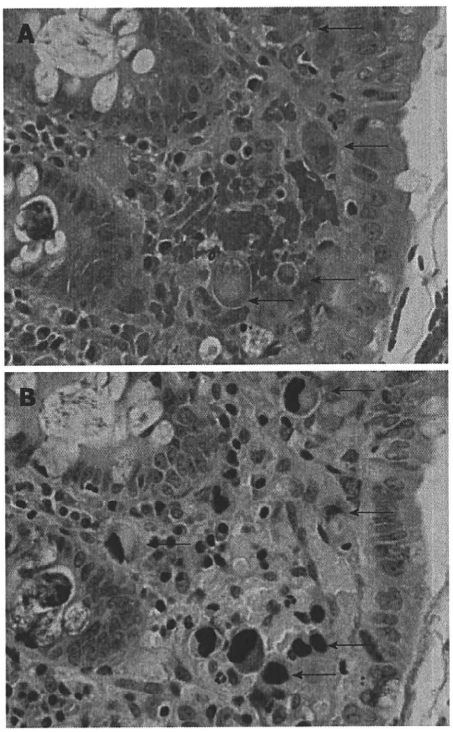

Figure 2 Pathological features in cytomegalovirus gastrointestinal disease. A: Large cells with intranuclear inclusions or associated with granular cytoplasmic inclusions (hematoxylin and eosin stain); B: Cytomegalovirus (CMV)-infected cells (arrows) show brown coloration in both nuclei and cytoplasm (immunohistochemical staining with anti-CMV).

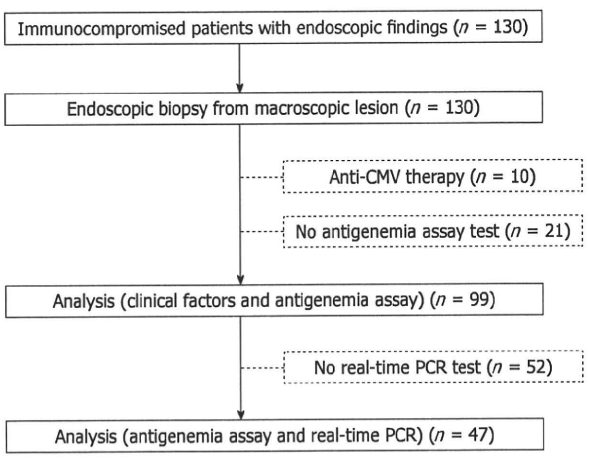

Figure 3 Study design. CMV: Cytomegalovirus; PCR: Polymerase chain reaction.

treatment, along with 21 patients who had not been examined using the CMV antigenemia assay. Thus, a total of 99 patients were retrospectively selected for analysis (Figure 3). Of the immunocompromised patients, 19 (19.1%) had malignant disease, 18 (18.1%) had autoimmune disease, 19 (19.1%) had disorders of biochemical homeostasis, three (3%) had undergone transplantation, and 45 (45.5%) had HIV infection. A total of 50 patients (50.1%) had received immunosuppressive therapy. No

Table 1 Clinical factors for cytomegalovirus gastrointestinal disease (univariate analysis)

	CMV-GID (n = 52)	Non-CMV-GID (n = 47)	P value
Age (yr, mean ± SD)	46.8 ± 16.2	56.6 ± 17.8	0.050
Male sex	30	41	0.098
Immunodeficiency disease			
HIV infection	33	12	< 0.001
Malignancy	9	10	0.617
Solid cancer	1	3	
Hematological cancer	8	7	
Autoimmune disease	7	11	0.200
Disorders of biochemical homeostasis	8	11	0.312
Chronic renal failure	1	2	
Liver cirrhosis	0	2	
Diabetes mellitus	7	7	
Transplantation	1	2	
Immunosuppressive therapy	25	25	0.611
Steroids	22	19	
Immunosuppressants	8	4	
Chemotherapy	4	4	
Positive CMV antigenemia	34	3	< 0.001
Leukopenia	35	21	0.023
With gastrointestinal symptoms	34	34	0.456

HIV: Human immunodeficiency virus; CMV: Cytomegalovirus; GID: Gastrointestinal disease.

patients had inflammatory bowel disease (IBD). Fifty-five patients were histologically diagnosed with CMV-GID. Univariate analysis (Table 1) identified HIV infection (P < 0.001), leukopenia (P = 0.023), and positive CMV antigenemia assay (P < 0.001) as being associated with CMV-GID. Multivariate analysis revealed HIV infection [odds ratio (OR), 6.57; 95% CI: 2.1-20.2, P = 0.001] and positive CMV antigenemia assay (OR, 33.3; 95% CI: 8.1-136.2, P < 0.001) as the only factors independently correlated with CMV-GID.

HIV-infected patients included 44 men (97.8%) and their mean age was 42.1 years (range, 25-74 years). Median CD4 count was 57 (interquartile range, 17-111). Patients with CMV-GID showed significantly lower CD4 counts than those without CMV-GID (median CD4 count; CMV-GID *vs* non-CMV-GID: 24 *vs* 150, P < 0.001).

Accuracy of CMV antigenemia assay for diagnosing CMV-GID

A positive CMV antigenemia assay showed low sensitivity and high specificity (Table 2). In a subgroup analysis, patients with leukopenia displayed low sensitivity and high specificity. Minimal differences in accuracy were seen among patients with or without leukopenia. HIV-infected patients displayed low sensitivity and high specificity. Accuracy barely differed between HIV-positive and -negative patients. In HIV-infected patients, CD4 count < 50 cells/μL resulted in low sensitivity and high specificity. Differences in accuracy among patients were minor, regardless of CD4 count.

In patients who had undergone both quantitative real-

Table 2 Diagnostic accuracy of cytomegalovirus antigenemia for detecting cytomegalovirus gastrointestinal disease

Subgroups	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
All patients (n = 99)	65.40% (55.4-74.9)	93.60% (87.3-97.7)	91.90% (84.7-96.4)	71.00% (60.7-79.4)
Patients with leukopenia (n = 56)	68.60% (54.0-79.7)	100% (93.6-100)	100% (93.6-100)	65.60% (52.2-78.2)
Patients without leukopenia (n = 43)	58.80% (42.1-73.0)	88.50% (74.9-96.1)	76.90% (61.4-88.2)	76.70% (61.4-88.2)
HIV-infected patients (n = 45)	63.60% (48.8-78.1)	100% (92.2-100)	100% (92.2-100)	50.00% (35.8-66.3)
Non-HIV-infected patients (n = 54)	68.40% (54.5-80.5)	91.40% (79.7-96.9)	81.30% (68.6-90.7)	84.20% (70.7-92.1)
HIV-infected patients with CD4 count < 50 (n = 22)	61.90% (40.7-82.8)	100% (84.6-100)	100% (84.6-100)	11.10% (1.12-29.2)
HIV-infected patients with CD4 count ≥ 50 (n = 23)	66.70% (42.7-83.6)	100% (85.2-100)	100% (85.2-100)	73.30% (51.6-89.8)

HIV: Human immunodeficiency virus; PPV: Positive predictive value; NPV: Negative predictive value.

Table 3 Comparison of diagnostic accuracy for detecting cytomegalovirus gastrointestinal disease between antigenemia assay and quantitative real-time polymerase chain reaction (n = 47)

	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
CMV real-time PCR	73.00% (57.4-84.4)	100% (92.5-100)	100% (92.5-100)	50.00% (36.1-65.9)
CMV antigenemia assay	64.90% (50.7-79.1)	100% (92.5-100)	100% (92.5-100)	43.50% (28.3-57.8)

CMV: Cytomegalovirus; PPV: Positive predictive value; NPV: Negative predictive value; PCR: Polymerase chain reaction.

time PCR and antigenemia assay (Table 3), real-time PCR was slightly more accurate in terms of sensitivity than the antigenemia assay; however, this difference was not statistically significant (P = 0.312).

DISCUSSION

CMV-GID is a major cause of morbidity and mortality in immunocompromised patients; therefore, diagnosis at an early stage is essential[1,2,5,8,9]. However, clinical diagnosis of this disease can be difficult, as physicians need to consider various underlying diseases and clinical presentations. Patients at high risk of CMV-GID have been reported as those with HIV infection or undergoing steroid therapy or cancer therapy[1]. The present study identified HIV infection as one of the independent factors in secondary immunodeficiency diseases. This is because the number of eligible subjects was small and included immunocompromised patients while excluding immunocompetent patients.

Among the various clinical manifestations, a positive CMV antigenemia assay was found to be a useful factor for diagnosing CMV-GID. The CMV antigenemia assay is one of the most widely used methods for detecting reactivation of CMV infection, but few studies have examined the diagnostic value for CMV-GID[3,21,22]. Our findings demonstrated 65% sensitivity and 94% specificity of the CMV antigenemia assay for diagnosing CMV-GID. Mori *et al*[3] reported that only four of 19 patients (21%) developed a positive CMV antigenemia assay before developing CMV-GID; however, all 19 patients subsequently tested positive for CMV antigenemia after diagnosis of CMV-GID. There is a possibility that patients with CMV-GID will develop a positive CMV antigenemia assay at follow-up, but our study did not assess this process after diagnosis of CMV-GID. Fica *et al*[21] also reported that the CMV antigenemia assay result was positive for 18 of 31 patients (58%) with CMV end-organ disease, with CMV-GID (71%) as the most frequent cause. However, these studies were limited in that the number of subjects was small and the specificity of the CMV antigenemia assay was unknown. Jang *et al*[22] recently reported that the sensitivity and specificity of the CMV antigenemia assay for diagnosing CMV-GID were 54% and 88%, respectively, in patients with secondary immunodeficiency disease. The reports mentioned above showed that the CMV antigenemia assay has low sensitivity for the diagnosis of CMV-GID, which is consistent with our results.

