験者も効果を判定する評価者（医師）も分からないようにしたのが無作為化二重盲検群間比較試験（randomized double-blind comparative trial）で、薬剤の効果を客観的に評価するためには最もよい試験であるとされている。

・プラセボとプラセボ効果

プラセボとは、臨床試験において比較対象の新規医薬品と外見上の区別は付かないが有効成分を含まないもののことをいい、偽薬とも呼ばれる。薬剤の効果を評価する際、「薬を飲んだ」ということだけで症状が軽くなった気になることがあり（プラセボ効果）、特に鎮痛薬や精神疾患の対処薬で見られることが多い。そのような思い込みを排除し、薬剤の真の効果を立証するために臨床試験ではプラセボが用いられる。臨床試験では、その種の思い込みのほかに、臨床試験に参加することで生活習慣が規則正しくなったり（試験によっては患者日誌を毎日つけることもある）暴飲暴食をしなくなったりということで症状が緩和されることもあり、それもプラセボ効果の一因となっている。疾患にもよるが、本文中で述べられているように、臨床試験ではプラセボ効果が思ったより大きいことが結構ある。しかしだからといってプラセボを処方すればよいとならないことはもちろんである。

・ハミルトンうつ病評価尺度

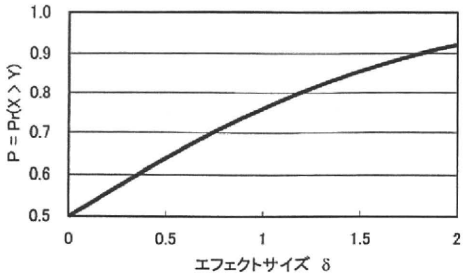
全世界で標準的に用いられているうつ病の評価尺度であり、17の項目の質問への回答によるものとなっている。各項目には0点から4点もしくは0点から2点の評価数字があり、点数の合計は最も軽度の0点から最も重度の52点となる。質問項目はたとえば「抑うつ気分はない」(0) から「抑うつ気分が言葉や言葉以外の行動の端々に現れる」(4) や、「落ち着いている」(0) から「手を動かす、爪を噛む、髪を抜く、唇を噛むなどの動作を行う」(4) となっている。医師が患者の病状の変化を評価するために利用されていて7点以下であれば治ったとされる。

・効果の大きさ（エフェクトサイズ）

エフェクトサイズの定義には種々のものがあるが、2つの群がそれぞれ分散は同じで平均値が異なる正規分布N（μ_1, σ^2)、N（μ_2, σ^2）で表されるとき、標準化された差$\delta=(\mu_1-\mu_2)/\sigma$とするのが最も一般的である。第1群および第2群からそれぞれ独立にm個、n個の観測値の観測値を得て、それらの標本平均$\bar{x}$、$\bar{y}$およびプールした分散sが求められたとすると、（母集団）エフェクトサイズdの推定値は$d=(\bar{x}-\bar{y})/s$となる。母集団平均の差の検定で用いられる2標本t統計量は$t=\sqrt{mn/(m+n)}\cdot d$であるので（m＝nのときは$t=\sqrt{n/2}\cdot d$)、エフェクトサイズdが大きいかもしくはmおよびnが大きい（あるいはその両方の）ときに検定は有意になる（統計的有意性が達成される)。エフェクトサイズが大きければ検定は有意になりやすいが、エフェクトサイズが小さくてもサンプルサイズが大きければ検定では有意となる。このように、統計的検定ではサンプルサイズが重要な役割を果たし、統計的有意性と実質上の有意性との間の乖離がしばしば問題とされる。統計手法を正しく理解していればこの種の混同はないのであろうが、実際上そうでないことも多い。そのため、エフェクトサイズはサンプルサイズと無関係に定義されるのである。

エフェクトサイズの解釈の一つとしてX～N（μ_1, σ^2）およびY～N（μ_2, σ^2）としたときP＝Pr(X＞Y)が用いられることがある。これは両群から1名ずつの被験者を無作為に選んだとき

に第１群の被験者の効果が第２群の被験者の効果よりも大きい確率を表わしていて、Ｐとエフェクトサイズδとの関係は以下のようである。本文中で言及されているδ＝0.5のときは大よそＰ＝0.64である。



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MAIN PAPER

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Sample size re-estimation for survival data in clinical trials with an adaptive design

