole PTH assays simultaneously. An EDTA-containing tube was collected before hemodialysis, kept on ice for 1 h, and immediately centrifuged at 1000 *g* for 10 min, separated and refrigerated. PTH has been shown to be stable for 72 h in refrigerated EDTA plasma (12). Specimens were stored at –20°C until they were thawed for analysis at DS Pharma Biomedical (Osaka, Japan).

A single incubation step immunoradiometric assay specific for whole PTH (1–84) was developed and optimized with the previously-mentioned assay reagents. Briefly, 200 μL of assay standards, controls, and patient samples were pipetted into appropriately labeled 12 mm × 7.5 mm polypropylene test-tubes. One hundred microliters of ^{125}I-labeled PTH-specific antibody tracer solution and one goat anti-PTH (39–84) polyclonal antibody-coated bead were added to all test-tubes (1–4). The immunochemical reaction was conducted at room temperature with shaking at 170 rpm for 18–22 h. During this assay incubation period, the immunochemical reaction forming the sandwich of (solid-phase goat anti-PTH (39–84) antibody)—(whole PTH (1–84))—(^{125}I-goat anti-PTH antibody) takes place in correlation with the amount or concentration of whole PTH (1–84) in the test sample. All beads in the test tubes, except the total count tube, were washed with the wash solution, and the radioactive signals from each bead were counted for 1 min using a gamma scintillation counter. The data were processed and calculated using nonlinear regression data reduction software.

Other laboratory measures

Other laboratory measures included measures of serum calcium, phosphate, albumin, alkaline phosphatase, total cholesterol, and HDL-cholesterol from dates that were closest available to the special lab draw date. Serum calcium was adjusted for albumin

using the formula: adjusted calcium = measured calcium – ((4.0 – serum albumin in g/dL)) (13). All calcium values reported and used in this analysis were corrected using the above formula. PTH values were log-transformed in some analyses due to their non-normal distributions.

Statistical analysis

Data are expressed as the mean ± SD. All statistical analyses were conducted using the DOCTOR SPSS II 18.0 program (SPSS Japan, Tokyo, Japan). Differences between groups were calculated using the Mann–Whitney *U*-test, the χ^2-test or ANOVA, where appropriate. Correlation coefficients were calculated by the Pearson method. The comparison of ranks between the whole and intact PTH values was performed using Student's *t*-test. A P value <0.05 was considered significant.

RESULTS

Baseline characteristics and laboratory data

As shown in Table 1, we studied 738 patients (58% male gender) with end-stage renal failure from five dialysis units. All patients underwent chronic hemodialysis treatment three times weekly. The median age of the patients was 60.0 years (range 9–103, mean 58.8 ± 12.6 years). The median vintage of hemodialysis was 132 months (range 2–1238, mean 145 ± 102 months). The underlying renal diseases were: chronic glomerulonephritis, 66%; diabetic nephropathy, 17%; hypertensive nephrosclerosis, 4%; and others, 13%. Of the patients, 24% had a history of cardiovascular disease. The ratio of use of vitamin D receptor activators (i.v. and oral) was 65%. The use of 3.0 mEq/L containing dialysate was 79% of the patients.

At entry, serum corrected calcium and phosphate values were 9.6 ± 0.9 mg/dL and 5.7 ± 1.4 mg/dL, respectively. Serum albumin and alkaline phosphatase concentrations were 3.8 ± 0.4 g/dL and 258 ± 118 IU/L, respectively. At baseline, serum total cholesterol and HDL-cholesterol values were 158 ± 35 mg/dL and 50 ± 16 mg/dL, respectively. The body mass index and Kt/V were 21.1 ± 3.2 kg/m^2 and 1.48 ± 0.28, respectively.

Distribution of serum whole and intact PTH levels

The median serum whole PTH level was 121 pg/mL (range 2–888, mean 164 ± 143 pg/mL; Table 1). The interquartile range of whole PTH was 63–223 pg/mL. The median iPTH was 210 pg/mL (range 3–1436, mean 259 ± 216 pg/mL), and the interquartile range was 111–346 pg/mL. The distribution of whole and iPTH was revealed in Figure 1. Both whole PTH and iPTH assays showed log-normal distribution. There were 224/738 (30.4%) patients with a whole PTH level between 35–105 pg/mL, which is the target range of the Japanese guidelines. Those with an iPTH level between 60–180 pg/mL numbered 221 of 738 (29.9%). Those with whole PTH level <35 pg/mL were 98/738 (13.3%), and those with an iPTH level <60 pg/mL numbered 106/678 (14.4%).