It has been reported that sufficient granulocytes are essential in evaluating CMV using the antigenemia assay. Previous studies using the antigenemia assay to diagnose CMV-GID have reported that most of the patients were transplant recipients and were mostly HIV-negative[3,21,22]. No studies have compared the assay among groups of HIV-positive/-negative patients and among groups with or without leukopenia. In patients with HIV infection, most cases of CMV-GID have known to occur with CD4 counts < 50 cells/μL[2,4]. However, whether the accuracy of the antigenemia assay is affected by the immunosuppressed state has not been elucidated. We suspected that such different groups would show differences in the accuracy of CMV antigenemia assay, but found little difference. This suggests that our results are applicable to these different groups in clinical practice.

Besides the CMV antigenemia assay, quantitative real-time PCR is also used for detecting reactivation of CMV infection, and is considered more useful for predicting CMV disease than the CMV antigenemia assay[23,24]. In our study, quantitative real-time PCR and CMV antigenemia assay were performed simultaneously on 47 patients. The PCR method showed a tendency toward slightly higher sensitivity, but no significant differences were evident. In Japan, the CMV PCR method has not been widely used in

clinical practice because of the higher costs compared to the antigenemia assay. We thus do not recommend use of PCR methods in the sub-diagnosis of CMV-GID, as the antigenemia assay is just as valid.

One limitation of this study was the single-center, retrospective nature of the investigation. A significant difference might not have been confirmed among independent factors due to the small number of patients. Further studies of more patients are needed. Another limitation is the verification bias, which is dependent on the physician's decision to perform the antigenemia assay.

The diagnosis of CMV-GID is considered as the gold standard for identifying CMV cells in tissue samples from endoscopic biopsy[1,2,13]. Various endoscopic findings are present in CMV-GID, such as ulcer and mucosal inflammation[25,26]; however, physicians may not perform a biopsy in cases only showing mucosal inflammation without ulcer. Even in cases of severe ulceration that is deep or bleeding, physicians may hesitate to perform a biopsy. In such cases, a diagnosis of CMV-GID may not be reached. Our results suggest that the CMV antigenemia assay is useful for the sub-diagnosis of CMV-GID in immunocompromised patients with endoscopic findings. Considering the high specificity of the test, the use of this method before endoscopy could potentially avoid complications due to biopsy. Positive antigenemia is also useful for evaluating improvements in CMV-GID after anti-CMV treatment. However, the low sensitivity means that if the antigenemia assay yields negative results, biopsy and immunohistochemical staining of specimens with anti-CMV will be required for diagnosis. Negative antigenemia assay results may require a repeat examination at a different time[3]. Moreover, the use of different non-invasive methods such as quantitative PCR should be considered.

In conclusion, the CMV antigenemia assay is highly useful for diagnosing CMV-GID. If the antigenemia assay provides positive results, the presence of endoscopic lesions should allow diagnosis of CMV-GID without biopsy. The accuracy of the test is unaffected by the presence of HIV infection or leukopenia.

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COMMENTS

Background

Cytomegalovirus (CMV) gastrointestinal disease (GID) is a major cause of morbidity and mortality in immunocompromised patients; therefore, diagnosis at an early stage is essential. However, clinical diagnosis of this disease can be difficult, as physicians need to consider various underlying diseases and clinical presentations.

Research frontiers

The diagnosis of CMV-GID requires an endoscopic biopsy, which is invasive and may lead to complications. While the CMV antigenemia assay is one of the most widely used methods for detecting reactivation of CMV infection, few studies have examined its diagnostic value for CMV-GID. In this study, the authors demonstrate that the CMV antigenemia assay was highly useful for diagnosing CMV-GID.

Innovations and breakthroughs

There were no studies of diagnosis on CMV-GID related factors using multivariate analysis. In this study, among the various clinical manifestations, human immunodeficiency virus (HIV) infection and positive CMV antigenemia assay were found to be a useful factors for diagnosing CMV-GID by multivariate analysis. As for accuracy of CMV antigenemia for diagnosing CMV-GID, recent reports have highlighted that the sensitivity and specificity were 54% and 88%, respectively, in patients with secondary immunodeficiency disease. However, no studies have compared the assay among groups of HIV-positive/-negative patients and among groups with or without leukopenia. In this study, the sensitivity, specificity, positive predictive value, and negative predictive value of antigenemia for CMV-GID were 65.4%, 93.6%, 91.9%, and 71.0%, respectively. In addition, its accuracy was not affected by the presence of HIV infection and leukopenia. These results are very useful for diagnosing CMV-GID by clinical physicians.

Applications

Considering the high specificity of the test, use of this method before endoscopy could potentially avoid complications due to biopsy. However, the low sensitivity means that if the antigenemia assay yields negative results, biopsy and immunohistochemical staining of specimens with anti-CMV will be required for diagnosis. Negative antigenemia assay results may require repeat examination at a different time. Moreover, the use of different non-invasive methods such as quantitative polymerase chain reaction should be considered.

Peer review

This paper is interesting and it could be valuable for other researchers.

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Factors Associated with Recovery of Renal Function in Patients with Multiple Myeloma Who Were Treated with Hemodialysis

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Key Words
Multiple myeloma · Hemodialysis · Prognostic factor · Serum Ca · β_2-microglobulin

Abstract
Background: The presence of renal failure in patients with multiple myeloma (MM) has been considered an ominous prognostic factor associated with a significantly decreased life expectancy. The prognostic factors have seldom been analyzed to predict discontinuation of hemodialysis (HD) therapy in MM patients with renal failure after HD initiation. It is clinically very important to predict whether HD can be discontinued after introducing HD in such patients. ***Methods:*** All medical and HD records were reviewed in MM patients who underwent HD in the National Center for Global Health and Medicine Hospital between January 1995 and May 2009. Thirty-two patients with MM had undergone HD. The clinical features and the factors that might be associated with recovery of renal function leading to discontinuation of HD in MM patients with severe renal failure were examined. ***Results:*** The factors associated with recovery of renal function and discontinuation of HD were: low International Staging System (ISS) score ($p = 0.0034$); high response to chemotherapy ($p = 0.036$); low serum Ca ($p = 0.006$); low Cr ($p = 0.019$), and low serum β_2-microglobulin ($s\beta_2M$) ($p = 0.002$). On multivariate analysis, low serum Ca and $s\beta_2M$ were significantly associated with HD discontinuation. Moreover, discontinuing HD was the significant factor associated with improved overall survival in MM patients who required HD at least once. ***Conclusion:*** $s\beta_2M$ and Ca were the laboratory parameters that were significant, independent prognostic factors for predicting the probability of recovery from severe renal failure and discontinuation of HD in MM patients who needed HD at least once.

Introduction

Multiple myeloma (MM) is a clonal B cell disease of proliferating plasma cells that mainly affects elderly patients. Although new aggressive treatments have been developed, such as autologous stem cell bone-marrow transplantation with high-dose chemotherapy, as well as thalidomide, the complete remission rate remains at 20–59%, with a median overall survival of 4.4–7.1 years and a median event-free period of 24–43 months [1].

Renal failure is one of the main causes of death in MM patients. Almost 25–50% of newly diagnosed patients present with renal dysfunction, and around 9% require hemodialysis (HD) [2–4]. Treatment-related mortality

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(29%) and morbidity (3.4%) are higher in patients with renal failure than in patients with normal renal function [5]. It has been reported that most patients with MM and severe renal failure die within the first 2–9 months after diagnosis [6, 7].

Clinicians must often decide whether HD should be introduced in MM patients who develop severe renal failure. When one considers the treatment plan and choices for such patients, it would be very useful to have a specific tool to evaluate the possibility of discontinuing HD in the near future. Although it is very important to estimate whether HD can be discontinued sooner and more safely, only a few papers have reviewed this problem [3, 8–11]. To identify prognostic factors associated with the recovery of renal function after initiating HD in MM patients, clinical and laboratory features were compared between groups of patients who could and could not discontinue HD.

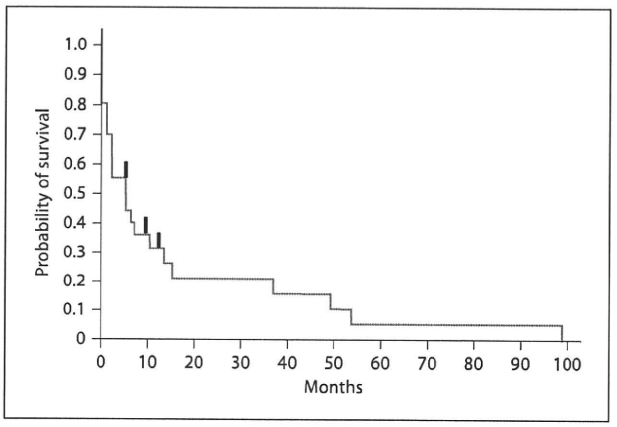

Fig. 1. Survival of all MM patients after initiation of HD. Mean and median survival periods from initiation of HD are 10.5 and 2.0 months, respectively. Three patients were alive at the time of the investigation; the mark on the right is a sensor.

Patients and Methods

The medical and HD records of MM patients who underwent HD between January 1995 and May 2009 were reviewed. The laboratory and demographic data, as well as additional treatments, were collected from our databases. All patients were admitted to the National Center for Global Health and Medicine Hospital, and HD was started for the previously diagnosed myeloma patients who later developed acute renal failure or acute-on-chronic renal failure after MM was diagnosed. These procedures were in accordance with the Helsinki Declaration, as updated in Seoul, October 2008. MM was diagnosed according to The International Myeloma Working Group (IMWG) criteria [5]. The primary outcome of interest in this study was discontinuation of HD in MM patients who had required HD. The prognostic variables of interest in this analysis were age, sex, time from onset of MM symptoms to initiation of HD, presence of nephrotic syndrome and heart failure, type of M protein, and light chain. The International Staging System (ISS) for MM and the Durie-Salmon staging system, as well as chromosomal abnormalities, were also assessed [12, 13]. Response to chemotherapy was defined as disease status during the course of therapy evaluated by the International Uniform Response Criteria [14] when HD was indicated. Laboratory data at the time of HD initiation, including uric acid (UA), total serum protein (TP), serum albumin, blood urea nitrogen (BUN), creatinine, calcium (Ca), hemoglobin (Hb), lactate dehydrogenase (LDH), C-reactive protein (CRP), serum β_2-microglobulin ($s\beta_2M$), and urinary protein, were also analyzed.