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In clinical trials with survival data, investigators may wish to re-estimate the sample size based on the observed effect size while the trial is ongoing. Besides the inflation of the type-I error rate due to sample size re-estimation, the method for calculating the sample size in an interim analysis should be carefully considered because the data in each stage are mutually dependent in trials with survival data. Although the interim hazard estimate is commonly used to re-estimate the sample size, the estimate can sometimes be considerably higher or lower than the hypothesized hazard by chance. We propose an interim hazard ratio estimate that can be used to re-estimate the sample size under those circumstances. The proposed method was demonstrated through a simulation study and an actual clinical trial as an example. The effect of the shape parameter for the Weibull survival distribution on the sample size re-estimation is presented. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: interim analysis; hazard ratio; sample size re-estimation; survival data

1. INTRODUCTION

In clinical trials, adaptive designs have been widely used over the past decade. Among the various adaptive designs, the design used to re-estimate the sample size on the basis of the observed treatment effect have been controversial form various viewpoints such as complicated inferential decisions, the possibility of resulting in clinically meaningless differences, and efficiency [1–3]. Nevertheless, the sample size re-estimation itself has attracted the attention of many investigators, as it is common that uncertainties remain in the critical assumptions about the effect size and extent of data variation during the design of a trial before its start. In the case of continuous and binary data, the methodology for sample size re-estimation has been intensively discussed for two decades. Proschan and Hunsberger [4] proposed a conditional power based on the treatment effect in an interim analysis and proposed critical values to preserve the type-I error rate for the test of a two-sample mean. Cui *et al.* [5] proposed a test statistic for testing the two-sample means that preserve the type-I error rate, using the ordinary critical values of fixed group sequential designs.

Clinical trials to compare the time to events such as death or heart failure tend to be large in size and very long in duration, and they contain fields with a great need for the sample size re-estimation based on interim analyses. As group sequential designs to terminate a trial early because of futility or success are frequently employed in trials with survival data, the design for re-estimating the sample size should allow the early termination of the trial.

There are various methods for using the survival data to control the inflation of the type-I error rate when the sample size is modified on the basis of the observed effect size, see, for example, References [6–9]. These procedures are sometimes complicated to be used in actual clinical trials. On the other hand, the procedure of Cui *et al.* [5] for testing two-sample means seems simple because it uses ordinary critical values in fixed group sequential designs. It would therefore be beneficial to use this procedure for the analysis of survival data. In this article, we employ a log-rank test statistic to which the method of Cui *et al.* [5] is applied, and investigate the performance of the test statistic from simulation studies.

In addition to inflation of the type-I error rate, the method used to calculate the sample size in an interim analysis should be carefully considered because the data in each stage are mutually dependent in trials with survival data. This article deals with methods to calculate the sample size as well as methods to calculate the target number of events, which is applicable in various practical trials. The hazard ratio estimated from the observed data is sometimes considerably higher than the hypothesized hazard ratio, but is not above the criterion for early termination. If the hypothesized hazard ratio is determined based on some evidence, the interim data may give an incorrect estimate. One solution for such cases is to balance the hypothesized and observed estimations. We propose a method to estimate hazards in order to re-estimate the sample size from this viewpoint. The sample size re-estimation is applied not only to internal pilot or seamless phase 2/3 studies but also pivotal confirmatory studies. The latter is more suitable to the proposed hazard estimation because the confirmatory studies are generally planned based on evidence such as the historical data of clinical

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studies for the same population as the planning study. For example, the standard error of the hazard ratio in the historical studies and interim results of the ongoing study can be utilized to weight the hypothesized and observed estimation in the proposed method. In addition to the hazard estimation, the shape parameter of survival distribution also has an effect on the sample size re-estimation when the survival distribution is the Weibull distribution. In the planning stage, the exponential distribution tends to be used as the survival distribution due to the lack of information. However, given the interim data, it is easier to estimate the shape parameter. We mention the effect of the shape parameter for the Weibull distribution on the sample size required to achieve the target number of events.