TABLE 1. *Patient baseline characteristics*

Characteristics	
N	738
Age (years)	59 ± 13
Gender (% male)	58
Duration of hemodialysis (months)	145 ± 102
Etiology of renal failure (%)	
Glomerulonephritis	66
Diabetes mellitus	17
Hypertension	4
Other	13
Comorbid conditions (%)	
Diabetes mellitus	18
Cardiovascular disease	24
Fracture	6
History of parathyroidectomy or PEIT	24
Treatment (%)	
Use of an anti-hypertension drug	74
Use of oral or injectable VDRA	65
Use of 3.0 mEq/L Ca-containing dialysate	79
Baseline laboratory tests	
Albumin (g/dL)	3.8 ± 0.4
Calcium (mg/dL)	9.4 ± 0.9
Corrected calcium (mg/dL)	9.6 ± 0.9
Phosphate (mg/dL)	5.7 ± 1.4
Alkaline phosphatase (U/L)	258 ± 118
Whole PTH (pg/mL)	164 ± 143
Median	121
Range	2–888
Intact PTH (pg/mL)	259 ± 216
Median	210
Interquartile range	3–1436
Total cholesterol (mg/dL)	158 ± 35
HDL cholesterol (mg/dL)	50 ± 16
Body mass index (kg/m^2)	21.1 ± 3.2
Kt/V	1.48 ± 0.28
nPCR (g/kg/day)	1.26 ± 0.49

HDL high-density lipoprotein; nPCR, normalized protein catabolic rate; PEIT, percutaneous parathyroid gland ethanol injection therapy; PTH, parathyroid hormone; VDRA, vitamin D receptor activator.

Correlation between serum whole PTH and iPTH levels

The association between whole and intact PTH is shown in Figure 2. The whole PTH assay had a very high correlation with iPTH, as shown below:

$$\text{Intact PTH} = \text{Whole PTH} \times 1.59$$
$$\text{Whole PTH} = \text{Intact PTH} \times 0.63$$
$$(r = 0.870,\ P < 0.001)$$

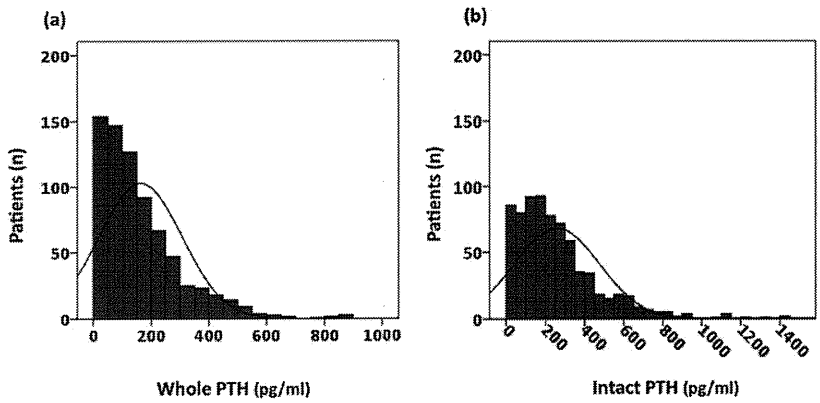

FIG. 1. Distribution of serum (a) whole parathyroid hormone (PTH), and (b) intact PTH levels. The median serum whole PTH level was 121 pg/mL (range 2–888, mean 164 ± 143 pg/mL). The median intact PTH was 210 pg/mL (range 3–1436, mean 259 ± 216 pg/mL). Both assays showed log-normal distribution.

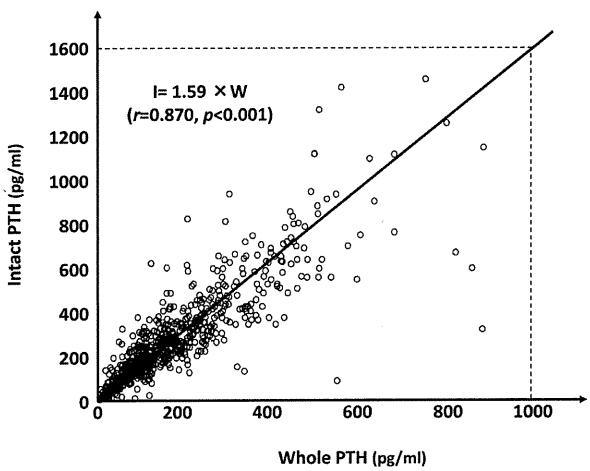

FIG. 2. Correlation between serum whole parathyroid hormone (PTH) and intact PTH levels. The whole PTH assay had a very high correlation with the intact PTH assay ($r = 0.870$, $P < 0.001$).

Then, we divided patients into three categories according to serum iPTH level: ≤60 pg/mL, 61–180 pg/mL, and >180 pg/mL (Fig. 3). In each category, the correlation coefficients were different. The correlation coefficient with iPTH below 60 pg/mL was 1.29, which was obviously lower than 1.59. As the serum PTH level increased, the correlation coefficient between whole and intact PTH levels increased.