Basically, HD was carried out initially using a central venous catheter and later with an internal arteriovenous fistula (AVF). One patient with IgD type MM already had an AVF. A hollow-fiber dialyzer (1.1–1.3 m^2) was used in most instances. Blood flow rates of 120–150 ml/min were maintained with a blood pump, and the dialysate flow rate was generally maintained at 500 ml/min. HD was performed for 3–4 h daily or every other day using DBG-03 (NIKKISO, Tokyo, Japan). Nafamostat mesylate was used as the anticoagulant. In the present study, renal dysfunction was defined using estimated glomerular filtration rate (eGFR) <60 ml/min/1.73 m^2, and discontinuation of HD was defined as dialysis-free 180 days after HD initiation [2, 15]. Patients who had died before 180 days were defined as the HD-dependent group. For all patients, it could be determined whether they belonged to the HD-independent or the HD-dependent group at the time of the investigation.

Statistical analysis was performed using JMP version 7.0 (SAS Institute Inc., Cary, N.C., USA). The associations between recovery from HD and patients' characteristics were tested using the Wilcoxon rank-sum test, the χ^2 test, and Fisher's exact test. The log-rank test was also used to compare survival times. Factors that were associated on univariate analysis were entered into multivariate logistic regression to further investigate whether they were independently associated.

Results

There were 32 patients who had been treated for MM with chemotherapy and newly hemodialyzed. Compared with the group that could not discontinue HD, the group that could discontinue HD had a significantly lower ISS score ($p = 0.004$), higher response to chemotherapy ($p = 0.036$), lower serum Cr ($p = 0.019$), lower Ca ($p = 0.0073$), and lower $s\beta_2M$ ($p = 0.0043$) (table 1).

Three patients were still alive at the time of the investigation. In the present study, 24 (80%) patients were treated with VAD therapy, a combination regimen of vincristine, adriamycin, and dexamethasone. Four patients (12.5%) were treated only with dexamethasone, and 2 patients (6%) were treated with MCNU-MP (ranimustine,

Table 1. Comparison of prognostic factors between those unable to discontinue HD and those able to discontinue HD

Prognostic factor	Continue HD (n = 20)	Discontinue HD (n = 12)	p value
Age, years	61.7 (56.7–66.6)	62.8 (55.7–69.9)	0.710
Sex, M:F	15:5	7:5	0.438
Symptom onset to HD, months	31.2 (14.2–48.1)	20.4 (5.60–35.2)	0.654
Nephrotic syndrome	35%	25%	0.550
EF, %	62.2 (58.3–66.1)	64.5 (59.2–69.7)	0.337
Type of MM	BJP 10 IgG 5 IgD 4 IgD + BJP 1	BJP 4 IgG 3 IgA 2 IgD 2 IgE 1	0.342
Type of light chain	λ10 κ10	λ8 κ4	0.354
ISS			
Stage 2	0	4	0.003*
Stage 3	20	8	
Durie-Salmon	II 4 III 18	II 2 III 10	0.916
Deletion (13)	66%	58%	0.503
Response			
CR, VGPR, PR	4 (12.5%)	8 (25%)	0.008*
SD, PD	16 (50%)	4 (12.5%)	
UA, mg/dl	8.50 (6.61–10.37)	9.20 (5.84–12.56)	0.694
TP, g/dl	6.34 (5.72–6.97)	7.12 (5.97–8.26)	0.226
Albumin, g/dl	3.17 (2.77–3.56)	3.45 (2.98–3.92)	0.266
BUN, mg/dl	82.1 (68.3–96.0)	65.1 (42.3–88.0)	0.206
Cr, mg/dl	7.43 (6.50–8.36)	6.30 (3.14–9.47)	0.019*
Ca, mg/dl	10.7 (9.61–11.9)	8.41 (7.39–9.45)	0.006*
Hb, g/dl	7.87 (6.92–8.81)	7.64 (6.46–8.82)	0.968
LDH, mg/dl	645.9 (247.5–1,044.5)	750.4 (205.6–1,295.2)	0.770
CRP, mg/dl	5.16 (1.72–8.60)	1.68 (0.27–3.08)	0.076
Serum β_2M, mg/dl	30.6 (23.6–37.6)	13.6 (4.76–22.5)	0.002*
Urine protein, g/day	3.30 (2.21–4.53)	2.26 (1.02–4.18)	0.460

EF = Ejection fraction; ISS = International Staging Score; CR = complete remission; VGPR = very good partial response; PR = partial response; SD = stable disease; PD = progression disease; UA = urinary acid; TP = total protein; BUN = blood urea nitrogen; LDH = lactate dehydrogenase; CRP = C-reactive protein.

All values in parentheses refer to 95% CI; *$p < 0.05$.

melphalan, prednisolone) therapy. Eighteen (56.2%) patients had renal dysfunction at their first presentation to the Department of Hematology, and 14 (43.7%) patients presented with renal failure during therapy. Thirteen (37.2%) patients underwent HD after chemotherapy, and 17 (53.1%) patients received both chemotherapy and HD at around the same time.

All 6 patients for whom autopsies were performed had myeloma kidney; histopathological examinations were not performed in other patients. All patients had received HD. The mean and median survival periods from HD initiation were 10.5 and 2.0 months, respectively (fig. 1). The main cause of death was infection concomitant with disseminated intravascular coagulation (56%). The mean time from initiation to discontinuation of HD was 2.02 months in the 12 patients who were able to discontinue HD. Mean survival time from HD initiation was significantly longer in the group that could discontinue HD than in the group that could not (32.7 ± 10.6 vs. 3.08 ± 0.99 months; $p = 0.0002$). Table 2 shows that serum Ca and sβ_2M were significantly associated with HD discontinuation on multivariate analysis. These results remained unchanged when the period was shortened (e.g. 90 days).

Table 2. Prognostic factors predicting discontinuation of HD (logistic regression model)

Prognostic factor	Odds ratio[1]	p value
Serum Ca	0.35 (0.68–0.96)	0.034*
Serum β_2M	0.83 (0.10–0.92)	0.019*

* $p < 0.05$.
[1] Prognostic factors were assessed as continuous variables (sCa and $s\beta_2M$: 1 mg/dl).

Discussion

The goal of this study was to assess the prognostic factors associated with HD independence in MM patients who had ever required HD. The results of our analysis indicate that several laboratory features (including low serum Cr, low Ca, and low $s\beta_2M$), ISS score, and response to chemotherapy appear to be important factors associated with HD discontinuation in MM patients with severe renal failure.

Based on the Durie-Salmon staging system, survival periods have been reported as 18, 6 and 2 months for stages I, II and III, respectively [16]. In the present study, almost all patients were classified as stage III, and the Durie-Salmon clinical staging system appeared to be of no use for assessing the possibility of discontinuing HD ($p = 0.916$). With regard to MM type, Irish et al. [17] reported that Bence-Jones protein (BJP) and IgD myeloma accounted for 46% of cases in patients with both MM and severe renal failure. Although the presence of light-chain/IgD myeloma did not correlate with HD dependence in the present study ($p = 0.14$), light-chain BJP/IgD myeloma was found in 20 of 32 patients (62.5%) in our study, which is consistent with previously reported studies that light-chain BJP/IgD myeloma was associated with significant renal damage [18, 19].

When MM patients present with high serum Cr, their prognosis is considered to be very poor. Furthermore, there has been a report that patients with MM initiating dialysis have an adjusted relative risk of death 2.5 times greater than that of dialysis patients without MM, and they have a significantly greater 2-year mortality rate (58 vs. 31%) [10].

$s\beta_2M$ plays an important role in regulating the elimination of tumor cells, which might activate $s\beta_2M$ as an apoptosis-inducing factor [20]. Needless to say, $s\beta_2M$ is also a general marker of tubulointerstitial dysfunction in renal failure. It has been pointed out that $s\beta_2M$, the light chain of the HLA class 1 antigen complex, is one of the few factors to have independent prognostic importance for survival on univariate and multivariate analyses and is part of the ISS staging system [21]. $s\beta_2M$ might reflect not only tumor mass and renal function but also other as yet unknown parameters, possibly including immune dysfunction [12].

High serum Ca appeared to be a disadvantage with respect to recovery of renal function in the present study. Hypercalcemia is most common in myeloma patients with the greatest tumor volume, irrespective of serum parathyroid hormone-related protein (PTHrP) status. The reasons for this are still unclear, but they may be related to the amount of bone-resorbing activity produced by myeloma cells, as well as glomerular filtration status [22]. One study reported that hypercalcemia and BJP excretion were predominant when specific disease features implicated in the pathogenesis of renal failure were examined in MM patients [23]. Another study reported that hypercalcemia (calcium 11–13.8 mg/dl) was the most common precipitating factor for acute renal failure in MM patients [24].

In one study, the response rate to chemotherapy (PR and/or better response) was 39% in patients with renal failure, which was significantly lower than the 56.4% observed in patients with normal renal function [8]. The response rate of patients with renal failure treated with a single alkylating agent, melphalan, plus prednisolone was 24%, whereas the response rate in patients who received combination chemotherapy (VAD) was 50% [25]. We could safely administer the VAD regimen in patients with renal failure without any dose reduction.

Other factors, such as serum albumin or chromosomal abnormalities, were not significantly associated with independence from HD in the present study. Serum albumin is an indirect indicator of IL-6 levels, and low serum albumin correlates with both rapid myeloma growth and the patient's performance status. However, in the present study, abnormal conventional cytogenetics, such as deletion 13q with G-band, did not affect the outcome, and any difference remains to be revealed in the future. On this subject, Fonseca et al. [26] demonstrated that 3 distinct staging groups can be defined by the presence of t(4;14)(p16.3;q32), t(14;16)(q32;q23), deletion 17p13, and deletion 13q by fluorescence in situ hybridization (FISH).

On multivariate analysis, serum Ca and $s\beta_2M$ were significantly associated with HD discontinuation in the present study. These two factors are not only very easy to measure, but they may also be important new predictors of independence from HD in MM patients.