In Section 2, we provide an outline of the clinical trial that we deal with in this study. One interim analysis is planned in order to re-estimate the sample size as well as to decide whether or not an early termination for futility or success will be made. We present the test statistic of Cui *et al.* [5] applied to the log-rank test and other methods to preserve the type-I error rate using the log-rank test. In Section 3, we describe a method to calculate the minimum number of events required in the second stage. Then, we present a method for calculating the sample size in order to observe the target number of events within the anticipated period. Furthermore, we propose a method to estimate hazards; this method is used to calculate the number of events and sample size in the interim analysis. Section 4 presents simulation results, which demonstrate the property of the method proposed in Section 3. The test statistics described in Section 2 is also compared. In Section 5, we present an example using the results of an actual trial in order to show how the proposed method is applied. Finally, in Section 6, we briefly discuss some issues that accompany the sample size re-estimation, including the upper/lower bounds of the sample size modified in the interim analysis.

2. GROUP SEQUENTIAL TRIALS WITH SAMPLE SIZE RE-ESTIMATION

2.1. Assumptions

We consider a two-arm clinical trial to compare an investigational treatment with a control, in which the endpoint is the time to event occurred first. Let X denote an indicator variable, i.e. $X=0$ for the control and $X=1$ for the investigational treatment. The hazard function can be expressed as

$$\lambda(t|X) = \lambda_0(t)\exp(-\theta X) \tag{1}$$

where $t \geqslant 0$, $\lambda_0(t)$ is the hazard for the control group, and the hazard ratio is $\exp(\theta)$. The null hypothesis of no treatment effect is thus expressed as

$$H0{:}\theta = 0 \text{ vs. } H1{:}\theta > 0$$

Suppose that we plan an interim analysis to decide whether to terminate the trial early for success or futility or to re-estimate the sample size for the second stage and continue the trial. Let z_1 be a certain test statistic to test the treatment effect on the basis of n_1 observations in the interim analysis, and let p_1 be the p-value corresponding to the z_1. Then, for the prescribed threshold values α_0 and α_1, we set the decision rules for the interim analysis as follows:

(A) if $p_1 < \alpha_1$, then reject H_0 (early termination for success),
(B) if $p_1 \geqslant \alpha_0$, then accept H_0 (early termination for futility),
(C) if $\alpha_1 \leqslant p_1 < \alpha_0$, continue the trial and enroll n_2 patients in the second stage.

We assume that the interim analysis is performed after d_1 events are observed. Let d_2 denote the preplanned numbers of events to be observed in the second stage of the trial. The target number of events, D $(=d_1+d_2)$, will be estimated at the outset so that the power of the test is over $1-\beta_1$, under a pre-chosen alternative hypothesis. In the interim analysis, the minimum number of events required after the interim analysis is re-estimated, and we denote it by d_2^*. Thus, the final analysis will be performed when D^* $(=d_1+d_2^*)$ events are observed. Figure 1 shows a schema of the trial considered here.

Although many papers (e.g. [6,7]) do not require to plan d_2 before the trial starts, most investigators and sponsors need to preplan d_2. The total number of patients is estimated on the basis of the number of events, $D=d_1+d_2$, which determines the budget and resources for the trial that need to be approved by their organization, and is sometimes required by health authorities and ethics committees. In this article, we assume that the overall sample size is specified at the planning stage prior to the trial.

Let S_j denote the survival function in each group $(j=0, 1)$, and $F_j=1-S_j$ be the corresponding distribution function. Suppose that the survival function follows a Weibull distribution with a distribution function $\exp[-(t\lambda)^\gamma]$, where t is the time from the start of the study, and λ and γ are the parameters of the distribution. The time of the interim analysis is denoted by t_1. The final analysis will be performed at time t_2, and the accrual period in the second stage is $t_1 \leqslant t \leqslant t_a$. Let r_i denote the duration from the start of the trial to the time of the enrollment of the ith subject.

2.2. Methods to preserve type-I error rate

We deal with the log-rank test, or the Fleming-Harrington G_ρ family with $\rho=0$, under the assumption of proportional hazard for the simplicity and practicality. Lawrence [10] showed that changing the value of ρ on the basis of interim results has more power than the log-rank test for non-proportional hazard.

Cui *et al.* [5] proposed a test statistic, called 'CHW statistic' hereafter, as a repeated test statistic for two-sample means or a test statistic following the Brownian motion process, which preserves the type-I error rate when the sample size is re-estimated at an interim analysis. The significance level of each test is determined in the same way as a fixed group sequential design by using an α-spending function [11]. Let $Z(t)$ be the test statistic at the information fraction of t $(0 \leqslant t \leqslant 1)$. The standardized version of the test statistic is given by

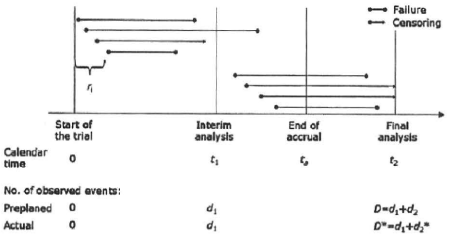

Figure 1. Schema of the supposed trial.