Non-1–84 PTH

Serum concentrations of large, truncated fragments of non-1–84 PTH can be determined by subtracting the whole PTH value from that measured with the iPTH assay (7,8). Theoretically, the serum PTH level measured with the iPTH assay is higher than that with the whole PTH assay; however, rare exceptions to this rule have been reported in some

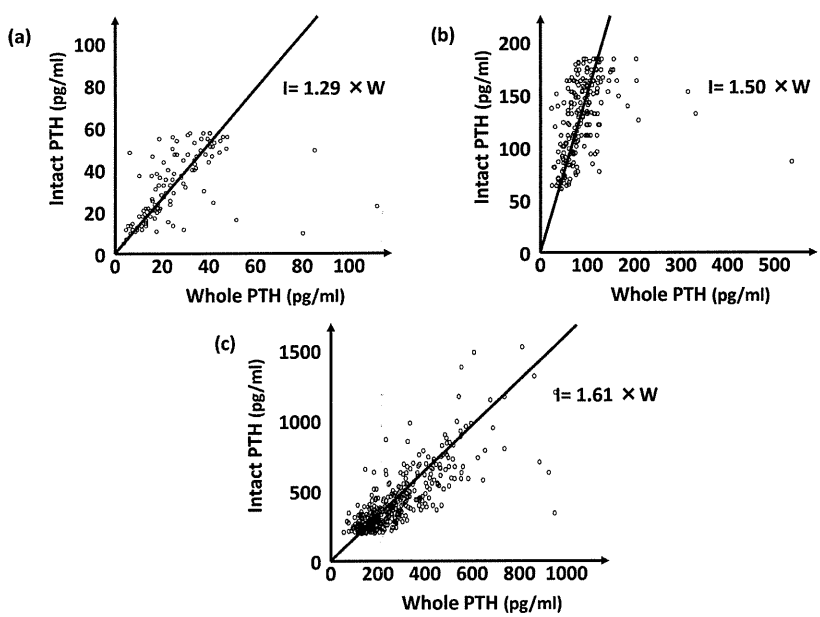

FIG. 3. Correlation between both assays in each intact parathyroid hormone (PTH) level: (a) ≤60 pg/mL; (b) 61–180 pg/mL; and (c) >180 pg/mL. In patients with low PTH levels (<60 pg/mL), the correlation coefficient was comparably low.

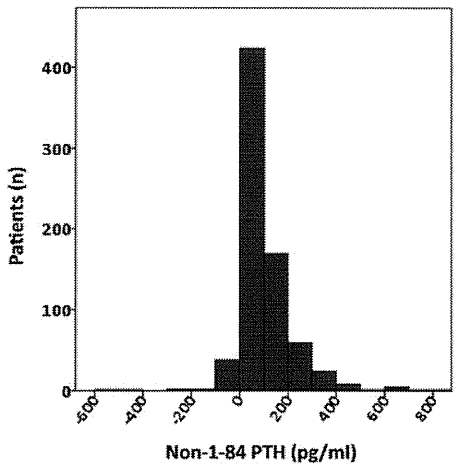

FIG. 4. Distribution of serum non-1–84 parathyroid hormone (PTH) level. There were 43/738 patients (5.8%) with (intact PTH – whole PTH) < 0.

patients, associated with N-PTH overproduction (14–16). As shown in Figure 4, the patients with (iPTH—whole PTH) < 0 numbered 43/738 (5.8%). The association between non-1–84 PTH and each assay is shown in Figure 5. Both assays were significantly correlated with non-1–84 PTH ($P < 0.001$). In particular, the iPTH assay had a very high correlation with non-1–84 PTH ($r = 0.791$).

Association of whole PTH, iPTH, and non-1–84 PTH with patient characteristics

The patients who were younger than 65 years and did not have diabetes mellitus, high calcium, high phosphate, or high alkaline phosphatase had higher whole and intact PTH levels, significantly (Table 2). Among these, serum calcium, phosphate, and alkaline phosphatase levels were significantly related with non-1–84 PTH.

In multivariate linear regression analysis, patient characteristics associated with greater whole PTH levels included male gender, dialysate calcium 3.0 mEq/L versus 2.5 mEq/L, higher corrected calcium, higher phosphate, and higher alkaline phosphatase levels (Table 3). These results were all the same as the iPTH assay. Patient characteristics associated with higher non-1–84 PTH levels included dialysate calcium 3.0 mEq/L, higher serum albumin, higher calcium, higher phosphate, and higher alkaline phosphatase levels.