Our study had several limitations. First, for compelling reasons, the number of MM patients who underwent HD was small, which makes it difficult to evaluate the factors associated with discontinuation of HD. To the best of our knowledge, the number of MM patients who underwent HD in the present study [32] was the fourth largest number reported in the English literature [4–8, 15, 19, 22, 31–38]. Second, pathological examination should have been done to disclose the cause of renal failure or cause of death if possible in all patients, but it was not always done. In the present study, 6 patients underwent autopsy examination; all of them had cast nephropathy and died within 5 months after HD was started. One of these patients died from ventricular fibrillation and amyloidosis was detected at autopsy. The other patients died from infection/sepsis.

In conclusion, this study showed that $s\beta_2M$ and Ca were the laboratory parameters that were significant, independent prognostic factors for predicting the probability of recovery from severe renal failure and discontinuation of HD in MM patients who needed HD at least once. Further studies are needed in patients with MM to confirm our results.

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ORIGINAL ARTICLE
Molecular Cardiology

Evaluation of Pharmacogenetic Algorithm for Warfarin Dose Requirements in Japanese Patients

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Background: Warfarin dosing is difficult to establish because of considerable interindividual variation. Thus, warfarin pharmacogenetics have attracted particular interest in relation to appropriate control of anticoagulation.

Methods and Results: The 200 eligible subjects were chosen from participants in a hospital cohort. Performance of a pharmacogenetic algorithm recently developed by the International Warfarin Pharmacogenetics Consortium (IWPC) was tested and compared with a clinical algorithm (without genotype data) by calculating the percentage of patients for whom the predicted dose deviated by less than 7 mg/week (1 mg/day) from the actual dose. The pharmacogenetic algorithm accurately identified a significantly ($P<0.05$) larger proportion of patients to achieve the target international normalized ratio than did the clinical algorithm (68% vs 36% for a low-dose group; and 21% vs 0% for a high-dose group). Also, an increase in warfarin dosage was found to be appropriate for the current status of alcohol drinking (4 mg/week, as against non-drinking) and smoking (3.3 mg/week, as against non-smoking).

Conclusions: The IWPC pharmacogenetic algorithm has clinical application, particularly in identifying Japanese patients who require a low dosage of warfarin and are at greater risk of excessive anticoagulation. (*Circ J* 2010; **74:** 977–982)

Key Words: Anticoagulation; *CYP2C9*; Pharmacogenetics; *VKORC1*; Warfarin

Warfarin is the most commonly prescribed oral anticoagulant drug for the prophylaxis and treatment of thromboembolic disorders, but the appropriate dose can be difficult to establish because it can vary substantially (>10 fold) among patients, in part because of differences in each patient's age, diet, race and genotype.[1–3] Incorrect doses contribute to a high incidence of adverse effects (ie, bleeding and thromboembolic events) when the effectiveness of warfarin, expressed as the international normalized ratio of prothrombin time (PT-INR), is above or below the therapeutic range.

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During the initial dosing period (ie, the first few months), patients are at the greatest risk of overanticoagulation. To reduce this risk, a number of warfarin dosing algorithms[4,5] and regimens[6,7] have been proposed, mostly incorporating clinical factors, demographic variables, and molecular variations in 2 genes: the warfarin metabolic enzyme *CYP2C9* and the warfarin target enzyme, vitamin K epoxide reductase complex subunit 1 (*VKORC1*). Regarding genetic factors, of note is the fact that, in 2007, the US Food and Drug Administration (FDA) added pharmacogenetic information to the warfarin product label.[3] Along this line, the International Warfarin Pharmacogenetics Consortium (IWPC) has recently developed a pharmacogenetic dose algorithm for warfarin using a large data set (involving a total of 5,052 patients) from diverse ethnic groups.[8] The IWPC algorithm appears to provide better predictive accuracy than the one that uses only clinical variables or a fixed-dose (5 mg/day) strategy.

In general, patients of Asian descent require a lower maintenance dose of warfarin for a similar degree of anticoagulation than patients of European descent.[5,9] Moreover, it has been reported that compared with Europeans, the incidence of thromboembolism is low in Japan, despite the less intensive regimen;[10,11] which indicates that adjusted low-dose warfarin (eg, PT-INR 1.6–2.6) is optimal for prevention of thromboembolism in Japanese patients.[12,13]

Considering these racial differences in the anticoagulation therapy, we attempted to validate the IWPC pharmacogenetic dose algorithm for warfarin in Japanese patients under low-

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Table 1. Characteristics of Study Subjects

	n=200
M/F, n	136/64
Age at entry, years	67.8±10.3
Height, cm	160.3±14.6
Body weight, kg	61.4±12.7
Daily warfarin dose, mg	3.05±1.20
Primary reason for anticoagulation, n (%)	
Atrial fibrillation	119 (59.5)
Prosthetic valve replacement	27 (13.5)
Deep vein thrombosis/pulmonary embolism	12 (6)
Other	42 (21)
Amiodarone use, n (%)	11 (5.5)
VKORC1 rs9923231 genotype, n (%)	
G/G:A/G:A/A	3 (1.5): 31 (15.5): 166 (83)
CYP2C9 genotype, n (%)	
*1/*1:*1/*3:*3/*3	195 (97.5): 5 (2.5): 0 (0)
Alcohol drinking	
Never (%)	33.5
Ex-drinker (%)	18
Current drinker (%)	48.5
Smoking	
Never (%)	38
Ex-smoker (%)	49.5
Current smoker (%)	12.5

VKORC1, vitamin K epoxide reductase complex subunit 1.

dose treatment. Also, we examined the impact of alcohol intake and smoking on warfarin dose requirements, aiming at refinements of the algorithm.

Methods

Study Population

A total of 200 eligible subjects were chosen from participants in the Hospital-Based Cohort Study in the International Medical Center of Japan (IMCJ), which was designed to investigate clinical epidemiology, pharmacogenetics and genetic susceptibility of lifestyle-related disorders such as diabetes, hypertension and cardiovascular diseases.[14] We collected information on demographic characteristics, the primary indication for warfarin treatment, the stable therapeutic dose of warfarin, the treatment INR (the INR achieved with a stable warfarin dose), the use of concomitant enzyme inducers (carbamazepine, phenytoin, rifampin, or rifampicin) and amiodarone. Anticoagulation of patients was stably controlled with a target PT-INR of 1.6–2.6 for the prevention or treatment of thromboembolic diseases. Characteristics of the patients are shown in **Table 1**. We largely divided them into 3 categories of alcohol drinking (never-drinker; ex-drinker; current drinker) and 3 categories of smoking status (never-smoker; ex-smoker; current smoker). Participants were asked to report their daily alcohol consumption, using a structured questionnaire that ascertained the consumption of typical alcoholic beverages (beer, wine, Japanese sake, shochu and spirits). Alcohol intake was denoted in terms of servings of sake (1 gou [180 ml] of Japanese rice wine is considered equal to 22 g of ethanol). As a variable of smoking conditions, the Brinkman Index was calculated from [the number of cigarettes smoked daily]×[smoking period] in addition to the categorical smoking status. All subjects were Japanese and gave written informed consent for participation in the study. The ethics committee of IMCJ approved the study protocol.

Genotyping of *CYP2C9* and *VKORC1* SNPs

Among the genetic variants of *CYP2C9* used for the IWPC algorithm (*1, *2 and *3), the *CYP2C9*2* allele (I359L) has not been reported in Asian populations.[5,15,16] Accordingly, we genotyped the *3=rs1057910 polymorphism in relation to the wild-type allele *1, thereby determining *1/*1, *1/*3 and *3/*3 genotypes at the *CYP2C9* locus. At the *VKORC1* locus, on the other hand, the −1639 G→A=rs9923231 polymorphism was genotyped, following the IWPC algorithm.[8] Both SNPs were characterized with the use of TaqMan assays (Applied Biosystems, Foster City, CA, USA).

Statistical Analysis

First, we performed multiple regression analysis to test the effects of predictor variables on interindividual variability of warfarin dose, with the square root of the warfarin dose in mg/week being used as a dependent variable, which was in accordance with the IWPC study.[8] We then evaluated the potential clinical value of 2 algorithms (the IWPC pharmacogenetic algorithm and a clinical algorithm without including genotype data) by calculating the percentage of patients whose predicted dose of warfarin was within 7 mg/week (1 mg/day) of the actual stable therapeutic dose. The IWPC pharmacogenetic algorithm for Japanese was: 5.4952−(0.2614×[age in decades])+(0.0087×[height in cm])+(0.0128×[weight in kg])−(0.8677×[*VKORC1* A/G])−(1.6974×[*VKORC1* A/A])−(0.9357×[*CYP2C9**1/*3])−(2.3312×[*CYP2C9**3/*3])+(1.1816×[enzyme inducer status])−(0.5503×[amiodarone status])=Square root of weekly warfarin dose.[8] In addition, we calculated the percentage of patients for whom the predicted dose according to each algorithm was at least 7 mg/week higher than the actual dose (overestimation) or at least 7 mg/week lower than the actual dose (underestimation). Here, we adopted 7 mg/week (1 mg/day) as a difference that clinicians would be likely to define as clinically relevant. With consideration of warfarin dose distribution in the study sample (**Figure S1**), the performance of the IWPC algorithm was assessed in 3 dose groups: low dose (≤10.5 mg/week), high dose (≥31.5 mg/week), and intermediate doses (between 10.5 and 31.5 mg/week) for stable therapeutic anticoagulation. These thresholds of 10.5 mg and 31.5 mg/week bracket the usual maintenance dose of 17.5–24.5 mg/week (2.5–3.5 mg/day) in Japanese patients.[5,15,17,18] The overall performance was measured as the coefficient of determination, R^2, which was the square of the sample correlation "R" between the predicted and therapeutic doses. Besides assessing the potential benefit of using the pharmacogenetic algorithm instead of the clinical algorithm, we computed the number needed to genotype (NNG: the number of patients who must be genotyped in order for 1 patient to have an improved dose estimate).

Furthermore, we evaluated the effects of alcohol drinking and smoking on warfarin dose requirements by multiple regression analysis in which 3 numerical models (2 categorical and 1 continuous trait models) were tested for each behavior.