$B(t) = Z(t) \cdot t^{1/2}$, which follows a Brownian motion process [12]. The CHW statistic applied to the log-rank test, and the statistic at the final analysis is

$$\begin{aligned} Z_{CHW} &= Z_1\sqrt{t_1(D)} + \frac{B(t_2(D)) - B(t_1(D))}{\sqrt{t_2(D) - t_1(D)}}\sqrt{1 - t_1(D)} \\ &= Z_1\sqrt{t_1(D)} + Z_2'\sqrt{1 - t_1(D)} \end{aligned} \quad (2)$$

where $Z_2' = \{B(t_2(D)) - B(t_1(D))\}/\sqrt{t_2(D) - t_1(D)}$, $t_1(D) = d_1/D$ and $t_2(D) = D^*/D$.

Here, $t_1(D)$ is the information fraction in the interim analysis, which is defined as the ratio between the variance of the log-rank statistics in the interim analysis and the one in the final analysis. The statistic Z_1 indicates the interim test statistic of the log-rank test. Compared with the expression (2), the unmodified log-rank test statistic in the final analysis can be expressed as

$$Z = Z_1\sqrt{t_1(D^*)} + Z_2\sqrt{1 - t_1(D^*)}$$

where $Z_2 = \{B(t_2(D^*)) - B(t_1(D^*))\}/\sqrt{t_2(D^*) - t_1(D^*)}$, $t_1(D^*) = d_1/D^*$, and $t_2(D^*) = D^*/D^*$.

The mathematical properties of the log-rank test show that it follows a Brownian motion process and has independent increments in repeated test: see References [6] and [13].

Li *et al.* [8] applies the critical values of Li *et al.* [14] for continuous data to the survival data. This method adjusts the critical value for the final test to maintain the overall type-I error rate of α, given the pre-specified α_0 and α_1. The final critical value, c_α is calculated by

$$\alpha_0 - \alpha = \int_{z_{1-\alpha_0}}^{z_{1-\alpha_1}} \Phi\left[\frac{c_\alpha\sqrt{d_1 + d_2} - z_1\sqrt{d_1}}{\sqrt{d_2}}\right]\varphi(z_1)\,dz_1 \quad (3)$$

where $\Phi(\cdot)$ and $\varphi(\cdot)$ are the distribution function and the density function of the standard normal distribution, respectively.

Desseaux and Porcher [9] applies the sample size re-estimation based on Fisher's criterion proposed by Bauer and Köhne [15] to survival data. Let p_1 and p_2 denote the p-values obtained from the interim and final log-rank test, respectively. If $p_1 \times p_2 < c_{\alpha_2}$ in the final analysis, the null hypothesis is rejected, where is calculated on the basis of the Fisher's criterion [16] as

$$c_{\alpha_2} = \exp\left(-\frac{1}{2}\chi^2_{4,1-\alpha_2}\right)$$

where $\chi^2_{4,1-\alpha_2}$ denotes the $(1-\alpha_2)$-quantile of the chi-square distribution with degrees of freedom of 4.

3. NUMBER OF EVENTS AND SAMPLE SIZE REQUIRED IN THE SECOND STAGE

3.1. Target number of events and sample size in the second stage

The target number of events for the second stage d_2^* is re-estimated in the interim analysis so that the conditional power becomes greater than $1-\beta_2$ under the prescribed alternative hypothesis. This can be expressed as $\text{Prob}(Z > z_{1-\alpha_2}|Z_1 = z_1, \theta) \geqslant 1 - \beta_2$, i.e.

$$1 - \Phi\left(\frac{z_{1-\alpha_2}\sqrt{d_1 + d_2^*} - z_1\sqrt{d_1} - d_2^*\theta/2}{\sqrt{d_2^*}}\right) \geqslant 1 - \beta_2 \quad (4)$$