Comparison between whole PTH and calculated 1–84 PTH levels

When the 1–84 fraction was calculated using 63% of the iPTH, 18% (131/738) of the total population was misclassified into a different Japanese guideline category. The percentage agreement was 82%. Stratified by Japanese guideline classifications, 24% (27/114) of those below target range were misclassified, while 28% (60/214) and 11% (44/410) of those within or above target range, respectively, were misclassified. The Bland–Altman plot, showing the difference between whole PTH and calculated 1–84 PTH plotted against the mean of the two values, reveals that at low PTH levels, there are a few significant differences between the two assays, but that, as the PTH levels increase, there is a large difference between the two measures (Fig. 6).

DISCUSSION

The first objective of the present study was to elucidate a difference between whole and intact PTH assays. At first, we investigated the distribution of both assays and, as shown in Figure 1, both assays revealed a log-normal distribution. Patients whose PTH level was within the Japanese target range were about 30% in both assays. In accordance with some previous reports (17–20), there was a strong correlation ($r = 0.870$) between both assays in the present

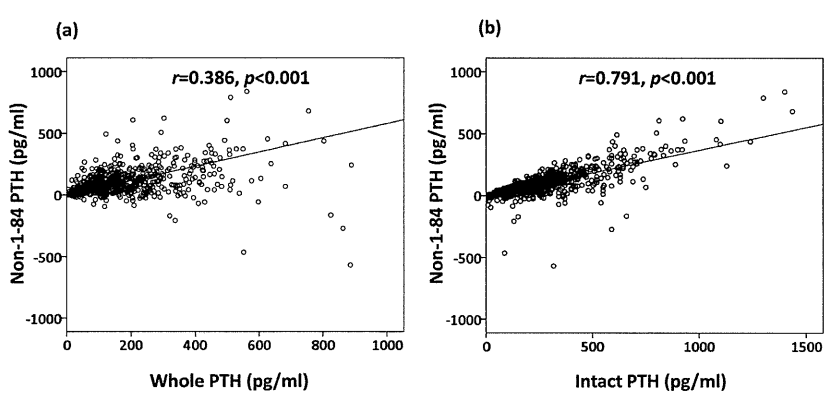

FIG. 5. Correlation between non-1–84 parathyroid hormone (PTH) and (a) the whole PTH and (b) intact PTH assays. Both assays were significantly correlated with non-1–84 PTH ($P < 0.001$). In particular, the intact PTH assay had a very high correlation with non-1–84 PTH ($r = 0.791$).

TABLE 2. *Whole parathyroid hormone (PTH), intact PTH and non-1–84 PTH by patient characteristics*

Characteristics	Whole PTH (pg/mL)		Intact PTH (pg/mL)		Non-1–84 PTH (pg/mL)	
	Median (IQR)	*P* value	Median (IQR)	*P* value	Median (IQR)	*P* value
Age						
<65 years (*N* = 498)	134 (70–238)	<0.01	224 (120–360)	<0.01	75 (27–147)	0.06
≥65 years (*N* = 240)	105 (43–204)		181 (85–280)		64 (23–110)	
Gender						
Male (*N* = 431)	130 (63–233)	0.18	219 (110–360)	0.32	68 (24–134)	0.85
Female (*N* = 307)	109 (60–212)		201 (102–336)		75 (27–137)	
Hemodialysis vintage						
<132 months (*N* = 369)	115 (54–209)	0.05	196 (101–318)	0.22	71 (31–133)	0.91
≥132 months (*N* = 369)	127 (70–239)		220 (119–355)		70 (19–142)	
Diabetes						
Absent (*N* = 603)	127 (66–237)	<0.01	221 (117–353)	<0.01	75 (24–144)	0.07
Present (*N* = 135)	101 (52–172)		160 (93–266)		58 (28–101)	
Corrected calcium						
<9.4 mg/dL (*N* = 359)	110 (59–204)	<0.01	180 (105–302)	<0.01	58 (25–108)	<0.01
≥9.4 mg/dL (*N* = 379)	133 (64–237)		242 (110–370)		86 (25–162)	
Phosphate						
<5.6 mg/dL (*N* = 351)	101 (49–177)	<0.01	180 (88–285)	<0.01	60 (22–115)	<0.01
≥5.6 mg/dL (*N* = 387)	150 (79–272)		244 (130–420)		77 (30–162)	
Alkaline phosphatase						
<234 U/L (*N* = 359)	104 (52–196)	<0.01	176 (91–290)	<0.01	58 (18–113)	<0.01
≥234 U/L (*N* = 353)	141 (76–245)		240 (140–386)		80 (34–162)	

IQR, interquartile range.