Results

The characteristics of the 200 participants in the present study are summarized in **Table 1**. Among them, the most common indications for warfarin use were atrial fibrillation

Table 2. Effect Size of Predictors on Warfarin Dose Among Japanese Subjects

Predictor	Regression of warfarin dose		Effect in the IWPC algorithm
	Effect (95%CI)	P value	
Intercept	4.940 (3.404, 6.477)	1.6E-09	3.798*
Age in decades	−0.215 (−0.334, −0.095)	5.0E-04	−0.261
Height in cm	0.001 (−0.009, 0.012)	0.82	0.009
Weight in kg	0.012 (−0.001, 0.025)	0.07	0.013
VKORC1 rs9923231			
AG vs AA	0.862 (0.545, 1.178)	2.2E-07	0.830
GG vs AA	1.677 (0.714, 2.640)	7.3E-04	1.697
CYP2C9 rs1057910			
*1/*3 vs *1/*1	−0.714 (−1.466, 0.038)	0.06	−0.936
Amiodarone status	−0.475 (−0.972, 0.022)	0.06	−0.550

Predictor variables in the multiple regression are same as those in the pharmacogenetic dosing algorithm proposed by the IWPC; enzyme inducer status is not shown because none of the subjects was taking any of the enzyme inducers listed in the IWPC algorithm (ie, carbamazepine, phenytoin, rifampin or rifampicin).
A dependent variable is the square root of the warfarin dose in mg/week.
*Intercept used for patients of Asian race with AA genotype at *VKORC1* rs9923231 and *1/*1 genotype at *CYP2C9* rs1057910.
CI, confidence interval; IWPC, International Warfarin Pharmacogenetics Consortium; *VKORC1*, vitamin K epoxide reductase complex subunit 1.

Table 3. Percentage of Patients With Ideal, Underestimated or Overestimated Dose of Warfarin, as Estimated by the Pharmacogenetic and Clinical Algorithms, in Patients Requiring Low, Intermediate or High Actual Doses of Warfarin

Actual dose required	No. of patients	Patients classified by performance of prediction			Difference between 2 algorithms, P value
		Ideal dose (error within ≤7 mg/week), %	Underestimated, %	Overestimated, %	
≤10.5 mg/week	25				0.046
Pharmacogenetic algorithm		68	0	32	
Clinical algorithm		36	0	64	
>10.5 to <31.5 mg/week	151				0.613
Pharmacogenetic algorithm		80	19	1	
Clinical algorithm		79	18	3	
≥31.5 mg/week	24				0.050
Pharmacogenetic algorithm		21	79	0	
Clinical algorithm		0	100	0	

The clinical algorithm involves clinical and demographic variables (age, height, weight, and medication), and the pharmacogenetic algorithm involves the same set of variables plus genotypes in 2 genes (*CYP2C9* and *VKORC1*).
Underestimate is the case where the predicted dose (by either pharmacogenetic or clinical algorithm) is lower than the observed dose; overestimate is the opposite.
VKORC1, vitamin K epoxide reductase complex subunit 1.

(n=119, 59.5%), prosthetic valve replacement (n=27, 13.5%), and deep vein thrombosis or pulmonary embolism (n=12, 6%). The minor allele frequencies of rs1057910 (*CYP2C9*) and rs9923231 (*VKORC1*) were 0.013 and 0.093, respectively, which were comparable to those previously reported in Japanese patients[5,15,17,18] or HapMap JPT (http://hapmap.ncbi.nlm.nih.gov/): 0.016–0.049 for rs1057910 and 0.075–0.088 for rs9923231. Each SNP was in Hardy-Weinberg equilibrium (P>0.05).

The effects of predictor variables on warfarin dose were first examined in the ordinary regression model (**Table 2**). The effect sizes of individual variables thus estimated were almost comparable to those in the IWPC algorithm.[8] When applied to the Japanese patients' data, the IWPC pharmacogenetic algorithm identified significantly (P<0.05) larger proportions of patients who required 10.5 mg or less per week (low-dose group) or those who required 31.5 mg or more per week (high-dose group) to achieve the target PT-INR than did the clinical algorithm (68% vs 36% in low-dose group; and 21% vs 0% in high-dose group; **Table 3**). We depicted the fair performance of the IWPC pharmacogenetic algorithm in the plots comparing the predicted dose and actual dose of warfarin (R^2=0.28, P=1.5×10^{-15}) (**Figure**).

A significant benefit of using genetics was further verified with the NNG analysis (**Table 4**). The NNG can be computed using the number needed to treat (NNT) method; the NNT is the inverse of the absolute risk reduction (ARR). The ARR was calculated as the absolute difference between the event rate (ER) for the pharmacogenetic algorithm and the ER for the clinical algorithm (ER=ratio of the number of patients for which an algorithm estimates a poor dose (more or less than 7 mg/week than the actual therapeutic dose) over the total number of patients). Despite different criteria for a poor dose estimate (ie, the criteria in the IWPC study[8] were >20% above or below the actual therapeutic dose), the NNG was in good agreement between the studies: 13.3 in the present study and 13.2 in the IWPC study.

Our data on Japanese patients also indicated that both

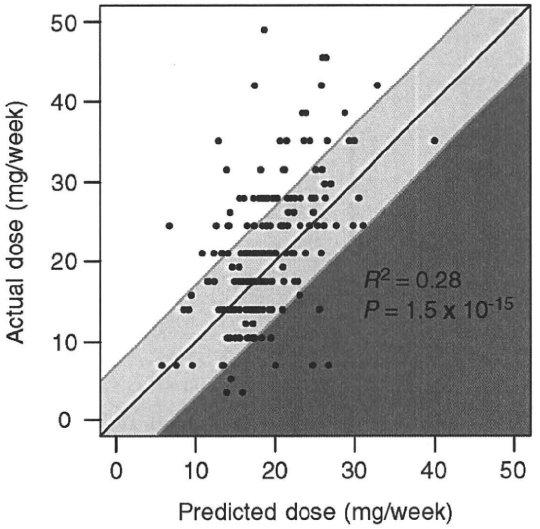

Figure. Predicted vs actual stable therapeutic warfarin dose (mg/week) for 200 Japanese patients in the present study. The diagonal solid (black) line indicates the perfect prediction, above and below which the gray lines indicate thresholds that the predicted dose according to the International Warfarin Pharmacogenetics Consortium pharmacogenetic algorithm is 7 mg/week lower than the actual dose (underestimation) or 7 mg/week higher than the actual dose (overestimation).

alcohol drinking and smoking significantly influence warfarin dose (**Table 5**). In the multiple regression model, the current status of drinking or non-drinking (ex-drinker+never-drinker) and that of smoking or non-smoking (ex-smoker+never-smoker) exerted approximate warfarin dose effects of 4 mg/week ($P=9.5\times10^{-5}$, $R^2=0.06$) and 3.3 mg/week ($P=0.03$, $R^2=0.02$), respectively. With these predictor variables being incorporated into the IWPC algorithm, its performance was augmented ($R^2=0.33$, $P=5.5\times10^{-19}$) (**Figure S2**).

Table 4. NNG Analysis: Clinical vs Pharmacogenetic Algorithm With ±7 mg/week Criterion

No. of events (n=200)	
Clinical >7 mg/week than actual	21
Clinical <7 mg/week than actual	51
Pharmacogenetic >7 mg/week than actual	10
Pharmacogenetic <7 mg/week than actual	47
Absolute risk reduction	0.075
NNG	13.3

NNG, number needed to Genotype.

Discussion

We have evaluated the IWPC pharmacogenetic algorithm in 200 Japanese patients under low-dose warfarin treatment. Although the target PT-INR (1.6–2.6) in the present study was slightly lower than the range (2.0–3.0) set in the IWPC study,[8] the performance of the tested algorithms in the 2 studies proved almost comparable: $R^2=0.28$ in the present study and $R^2=0.33$–0.34 for Asians in the IWPC study. Besides the reproducible performance in the whole study sample, of particular note is the fact that among patients in a low-dose group (≤10.5 mg/week), the percentage of overestimation was significantly smaller when the warfarin dose was predicted with the pharmacogenetic algorithm (32%) than with the clinical algorithm (64%) (**Table 3**), thus enabling us to appreciably reduce the risk of overanticoagulation. Furthermore, we demonstrated the substantial influence of alcohol drinking and smoking on warfarin dose requirements, which used to be anticipated but has not been evaluated in detail thus far.[2]

The incidence of major bleeding (eg, intracranial hemorrhage) has been reported as higher in Japanese patients (6.6% per year) than in European patients (1.6–2.5% per year) with adjusted standard-dose warfarin therapy: a target PT-INR of 2.2–3.5 in the Japanese and 2.0–4.5 in Europeans.[19–22] Because of such racial differences in bleeding tendency under warfarin, the necessity of customizing warfarin therapy has been argued.[10] Recently, a prospective study of 4,202 patients

Table 5. Effects of Alcohol Drinking and Smoking on Warfarin Doses in Different Regression Models

Model	Tested predictors	Approximate effect on warfarin dose in mg/week*		
		Effect (95%CI)	P value	R^2
Alcohol drinking				
Model 1	Stopped vs Yes	−4.1 (−6.6, −1.5)	0.003	0.06
	No vs Yes	−3.9 (−6.0, −1.7)	6.7E-04	
Model 2	Stopped/No vs Yes	−4.0 (−5.8, −2.1)	9.5E-05	0.06
Model 3	Alcohol unit (gou) per week†	0.6 (−0.05, 1.2)	0.070	0.01
Smoking				
Model 1	Stopped vs Yes	−3.5 (−6.4, −0.4)	0.03	0.02
	No vs Yes	−2.9 (−6.0, 0.4)	0.09	
Model 2	Stopped/No vs Yes	−3.3 (−6.1, −0.3)	0.03	0.02
Model 3	Brinkman index [amount per day×years]†	−0.02 (−0.1, 0.1)	0.56	0.001

Alcohol drinking was categorized into 3 groups: current drinker (yes), abstainer (stopped), and never-drinker (no), based on the self-reported questionnaire. Likewise, smoking status was categorized into 3 groups: current smoker (yes), ex-smoker (stopped) and never-smoker (no).
*Predictors in Table 2 were included as covariates in the tested regression model.
†The square root of the value was used for the regression analysis.
CI, confidence interval.

showed an optimal PT-INR of 3.0–3.5 in the Dutch,[23] whereas the Japanese Guidelines for Pharmacotherapy of Atrial Fibrillation[24–26] (JCS 2008) have set a PT-INR of 2.0–3.0 as the therapeutic range, except for the elderly (≥70 years of age), in whom a lower dose of warfarin (PT-INR 1.6–2.6) is recommended for prevention of thromboembolism and safety from bleeding complications.[27] The intensity of warfarin control (ie, optimal PT-INR) in the Japanese remains to be further defined according to the balance between risks and benefits under individual conditions. Among the primary indications for warfarin use, the optimal therapeutic range has been debated for patients with prosthetic valve replacement,[13] corresponding to 13.5% of the current subjects (**Table 1**). Partly because of the risk of eventual valve failure of bioprosthesis, and resultant reoperation, there seems to be a tendency for increased use of prosthetic valves in Japan, with its population's long life expectancy, as compared with the USA and Europe. Including patients with prosthetic valve replacement, because the target PT-INR is often set at 1.6–2.6 in outpatient clinics in Japan,[12] our findings obtained in equivalent clinical setting should encourage clinicians to apply the IWPC algorithm to their patients. Nevertheless, in cases where the optimal therapeutic range is set differentially according to the primary disease condition, some modification of the IWPC algorithm may be required.