([4]). The statistic z_1 is an ordinary log-rank test statistic without any modification as mentioned in Section 2.2; it is obtained from the observed data in the interim analysis. Since θ in inequality (4) is unknown, the estimated θ^* from the observed data can be used. In the interim analysis, θ^* can be estimated based on the proportional hazard model of Equation (1) using the method of partial likelihood [17]. This method of estimating θ^* in the interim analysis is the same as that in the final analysis. Although the estimate from the interim data θ^* is commonly used for θ, we propose another approach in Section 3.2. The number of events d_2^* in inequality (4), which we want to re-estimate, cannot be expressed in a closed form and is solved by a numerical calculation. The target number of events based on the unconditional power is given by

$$d_2^* = \frac{4(z_{1-\alpha_2} + z_{\beta_2})^2}{\theta_1^{*2}} - d_1 \quad (5)$$

Li *et al.* [8] proposed an equation modified from inequality (4), which is consequently the same as Equation (5). In Section 4, we present the effect on the sample size based on the unconditional power instead of the conditional power.

The number of events observed in the second stage consists of the events in subjects enrolled in the first stage and those at risk in the interim analysis, denoted by $S\{R\}$, along with the events in subjects enrolled in the second stage. Under the assumption of a constant accrual rate, we have

$$d_2^* = \sum_{j=0,1}\left[\sum_{i\in S\{R\}}\frac{F_j(t_2 - r_i) - F_j(t_1 - r_i)}{1 - F_j(t_1 - r_i)} + \frac{n_2}{2(t_a - t_1)}\int_{t_1}^{t_a} F_j(t_2 - y)\,dy\right] \quad (6)$$

which is used to calculate n_2.

When the survival function is Weibull, the number of events from $S\{R\}$ on the right-hand side of Equation (6), which is denoted by d_{2f1}, is given by

$$\begin{aligned} d_{2f1} &= \sum_{j=0,1}\sum_{i\in S\{R\}}\frac{F_j(t_2 - r_i) - F_j(t_1 - r_i)}{1 - F_j(t_1 - r_i)} \\ &= \sum_{j=0,1}\sum_{i\in S\{R\}}\frac{1 - \exp(-(t_2 - r_i)^\gamma\lambda_j^\gamma) - \{1 - \exp(-(t_1 - r_i)^\gamma\lambda_j^\gamma)\}}{\exp(-(t_1 - r_i)^\gamma\lambda_j^\gamma)} \\ &= \sum_{j=0,1}\sum_{i\in S\{R\}}\{1 - \exp(-((t_2 - r_i)^\gamma - (t_1 - r_i)^\gamma)\lambda_j^\gamma)\} \end{aligned}$$

When $\gamma = 1$ (i.e. an exponential distribution),

$$\begin{aligned} d_{2f1} &= \sum_{j=0,1}\sum_{i\in S\{R\}}\{1 - \exp(-(t_2 - t_1)\lambda_j)\} \\ &= \sum_{j=0,1}\sum_{i\in S\{R\}}F_j(t_2 - t_1) \end{aligned} \quad (7)$$

Equation (7) does not include r_i. Although $\gamma = 1$ is used in general when designing a trial as well as the sample size, the actual hazard may increase ($\gamma > 1$) or decrease ($\gamma < 1$) over time in the clinical field. If $\gamma = 1$ is supposed against the true survival distribution of the Weibull distribution with $\gamma = 2$, d_{2f1} will be underestimated by the following difference:

$$\begin{aligned} &\sum_{j=0,1}\sum_{i\in S\{R\}}\{1 - \exp(-(t_2 - t_1)\lambda_j)\} \\ &\quad - \sum_{j=0,1}\sum_{i\in S\{R\}}\{1 - \exp(-((t_2 - r_i)^2 - (t_1 - r_i)^2)\lambda_j^2)\} \\ &= -\sum_{j=0,1}\sum_{i\in S\{R\}}\{S_{\gamma=1}(t_2 - t_1)F_{\gamma=1}((t_2 - r_i) + (t_1 - r_i))\} < 0 \end{aligned}$$

Consequently, superfluous subjects are enrolled in the second stage of the trial. To mitigate this risk, the interim estimate of γ can be used for calculating d_2^*, although it should be considered carefully if the interim estimate of $\gamma<1$ (or >1) against the anticipation of $\gamma>1$ (or <1).