study. The correlation coefficient was 1.59, which was almost the same as that of the Japanese guidelines (21). When we divided patients into three categories according to the serum iPTH level, the correlation coefficient with iPTH below 60 pg/mL was 1.29, which was obviously lower than 1.59. This difference of the coefficient would be due to N-PTH overproduction or some PTH fractions, such as 7–84 PTH fragment. During hypercalcemia, the proportional decrease in carboxyl terminal fragments is less than that of 1–84 PTH (22–24). In hemodialysis patients, the percent of non-1–84 PTH was shown to directly correlate with the predialysis serum calcium concentration, with an increase in the serum calcium concentration associated with a reduction in the ratio of 1–84 PTH/non-1–84 PTH (7,8). These results suggest that hypercalcemia or calcium load would induce the fragmentation of 1–84 PTH and lead to the increase of the non-1–84 PTH value.

In the category with iPTH <60 pg/mL, iPTH is considered to be underestimated. Twenty-four percent of the low iPTH category showed serum whole PTH levels within the Japanese target range (35–105 pg/mL). In the category with iPTH 61–180 pg/mL, 22% of the patients showed high whole PTH levels (>105 pg/mL). On the other hand, in the high iPTH category, 11% of the patients revealed serum whole PTH levels within the Japanese target range (35–105 pg/mL). The distribution of both assays was alike and the correlation between both assays was high, although there were a considerable number of misclassifications.

We elucidated an association between each PTH assay and the patient characteristics. In multivariate

TABLE 3. *Adjusted differences in whole parathyroid hormone (PTH), intact PTH and non-1–84 PTH by patient characteristics*

Characteristics	Whole PTH (pg/mL)		Intact PTH (pg/mL)		Non-1–84 PTH (pg/mL)	
	β	P value	β	P value	β	P value
Age, per 10 years	−0.06	0.15	−0.03	0.51	0.21	0.58
Female vs. male	−0.07	0.04	−0.06	0.09	−0.21	0.57
Hemodialysis vintage, per 10 months	0.02	0.60	0.003	0.94	−0.17	0.66
Diabetes mellitus vs. no diabetes mellitus	−0.05	0.22	−0.04	0.29	−0.16	0.68
Dialysate calcium 3.0 mEq/L vs. 2.5 mEq/L	0.09	0.01	0.11	<0.01	0.87	0.02
Albumin, per g/dL	−0.05	0.21	0.05	0.20	0.18	<0.01
Corrected calcium, per mg/dL	0.12	<0.01	0.13	<0.01	0.09	0.03
Phosphate, per mg/dL	0.27	<0.01	0.28	<0.01	0.19	<0.01
Alkaline phosphatase, per U/L	0.19	<0.01	0.23	<0.01	0.20	<0.01

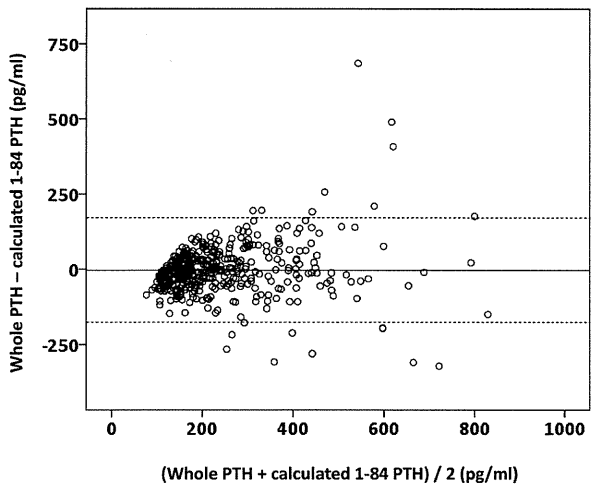

FIG. 6. Bland–Altman plot of whole parathyroid hormone (PTH) versus calculated 1–84 PTH. At low PTH levels, there are a few significant differences between two assays; however, as the PTH levels increase, there is a large difference between the two measures (calculated 1–84 PTH = intact PTH × 0.63).

analysis using a linear regression model, the patients with both greater whole and intact PTH levels include higher dialysate calcium concentrations and higher serum calcium levels. Hypercalcemia usually inhibits PTH secretion. Elevation of the serum calcium level with a high PTH state was considered due to medical therapy, such as with vitamin D or calcium carbonate. The serum non-1–84 PTH level significantly correlated with dialysate calcium 3.0 mEq/L, higher serum albumin, higher calcium, higher phosphate, and higher alkaline phosphate levels. These results indicated that a high serum calcium level or calcium overload would cause an increase of non-1–84 PTH production.