We have found that the average dose of warfarin in the Japanese patients is 21 mg/week (3 mg/day), which is less than the standard dose (35 mg/week) in Europeans, and in the present study one-eighth (12.5%) of the participants were categorized into a low-dose group (≤10.5 mg/week). If a fixed dose of 3 mg/day is given to these patients without conscientious monitoring of PT-INR, there is a high risk of overanticoagulation, leading to fatal bleeding events. The use of the IWPC algorithm will enable clinicians to detect approximately two-thirds (68%) of the Japanese patients in this low-dose group, which is twice as large as the proportions (36%) attainable with the clinical algorithm (**Table 3**). Although the value of adding genotype to clinical (and demographic) information seems to be less modest, benefits also accrue to Japanese patients in the high-dose group (≥31.5 mg/week); 21% of the patients were identified with the pharmacogenetic algorithm, but none (0%) with the clinical algorithm (**Table 3**).

Since the US FDA changed the labeling of warfarin to suggest that clinicians consider using genetic tests to guide dosing,[3] warfarin pharmacogenetics has drawn substantial attention towards "personalized" patient care. Although more than 30 genes may contribute to the net warfarin effect, *CYP2C9* and *VKORC1* are known to exert the most influence.[28–30] The pharmacogenetic algorithm involving these polymorphisms, developed by the IWPC, can predict approximately one-third of all dosing variations at most.[8] The question of whether the knowledge of genetic information is cost-effective in reducing bleeding and thrombotic complications is under debate.[31] Many clinical factors influence warfarin dose requirements, including diet (in particular, the vitamin K content) and concomitant drug administration, besides a list of variables that have been incorporated into the IWPC algorithm. As the clinical factors often change in individual patients, appropriate alterations in warfarin dosing must be made, regardless of genetic information. In this respect, it is important to quantitatively evaluate the individual contribution of clinical factors, as has been performed for alcohol drinking and smoking in the present study, toward refining warfarin pharmacogenetic testing for not only initial but also maintenance dose requirements.

In summary, we report the usefulness of the IWPC pharmacogenetic algorithm in its clinical application, particularly for identifying Japanese patients who require a low dose of warfarin. However, it has to be kept in mind that considering the current limitations of its application to clinical medicine, this testing alone does not make conscientious PT-INR monitoring unnecessary. When the genotype cost falls to a more reasonable level, we expect that the use of pharmacogenetic-based initial dosing will become routine clinical practice.

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Supplementary files

Figure S1. Histogram of warfarin dose.

Figure S2. Actual dose vs predicted dose with information on alcohol drinking and smoking incorporated into the International Warfarin Pharmacogenetics Consortium (IWPC) pharmacogenetic algorithm.

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PLoS one

Deletion of CDKAL1 Affects Mitochondrial ATP Generation and First-Phase Insulin Exocytosis

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Abstract

Background: A variant of the *CDKAL1* gene was reported to be associated with type 2 diabetes and reduced insulin release in humans; however, the role of CDKAL1 in β cells is largely unknown. Therefore, to determine the role of CDKAL1 in insulin release from β cells, we studied insulin release profiles in *CDKAL1* gene knockout (*CDKAL1* KO) mice.

Principal Findings: Total internal reflection fluorescence imaging of *CDKAL1* KO β cells showed that the number of fusion events during first-phase insulin release was reduced. However, there was no significant difference in the number of fusion events during second-phase release or high K^+-induced release between WT and KO cells. CDKAL1 deletion resulted in a delayed and slow increase in cytosolic free Ca^{2+} concentration during high glucose stimulation. Patch-clamp experiments revealed that the responsiveness of ATP-sensitive K^+ (K_{ATP}) channels to glucose was blunted in KO cells. In addition, glucose-induced ATP generation was impaired. Although CDKAL1 is homologous to cyclin-dependent kinase 5 (CDK5) regulatory subunit-associated protein 1, there was no difference in the kinase activity of CDK5 between WT and *CDKAL1* KO islets.

Conclusions/Significance: We provide the first report describing the function of CDKAL1 in β cells. Our results indicate that CDKAL1 controls first-phase insulin exocytosis in β cells by facilitating ATP generation, K_{ATP} channel responsiveness and the subsequent activity of Ca^{2+} channels through pathways other than CDK5-mediated regulation.

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Introduction

Type 2 diabetes is a multifactorial disease characterized by decreased insulin secretion and decreased insulin action at target tissues. While the primary molecular defects in type 2 diabetes remain largely unknown, genetic factors in combination with environmental factors are thought to be involved in the onset and development of the disease. Recent genome-wide association studies have identified *CDKAL1* (cyclin-dependent kinase 5 regulatory subunit associated protein 1-like 1) as a susceptibility gene for type 2 diabetes, which has been replicated in several populations [1–5]. The *CDKAL1* gene is located on chromosome 6p22.3 and encodes a 65-kDa protein (CDKAL1). The expression of *CDKAL1* mRNA has been detected in human tissues including pancreatic islets, skeletal muscle and brain [1,4]. Although the function of CDKAL1 is still unclear, CDKAL1 is similar to cyclin-dependent kinase 5 regulatory subunit-associated protein 1 (CDK5RAP1), which is expressed in neuronal tissues, and inhibits cyclin-dependent kinase 5 (CDK5) activity by binding to the CDK5 activator p35 [6]. CDK5 was previously implicated in islet function [7–9], suggesting that CDKAL1 plays a role in β cell function by inhibiting CDK5 kinase activity [1–4].

Genome-wide association studies have shown that several single-nucleotide polymorphisms (SNPs) in intron 5 of the *CDKAL1* gene are associated with type 2 diabetes [1–4]. An association between SNPs and insulin release has also been suggested [2,3]. Recently, Groenewoud et al. [10] and Stancakova et al. [11] reported that a *CDKAL1* variant (rs7754840) decreased first-phase insulin secretion but not second-phase insulin secretion during hyperglycemic clamps and intravenous glucose tolerance tests, respectively. Furthermore, the gene variants were not associated with the insulin sensitivity index [10,11], suggesting that *CDKAL1* variants influence the risk of type 2 diabetes by impairing first-phase insulin secretion. However, the molecular

mechanisms through which CDKAL1 modulates insulin release in pancreatic β cells are unknown.

To understand the role of CDKAL1 in β cells, we studied the phenotype of mice deficient in CDKAL1 expression, which was established using a gene-trap technique (*CDKAL1* KO mice). Our data demonstrate that CDKAL1 has a role in first-phase insulin exocytosis in β cells by facilitating ATP generation, K_{ATP} channel responsiveness and subsequent Ca^{2+} channel activity; surprisingly, these effects were independent of CDK5 activity.

Results

Expression and histological examination of *CDKAL1* KO mice

To explore the potential role of CDKAL1 in β cells, we studied *CDKAL1* KO β cells. *CDKAL1* KO mice did not express *CDKAL1* mRNA in pancreatic islets or the brain (Fig. 1A). In immunoblot analysis (Fig. 1B), CDKAL1 protein was not detected in KO islets or KO brain, whereas it was more highly expressed in WT islets than in WT brain. We next morphologically examined the pancreatic islets. Immunostaining of pancreatic sections with insulin antibodies revealed no significant difference in islet architecture, including β cell area per pancreas, between WT and KO mice (Fig. 2A–B). Electron microscopy of the pancreatic β cells also revealed that cell size, total number of granules per section and mean granule diameter were similar in WT and KO β cells (Fig. 2C–F). Furthermore, pancreatic insulin content, islet DNA content and islet insulin content of *CDKAL1* KO mice were similar to those of WT mice (Fig. 2G–I). Thus, *CDKAL1* KO β cells displayed specific CDKAL1 protein depletion but their characteristics were similar to those of WT cells.

Effects of *CDKAL1* KO on glucose-induced biphasic insulin exocytosis

We next investigated the effects of *CDKAL1* KO on glucose-induced insulin release from β cells. Batch experiments showed that 16.7 mM glucose-induced insulin release (for 30 min) from *CDKAL1* KO β cells was decreased (~19%) relative to that from WT β cells (P=NS) (Fig. 3A). To evaluate in detail the effects of CDKAL1 deficiency on glucose-evoked biphasic insulin exocytosis, the dynamic motion of individual insulin granules tagged with green fluorescent protein (GFP) was observed under total internal reflection fluorescence (TIRF) microscopy. As previously reported [12], stimulation with 22 mM glucose evoked biphasic insulin granule exocytosis through fusion of two types of granules (previously docked granules and newcomers) in WT β cells (Fig. 3B, top). During the first phase (within 5 min), the fusion events mostly occurred from the previously docked granules with some of the fusion events from newcomers while continuous fusion was observed thereafter, mostly from newcomers during the second phase (over 15 min). In contrast, *CDKAL1* KO β cells showed abnormalities in biphasic insulin granule exocytosis (Fig. 3B, bottom) because the number of fusion events during the first phase was significantly reduced in KO cells [WT (n = 16) vs. KO (n = 14), 43.0±6.2 vs. 12.7±3.8, P<0.001]. In contrast, during the second phase, the number of fusion events tended to be increased but there was no significant difference [WT (n = 16) vs. KO (n = 14), 87.3±6.4 vs. 105.6±8.0, P = NS].