3.2. Method to estimate hazards

When the conditional power is calculated at the interim analysis, the hazard ratio estimated from the observed data is commonly used. However, if the estimated hazard ratio is considerably higher than the hypothesized hazard ratio but is not above the criterion for early termination, the observed data in the first stage may not represent the population well. In that case, the required number of events will probably exceed the pre-specified upper bound. If the hypothesized hazard ratio is determined based on some evidence, one remedial solution is to balance both sources of information. We propose a method to estimate the hazard ratio using such a view.

Let $S_{aj}(t)$ denote the survival function anticipated before the trial starts and $S_{nj}(t)$ denote the survival function estimated from the observed data in each treatment group ($j=0, 1$). Suppose that the survival function follows a Weibull distribution $\exp[-(t\lambda)^\gamma]$, and also suppose that $S_{aj}(t)$ and $S_{nj}(t)$ have the same parameter γ_j. The difference between them in a log–log scale, ϕ_j, following the notation of Whitehead *et al.* [18], is given by

$$\begin{aligned}\phi_j &= -\log(-\log S_{nj}(T)) + \log(-\log S_{aj}(T)) \\ &= -\log(-\log exp[-(T\lambda_{nj})^{\gamma_j}]) + \log(-\log \exp[-(T\lambda_{aj})^{\gamma_j}]) \\ &= -\gamma_j \log T - \gamma_j \log \lambda_{nj} + \gamma_j \log T + \gamma_j \log \lambda_{aj} \\ &= \gamma_j \log \lambda_{aj}/\lambda_{nj}\end{aligned}$$

We propose an interim estimation of the survival function $S_j^*(t) = \exp[-(t\lambda_j^*)^{\gamma_j}]$ as

$$-\log(-\log S_j^*(t)) = -\log(-\log S_{aj}(t)) + c\phi_j \qquad (8)$$

where c is a constant within (0, 1). The survival function $S_j^*(t)$ is consistent with $S_{nj}(t)$ if $c=1$; consistent with $S_{aj}(t)$ if $c=0$; and intermediate between them if $c=1/2$. The interim estimate of the hazard is given by $\lambda_j^* = \lambda_{aj}^{1-c}\lambda_{nj}^c$ from Equation (8). The hazard λ_{nj} from the observed data can be estimated using the method of maximum likelihood. In the example presented in Section 5, we estimated the hazard using the statistical package of the SAS LIFEREG procedure. Once λ_j^* is estimated, $\theta^* = -\log(\lambda_1^*/\lambda_0^*)$ can be used for θ in inequality (4) on which the target number of events for the second stage was re-estimated.

The different parameter γ can be assumed for $S_{aj}(t)$ and $S_{nj}(t)$ as ϕ_j is replaced with $\phi_j(t) = (\gamma_{aj}-\gamma_{nj})\log t + \gamma_{aj}\log\lambda_{aj} - \gamma_{nj}\log\lambda_{nj}$ in Equation (8), where γ_{aj} and γ_{nj} are γ_j for $S_{aj}(t)$ and $S_{nj}(t)$, respectively.

4. SIMULATION

A simulation study was performed to demonstrate the property of the method proposed in Section 3. Before that, comparisons for the test statistics described in Section 2.2 and the approach to d_2^* based on conditional and unconditional power are presented.

In the simulation study, random events were generated under the assumptions that the hypothesized hazard ratio was 0.8, the 1-year event rate for the control group was 0.2, and the survival function was a Weibull distribution with $\gamma=1$. The accrual period and the minimum period of follow-up were both 2 years, and the additional accrual period after the interim analysis was 1 year. The accrual was performed uniformly in this period. The power to be achieved was 0.9, which did not take into account of the inflation in the type-II error rate caused by an early termination for futility because of its negligible effect. The overall significance level of α was 0.025 (one-sided). The constants α_0 and α_1 were determined by the O'Brian–Fleming type of α-spending function, and d_2^* was calculated using the equation presented in Section 3.1 as Equation (6) to compare the overall power among the methods. Here, we set the upper bound for D^* as four times of D, where D was 849. It was not allowed to be decreased from the preplanned sample size in the modification of the sample size.