The amount of 1–84 PTH was calculated using 63% of the iPTH. As a whole, 18% of the total population was misclassified into a different Japanese guideline category. Stratified by Japanese guideline classifications, 28% of patients within the iPTH target range were misclassified. The Bland–Altman plot (Fig. 6), showing the difference between whole PTH and calculated 1–84 PTH (=iPTH × 0.63) plotted against the mean of the two values. There is not a systematic bias in this comparison. As the PTH levels increase, there is a large difference between the two measures. There were many outliers in the whole PTH side (the upper part of the graph). It is probably due to N-PTH overproduction. Some outliers in the calculated 1–84 PTH side (the lower part of the graph) would reveal PTH fragmentations.

There are several limitations in the present study. The first is that we did not have data for mortality, onset of cardiovascular disease, or fracture. If possible, we should elucidate an association between the serum PTH level and mortality or fracture in each PTH assay. Melamed et al. (25) had reported that an elevated 1–84 PTH value was significantly associated with an increased risk of death, whereas iPTH was not significantly associated with mortality. Lehmann et al. (26) took bone biopsies from 132 patients with chronic kidney disease in stages 3–5 and evaluated the association of bone histomorphometry with the iPTH and Bio-Intact PTH assays. Both assays effectively identified patients with reduced bone turnover. There was no difference between both assays. According to these reports, the difference of the PTH assays may not have much influence on the risk of either mortality or fracture.

In summary, in this cohort of dialysis patients, although the distribution of both assays was alike and the correlation between both assays was high, there were some misclassifications. Eighteen percent of the total population was misclassified into a different Japanese guideline category. As the PTH levels increased, there was a large difference between the two measures. In conclusion, both PTH assays were strongly correlated, although, the whole PTH assay may be more useful for precise evaluation of PTH activity than the iPTH assay.

Conflict of interests: The authors have no conflict of interests.

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ORIGINAL RESEARCH

Nonenzymatic Cross-Linking Pentosidine Increase in Bone Collagen and Are Associated with Disorders of Bone Mineralization in Dialysis Patients

Jun Mitome · Hiroyasu Yamamoto · Mitsuru Saito · Keitaro Yokoyama · Keishi Marumo · Tatsuo Hosoya

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Abstract Disorders of bone and mineral metabolism are common complications in chronic kidney disease (CKD) patients and lead to significantly increased fracture risk, morbidity, and mortality of cardiovascular disease due to ectopic calcifications, contributing to a worsening prognosis. Bone strength is determined by not only bone mineral density but also bone quality, which is dependent on bone collagen cross-links. Collagen cross-links are classified into enzymatic immature and mature types and nonenzymatic advanced glycation end products (AGEs). Pentosidine is well established as one of the AGEs that accumulates markedly in CKD patients. The chemistry, function, and clinical relevance of cross-links have been revealed, whereas bone quality and the relationship with bone mineralization in CKD patients are not clear. We performed transiliac bone biopsies on 22 dialysis patients (mean age 56 ± 9 years) with severe secondary hyperparathyroidism and measured cross-links by evaluating bone histomorphometry. Cross-links data were compared with age-matched non-CKD subjects (mean age 58 ± 8 years, $n = 17$). Enzymatic collagen cross-links were formed to a similar extent compared with non-CKD subjects and showed a positive correlation with plasma intact parathyroid hormone. Pentosidine was remarkably increased in dialysis patients and inversely correlated with bone-formation rate/bone volume and mineral apposition rate. This study suggests that AGE collagen cross-links strongly associate with disorders of bone metabolism in dialysis patients.

Keywords Collagen cross-link · Advanced glycation end products · Dialysis patient · Mineralization · Hyperparathyroidism

Bone disorders are common complications in chronic kidney disease (CKD) patients. Recently, renal osteodystrophy (ROD) has been redefined as CKD-mineral and bone disorder (CKD-MBD) [1]. The disorders of bone and mineral metabolism associated with CKD-MBD lead to a significantly increased fracture risk [2]. Deterioration in bone calcification and subsequent ectopic calcification such as vascular calcification are common complications. Furthermore, in dialysis patients, bone fragility and increased fracture risk affect quality of life and prognosis [3].

Collagen enzymatic and nonenzymatic cross-links in bone affect not only the mineralization process but also bone material properties [4–7]. In fact, impaired enzymatic cross-linking and/or an increase in nonenzymatic cross-links in bone collagen have been proposed to be determinants of impaired bone quality in aging, osteoporosis, and diabetes mellitus [8]. However, little is known about collagen cross-link formation and its relationship with bone mineralization in CKD patients. Collagen cross-links can be classified into enzymatic types, such as lysyl oxidase (LOX)-mediated cross-links, and nonenzymatic types, such as advanced glycation end products (AGEs) according to mechanism of formation and functional differences [4, 8]. Generally, enzymatic cross-link formation is affected by