On the other hand, high K^+-induced granule fusion was normal in KO cells (Fig. 3C), suggesting that the exocytotic steps after the elevation of intracellular calcium concentration ($[Ca^{2+}]_i$) were not impaired in KO cells. In fact, the number of morphologically docked insulin granules observed by TIRF and electron microscopy (Fig. 4A and B), and fusion kinetics (data not shown) were not altered in *CDKAL1* KO β cells. In addition, the expression levels of SNARE proteins (syntaxin1A, SNAP25 and VAMP2), important mediators of insulin granule docking and fusion [12], were similar in WT and KO β cells (Fig. 4C). These results indicate that granule docking and Ca^{2+}-triggered fusion are not impaired in *CDKAL1* KO β cells.

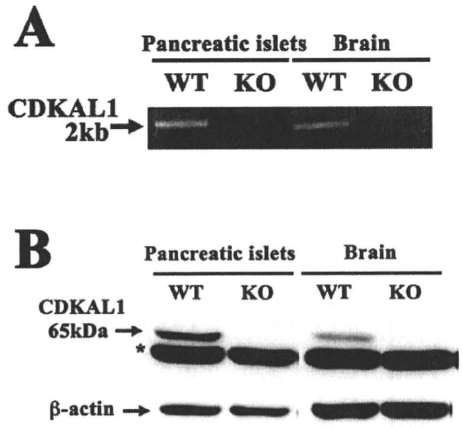

Figure 1. CDKAL1 expression is absent in *CDKAL1* KO mice. **A.** RT-PCR analysis of pancreatic islets and the whole brain of WT and *CDKAL1* KO mice. The *CDKAL1* transcript was not detected in *CDKAL1* KO mice. **B.** Immunoblot analysis. Homogenates of mouse pancreatic islets and whole brain (30 μg) were subjected to SDS-PAGE and immunoblotted with anti-CDKAL1 antibody. The protein band below the CDKAL1 protein (*) is a nonspecific protein band detected by the anti-CDKAL1 antibody.
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Changes in the $[Ca^{2+}]_i$ increase in KO cells

To determine whether the impairments in glucose-induced first-phase insulin exocytosis were associated with changes in the $[Ca^{2+}]_i$ increase, we monitored the time-course of the glucose-induced $[Ca^{2+}]_i$ increase using Fura-2 methods. *CDKAL1* KO resulted in a delayed and slow increase in $[Ca^{2+}]_i$ during high glucose stimulation with a similar time-course to insulin exocytosis in KO cells. In contrast, a rapid and marked $[Ca^{2+}]_i$ rise was observed in WT cells (Fig. 5A). The mean lag time (Δ time from stimulation to onset) and rise time (Δ time from onset to peak) were slower in KO cells than in WT cells [WT vs. KO (n = 12 each) lag time: 1.2±0.1 min vs. 3.0±0.1 min, P<0.0001; rise time: 3.0±0.2 min vs. 4.1±0.2 min, P<0.001]. The magnitude of the $[Ca^{2+}]_i$ rise was not different between KO and WT cells. Furthermore, there was no difference in the high K^+-induced $[Ca^{2+}]_i$ rise between KO and WT cells (Fig. 5B). These data suggest that specific steps in glucose-induced early signal transduction that precede the $[Ca^{2+}]_i$ rise were impaired in KO cells.

The responsiveness of the K_{ATP} channel current to glucose was impaired in *CDKAL1* KO β cells

We next investigated the function of K_{ATP} channels in *CDKAL1* KO cells. As shown in Fig. 6A, there were no differences in the expression of the K_{ATP} channel components Kir6.2 and SUR1 between WT and KO islets. The K_{ATP} channel currents evoked by the voltage ramp from −100 to −50 mV in WT and KO mice are expressed as the current density determined as the calculated conductance (pS) divided by the cell capacitance (pF). The K_{ATP} currents were determined in the presence of 2.8 and 8.3 mM

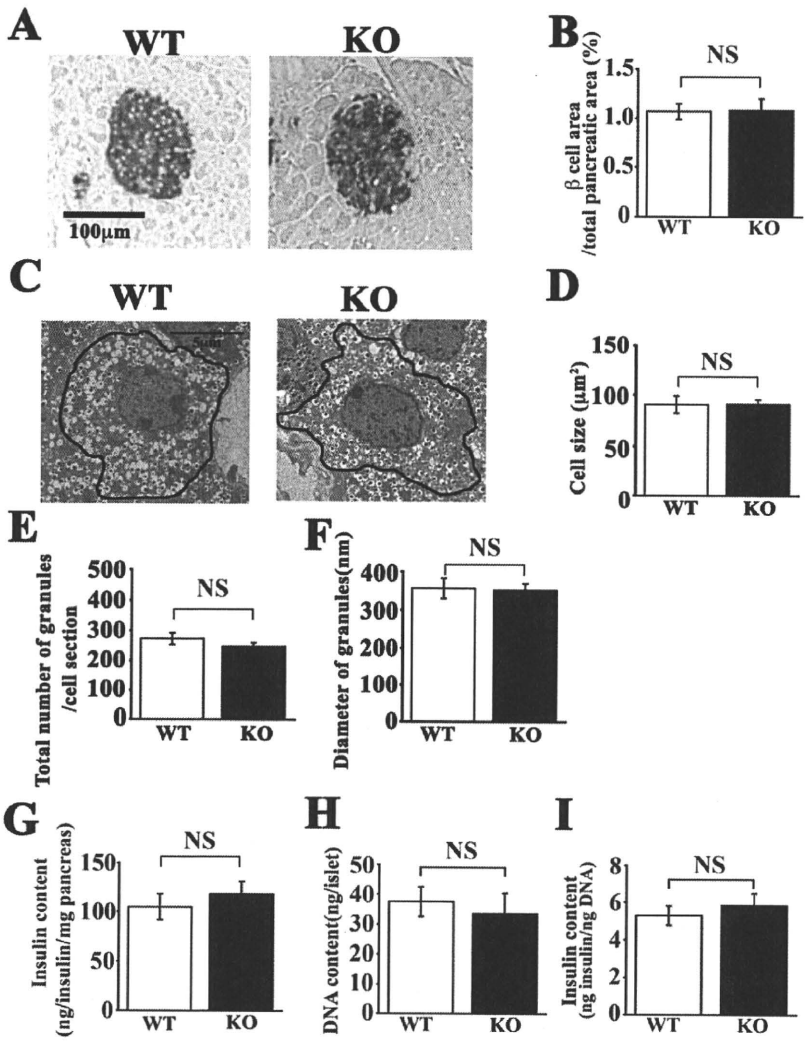

Figure 2. Pancreatic histology and insulin content in *CDKAL1* KO mice. A. *CDKAL1* KO mice have normal islet architecture. Pancreatic sections were peroxidase stained for insulin. Scale bar: 100 μm. **B.** Relative area occupied by β cells (percentage of total pancreatic area). Random sections of the entire pancreas from WT and *CDKAL1* KO mice were immunostained (as shown in **A**) and analyzed (60 sections from each of three mice per group). **C–F.** Electron micrographs of pancreatic tissue sections. **C** Representative sections (scale bar: 5 μm), **D** β cell size, **E** total number of granules per cell section, and **F** mean granule diameter in ultra-thin sections (100 nm) (n = 20 cells per group) of *CDKAL1* KO and WT β cells. **G–I.** Insulin content in *CDKAL1* KO mice. **G** Pancreatic insulin content measured in acid-ethanol extracts from WT and KO mice by ELISA (n = 6 per group). **H** DNA content per islet and **I** islet insulin content per DNA from WT and KO mice (n = 6 per group). Results are means±SEM.
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glucose in WT and KO mice (Fig. 6B and C). The current densities at 2.8 mM glucose were 392.9±121.6 pS/pF in WT (n = 6) and 639.5±145.1 pS/pF in KO cells (n = 8) (P>0.05). In WT cells, the K_{ATP} current density decreased significantly by increasing the glucose concentration from 2.8 to 8.3 mM (Fig. 6D; P<0.02), while there was a relatively small, non-significant decrease in the current density in KO mice (Fig. 6E; P>0.05). In addition, the time to the most inhibited level of K_{ATP} currents after exposing β cells to 8.3 mM glucose tended to be longer in KO cells (4.73±1.16 min, n = 5) compared with that in WT cells (3.11±0.48 min, n = 6; P>0.05). Thus our data indicate that the K_{ATP} channel responsiveness to glucose stimulation was blunted in *CDKAL1* KO β cells compared with WT cells.

On the other hand, we did not observe any differences in the ATP sensitivity of the channel in studies using inside-membrane patches. The half-maximal concentration of channel inhibition for ATP was 2.6 μM (n = 9) in *CDKAL1* KO mice (data not shown), which is less than those previously reported in control mice [13,14]. These results suggest that changes in K_{ATP} channel activity in KO cells were not responsible for the reduced ATP sensitivity of these channels.