First, we compare the overall power of the CHW statistic with the methods of Li *et al.* [8] and one of Desseaux and Porcher [9] in the simulation study. The overall power is the probability of accepting the alternative hypothesis in the interim analysis or in the final analysis. All the three methods showed some elasticity in the overall power, while the overall power in the fixed design without sample size re-estimations fell off as the expected hazard ratio differed from the true one (Figure 2). The overall power under the null hypothesis (i.e. true hazard ratio = 0) was 2.5% for the CHW method and 2.6% for the method of Li *et al.* [8]. In the method of Desseaux and Porcher, the overall power was slightly higher than that in the CHW method. As the results of our simulation, the observed overall power of 3.5% under the null hypothesis was over the target type-I error rate of 2.5%, which was consistent with the simulation results of Desseaux and Porcher [9], since they did not intend for the type-I error rate to always be below the nominal level. In conclusion, the CHW method is comparable to the other methods in terms of its overall power and ability to control the type-I error rate.

Second, we compare the approach to d_2^* based on the conditional power of the Equation (4) with the unconditional power of Equation (5). Figure 3(a) shows the overall power of each approach. The type-I error rate was controlled by the CHW method. Although the mean number of events required in the second stage (d_2^*) based on the conditional power was less than

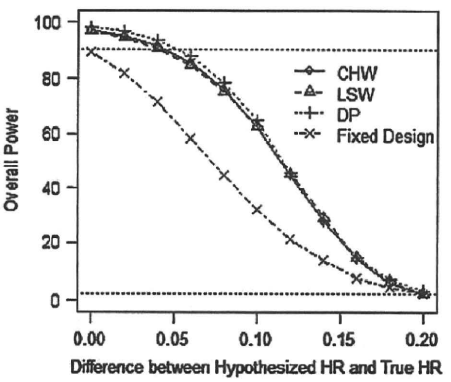

Figure 2. Overall power against the difference between the hypothesized hazard ratio and the true hazard ratio at the hypothesized hazard ratio of 0.8. CHW: Method applied from Cui *et al.* [5], LSW: Method of Li *et al.* [8], DP: Method of Desseaux and Porcher [9], Fixed Design: Overall power based on a study design with an interim analysis for the early termination but without sample size re-estimation.

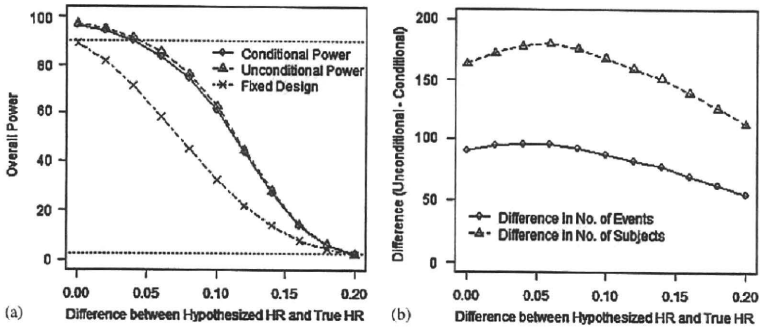

Figure 3. Comparison of simulation results between conditional power and unconditional power at the hypothesized hazard ratio of 0.8. (a) Overall powers for the sample size modified in interim analysis based on conditional power and unconditional power, and the overall power for the fixed design (without sample size modification). (b) Solid line: Differences between the mean number of events required in the second stage (d_2^*) based on the conditional power and that based on the unconditional power (i.e. d_2^* based on unconditional power – d_2^* based on conditional power); Dotted line: Differences between the mean number of subjects required in the second stage (n_2) based on conditional power and that based on unconditional power.

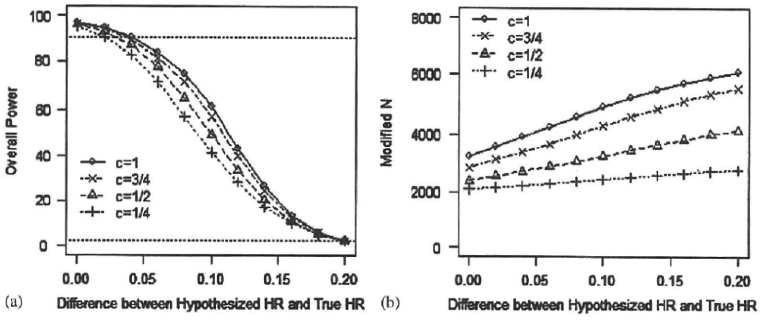

Figure 4. Comparison of simulation results in sample size re-estimation based on the hazard estimated by Equation (8) with c = 1, 3/4, 1/2, and 1/4 at the hypothesized hazard ratio = 0.8. (a) Overall powers for the sample size modified in the interim analysis. (b) Mean sample sizes modified in the interim analysis.

that based on the unconditional power (see Figure 3(b)), the overall power was almost the same in the two methods. As a natural result, the difference in the sample size for the second stage between the conditional power and unconditional power was further increased. Since the enrollment number of subjects is never trivial, it can be concluded that the conditional power is more effective in the calculation of the number of events.