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J. Mitome (✉) · H. Yamamoto · K. Yokoyama · T. Hosoya
Division of Kidney and Hypertension, Department of Internal Medicine, The Jikei University School of Medicine, 3-25-8, Nishi-shinbashi, Minato-ku, Tokyo, Japan
e-mail: mitome@jikei.ac.jp

M. Saito · K. Marumo
Department of Orthopedic Surgery, The Jikei University School of Medicine, Tokyo, Japan

LOX activity and tissue maturation [4], whereas accumulation of nonenzymatic cross-links depends on the degree of glycation and/or oxidation [5, 7, 9]. Although pentosidine is now well established as an AGE, which is formed by glycosylation and oxidation and accumulates markedly in the skin and serum from patients with CKD [5, 10, 11], there are no available data regarding enzymatic and AGE types of cross-link formation in bone collagen.

The aim of this study was to clarify collagen enzymatic and nonenzymatic cross-link formation and the relationship between cross-link formation and the bone-mineralization process in dialysis patients with secondary hyperparathyroidism (2HPT).

Materials and Methods

Patients

Twenty-two patients with severe 2HPT that was resistant to vitamin D therapy participated in this study. Their plasma intact parathyroid hormone (iPTH) concentration persisted at extremely high levels at a similar degree for at least 6 months. They were undergoing regular dialysis therapy; 21 patients were on standard hemodialysis and one was on continuous ambulatory peritoneal dialysis. They consisted of 15 males and seven females; mean age was 56 ± 9 years (range 35–72), and mean dialysis duration was 139 ± 81 months (range 3–357). We performed transiliac bone biopsies, immediately after parathyroidectomy (PTx) under general anesthesia. None of the patients had a history of fracture, were currently using corticosteroids, or were receiving estrogen-replacement therapy. Primary kidney disease diagnoses included nephrosclerosis due to hypertension (two patients), chronic glomerulonephritis (12 patients), diabetic nephropathy (four patients), and other causes (four patients). Blood samples for the determination of biochemical parameters were obtained after an overnight fast. All patients underwent a hemodialysis session the day before PTx.

Bone Biopsy and Bone Histomorphometry

All patients were submitted to a transiliac bone biopsy to evaluate bone histomorphometry and to measure bone collagen cross-links. Of these patients, ten were administered a course of double-labeling tetracycline using demethylchlortetracycline hydrochloride: 300 mg/day for 2 days, with an interval of 5–7 days between treatments. The biopsy was performed 2–7 days after the last dose of tetracycline by the same operator. Undecalcified bone specimens were submitted to standard processing for histological studies at Niigata Bone Science Institute (Niigata Prefecture, Japan). We evaluated cancellous bone histomorphometry of bone volume/tissue volume (BV/TV), osteoid volume/bone volume (OV/BV), fibrosis volume/tissue volume (Fb.V/TV), bone-formation rate/bone volume (BFR/BV), and mineral apposition rate (MAR).

Characterization of Enzymatic and Nonenzymatic Collagen Cross-Links

The reduction of collagen in bone with sodium borohydride ($NaBH_4$; Sigma-Aldrich, St. Louis, MO) and measurement of cross-links were carried out as previously described [12]. Briefly, each bone powder was demineralized twice with 0.5 M EDTA in 50 mM Tris buffer, pH 7.4, for 96 h at 4°C. Demineralized bone residues were then suspended in 0.15 M potassium phosphate buffer, pH 7.6, and reduced at 37°C with $NaBH_4$. Reduced specimens were hydrolyzed in 6 N hydrochloric acid at 110°C for 24 h. Hydrolysates were then analyzed for cross-links on a Shimadzu LC9 HPLC fitted with a cation exchange column (0.9 × 10 cm, Aa pack-Na; Jasco, Tokyo, Japan) linked to an online fluorescence flow monitor (RF10AXL; Shimadzu, Shizuoka, Japan). We determined the content of enzymatic immature reducible and mature nonreducible cross-links. In addition, we determined that pentosidine was one of the well-characterized AGE type of cross-links to evaluate nonenzymatic glycation-induced cross-links. LOX-mediated reducible immature cross-links, dehydro-dihydroxylysinonorleucine (deH-DHLNL), dehydro-hydroxylysinonorleucine (deH-HLNL), and dehydro-lysinonorleucine (deH-LNL) were identified and quantified according to their reduced forms (DHLNL, HLNL, and LNL, respectively). Immature cross-links, such as deH-DHLNL and deH-HLNL, are unstable Schiff bases. In fact, deH-DHLNL and deH-HLNL are present mainly as more stable keto forms such as hydroxylysino-5-keto-norleucine (HLKLN) and lysino-5-keto-norleucine (LKNL), respectively. Our established high-performance liquid chromatography (HPLC) system enables us to determine enzymatic and nonenzymatic cross-link contents within a linear range of 0.2–600 pmol in bone specimens. Enzymatic nonreducible mature cross-links, such as pyridinoline (Pyr) and deoxypyridinoline (Dpyr), and pentosidine were detected by natural fluorescence. The contents of each cross-link were expressed as mole/mole of collagen. In this study, we confirmed that hydrolysates of demineralized and reduced bone specimens consisted of glycine in the range of 322–310 residues per 1,000 amino acids and more than 80 residues of hydroxyproline per 1,000 amino acids, which is in accordance with the known amino acid composition of type I collagen.