ATP generation was impaired in KO cells

It is conceivable that the reduced K_{ATP} channel responsiveness to glucose in KO cells might be due to a defect in ATP production following glucose metabolism. Therefore, we measured ATP content in islets from WT and KO mice after incubation with 2.2 and 22 mM glucose using high-performance liquid chromatography (HPLC) with a reverse-phase column. As shown in Fig. 7A, ATP content was significantly increased in WT islets by stimulation

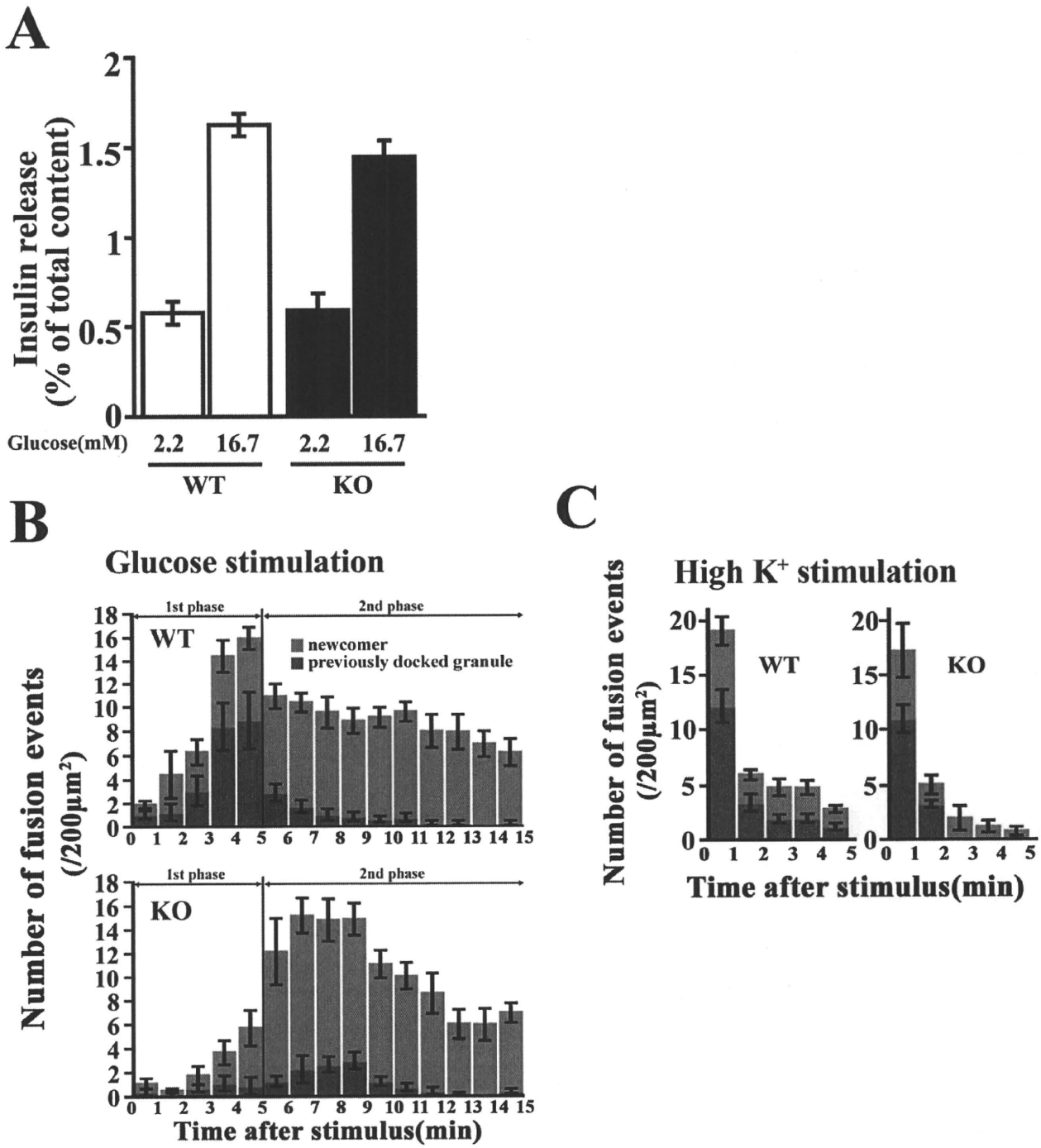

Figure 3. Effects of *CDKAL1* KO on glucose-induced biphasic insulin exocytosis. A. Insulin release (for 30 min) in batch-incubated WT and *CDKAL1* KO β cells in the presence of 2.2 mM or 16.7 mM glucose (n = 8 per group). **B.** Histogram showing the number of fusion events from GFP-tagged granules in wild-type (WT) and *CDKAL1* KO β cells (per 200 μm^2) at 1-min intervals after stimulation with 22 mM glucose and measured by TIRF microscopy. Data are mean±SEM (WT, n = 16 cells; KO, n = 14 cells). Time 0 indicates the addition of 22 mM glucose. The red column shows fusion events from previously docked granules, and the green column shows those from newcomers. **C.** Histogram showing the number of fusion events in WT and KO β cells (per 200 μm^2) at 1-min intervals after 40 mM high K^+ stimulation measured by TIRF microscopy (n = 8 cells per group).
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with 22 mM glucose ($P<0.001$; n = 7). In contrast, the ATP levels in the KO islets showed a small but not statistically significant increase, indicating impaired glucose metabolism in KO islets.

In line with glucose metabolism, we measured changes in the mitochondrial membrane potential in WT and KO cells during glucose stimulation using tetramethyl rhodamine ethyl ester

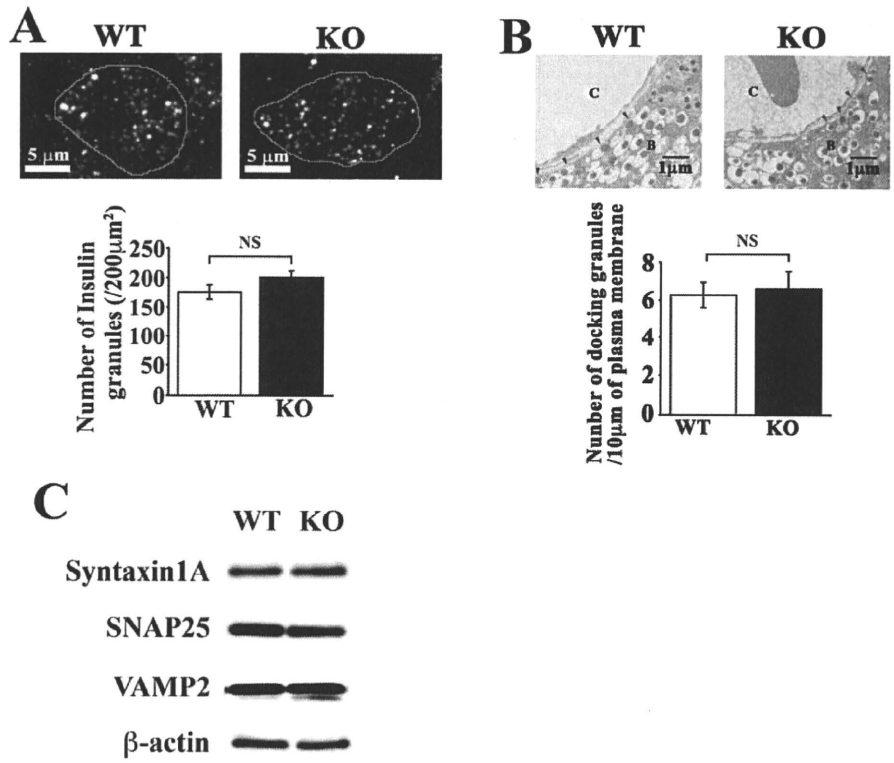

Figure 4. *CDKAL1* KO did not affect the number of morphologically docked granules or granule fusion kinetics. A. Total internal reflection fluorescence (TIRF) microscopy of insulin granules morphologically docked to the plasma membrane. (top) Typical TIRF images of docked insulin granules in WT and *CDKAL1* KO β cells. The surrounding lines represent the outline of cells attached to the cover glass. Scale bar: 5 μm. Pancreatic β cells were prepared from WT and KO mice, fixed, and immunostained for insulin. (bottom) Number of insulin granules morphologically docked to the plasma membrane. Individual fluorescent spots shown in the TIRF images were manually counted per 200 μm^2 in 15 cells per group. **B.** Electron micrograph of β cell sections. (top) Typical images of the plasma membrane area facing the blood capillary (C) of WT and KO β cells (B). Bar: 500 nm. (bottom) Number of morphologically docked insulin granules per 10 μm of the plasma membrane. Granules at their shortest distance of <10 nm from the plasma membrane were defined as morphologically docked granules (red arrowheads). Results are means±SEM. **C.** Expression of SNARE proteins in wild-type (WT) and KO islets by immunoblotting. Equal amounts of islet protein (30 μg) were separated by SDS-PAGE and immunoblotted. β-actin was used as a loading control.
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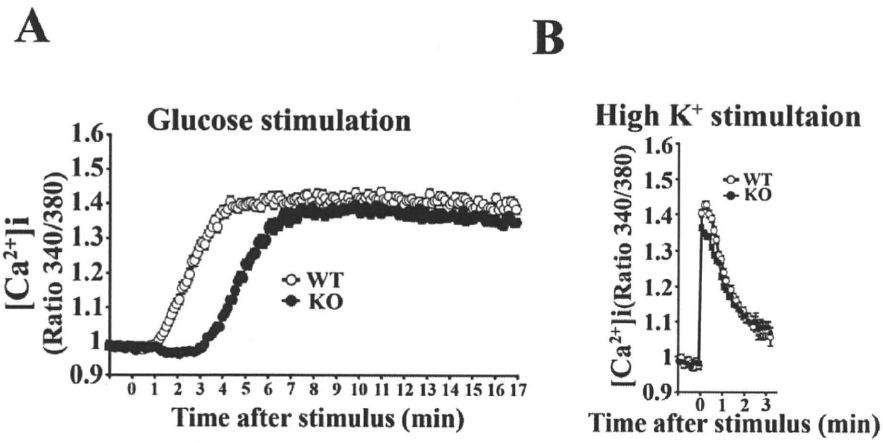

Figure 5. Effects of *CDKAL1* KO on changes in intracellular free calcium ($[Ca^{2+}]_i$). A 22 mM glucose- and **B** 40 mM high K^+-induced changes in $[Ca^{2+}]_i$ in WT and *CDKAL1* KO β cells. Changes in $[Ca^{2+}]_i$ were measured by Fura-2 acetoxymethyl (2 μM). Time 0 indicates when the stimulants were added. The fluorescence ratio (340/360) at time 0 was taken as 1. Results are means±SEM (n = 12 cells per group).
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