We compared the cross-link data from CKD patients to our previously reported Japanese age-matched cadaveric bone study from ilium (mean age 58 ± 8 years, range

40–69, male, $n = 17$, control group) without CKD, diabetes, metabolic disorders, and medications such as vitamin D, vitamin K, and bisphosphonate [8, 12, 13]. Bone specimens of control subjects were also used in our previous study into age-related changes in the biochemical characteristics of collagen from human subjects [13], approved by an independent ethics committee with consent obtained from relatives. Furthermore, the relationship between pentosidine and bone mineralization and the influence of iPTH concentration on bone cross-links in dialysis patients were studied.

Informed Consent

Informed consent to use blood samples, bone specimens, and clinical data for research was given at the time of admission for surgery by the patients themselves. The local official scientific and ethical committee on medical research and the hospital director approved the study.

Statistical Analysis

All values are listed as means with their standard deviation (SD) in the text and tables. For comparison of cross-link parameters between the dialysis patient group and the control group, Student's t-test or a Wilcoxon rank sum test was used, as appropriate. Correlations between cross-link parameters with iPTH, BFR/BV, and MAR were analyzed by univariate analysis. All P values were defined as significant at $P < 0.05$. Statistical analyses were performed using JMP, version 5.1.1 for Windows (SAS Institute, Cary, NC).

Results

Patient Characteristics

The clinical characteristics of the dialysis patients are shown in Table 1. All patients had bone and mineral metabolism disorders due to severe 2HPT, regardless of the consequence of conservative therapy such as dietary phosphate limitation, administration of oral phosphate binders, and active vitamin D analogues. None of the patients had been administered calcimimetics. Serum calcium, phosphate, and β_2-microglobulin concentrations were permissive as maintenance dialysis patients. Meanwhile, iPTH and bone alkaline phosphatase (BAP) reflected the severity of increased bone turnover at the time of PTx. Mean values of bone mineral density (BMD) in the total body and the young adult mean (YAM) were 1.023 ± 0.102 g/cm^2 and $88 \pm 9\%$ (range 63–101%), respectively.

Bone Histomorphometry

The results of bone histomorphometry in 22 patients obtained by transiliac bone biopsy are shown in Table 2. According to the classification of ROD [1, 14], 14 patients were mildly affected and eight patients had osteitis fibrosa (OF). None of them had adynamic bone disease. In addition, we evaluated BFR/BV and MAR in ten patients. The mean values of BFR/BV and MAR also indicated high-turnover bone.

Comparison of Bone Collagen Cross-Links

Comparisons of bone collagen cross-links between dialysis patients and the control group are shown in Fig. 1. There was no significant difference in total immature cross-links (the sum of DHLNL, HLNL, and LNL) (Fig. 1a), while total mature pyridinium cross-links (the sum of Pyr and Dpyr) significantly decreased in dialysis patients (Fig. 1b). The total amount of immature and mature pyridinium cross-links showed no difference between the dialysis and control groups (Fig. 1c). The relative ratio of immature cross-links to mature cross-links was significantly higher in dialysis patients (dialysis vs. control 3.41 ± 1.41 vs. 2.62 ± 0.67, $P = 0.048$). Pentosidine content in bone was significantly higher than that in the control group (Fig. 1d).

Correlation Between Bone Collagen Cross-Links and Plasma iPTH Concentration

We studied the correlation between bone collagen cross-links and plasma iPTH concentrations using univariate analysis (Fig. 2). Plasma iPTH concentration significantly and positively correlated with immature cross-links and total amount of immature and mature pyridinium cross-links, whereas neither mature pyridinium cross-links nor pentosidine correlated with iPTH concentration. The relative ratio of immature cross-links to mature cross-links also significantly and positively correlated with plasma iPTH concentration ($Y = 1.942 + 0.002 \times X$; $R^2 = 0.214$, $P = 0.03$).

Correlation Between Pentosidine and Bone Mineralization

Figure 3 shows the correlation between pentosidine and histomorphometric parameters regarding bone mineralization using univariate analysis. Both BFR/BV and MAR negatively correlated with pentosidine.

Discussion

In this study, we evaluated bone collagen cross-links and bone histomorphometry in a total of 22 dialysis patients