Finally, we applied the hazard estimation based on Equation (8) to re-estimate the sample size in the simulation study. The sample size was re-estimated based on the conditional power. The overall power and mean sample size were compared using Equation (8) with $c=1$ (i.e. the hazard estimated from the observed data in the first stage), 3/4, 1/2 (i.e. the intermediate between the hypothesized and the observed hazards), and 1/4 (Figure 4). As c was larger, the overall power and mean sample size were larger. However, the increase in mean sample size was remarkably large compared with the increase in overall power. For example, when the difference between the hypothesized and true hazard ratios was 0.1, the overall power with $c=1$ increased by 12% compared to that with $c=1/2$, but the mean sample size with $c=1$ increased by 1658. When there was no difference between the hypothesized and true hazard ratios, the overall power with $c=1$ increased by 1% compared to that with $c=1/2$, but the mean sample size with $c=1$ increased by 870. In addition, the percentage of cases where the re-estimated sample size was greater than the upper bound was 10–33% when $c=1$, while the percentage was zero when $c=1/2$. Investigators and sponsors could choose c to be less than 1 to avoid the considerable increase in the sample size, in exchange for some loss in the overall power. We provide an example of setting c to less than 1 in the next section.

5. EXAMPLE

The Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study (EPHESUS) was a double-blind, placebo-controlled clinical study evaluating the effect of eplerenone, a selective aldosterone blocker, on morbidity and mortality among patients with acute myocardial infarction complicated by left ventricular dysfunction and heart failure [19]. The co-primary endpoints were the time to death from any cause and the time to death from cardiovascular causes or the first hospitalization for a cardiovascular event. The trial was designed to enroll 6200 patients and to continue until 1012 deaths occurred. The target number of deaths was determined to have an 88% power to detect an 18.5% reduction from the placebo in the hazard of death using a log-rank test at a significance level of 0.04. As the results, 478 of the 3319 patients in the eplerenone group and 554 of the 3313 patients in the placebo group died. The hazard ratio for death was 0.85 (95%

confidence interval, 0.75–0.96). The difference in the hazard ratio between the anticipation and result was 0.035. Although the power to detect the reduction in the hazard ratio was overestimated, the reduction was statistically significant because of the original high power of 88%.

Here, we suppose that the hazard ratio of 0.8 was anticipated against the true hazard ratio of 0.85. The target number of deaths D is 897 to achieve a power of 90% under the hypothesized hazard ratio, where an interim analysis for early termination for futility or success is planned after 50% of the preplanned number of deaths has occurred. With 897 deaths, the probability of failing to detect a significant reduction in the hazard ratio is 36% (i.e. 64% power) under the true hazard ratio if no sample size modification is performed. We suppose that sample size re-estimation is planned in the interim analysis in addition to the early termination.

We conducted a simulation study based on the above conditions. In order to determine c in Equation (8), the standard error of the hazard ratio in a historical study and the interim results of the ongoing study were used. The historical study used was the TRACE study [20], which evaluated the effect of an ACE inhibitor against a placebo in patients with left ventricular dysfunction after myocardial infarction, resulted in a hazard ratio of 0.78 (95% confidence interval, 0.67–0.91) in the event of death. The hazard and its standard error based on the observed data in the first stage were estimated by the SAS LIFEREG procedure. When c was based on the inverse standard error of the hazard ratio in the interim analysis and TRACE study, it was approximately 1/2 in the simulation study. Table I shows the estimates from the observed data and pre-specified values in the simulation study, which were used to calculate the weighted hazard ratio with c in Equation (8). This weighted hazard was used for θ in inequality (4), on which the target number of events for the second stage d_2^* was re-estimated. The log-rank test statistic z_1 in inequality (4) was calculated by the SAS LIFETEST procedure from the observed data in the first stage. The minimum d_2^* that satisfied inequality (4) with 0.9 of $1-\beta_2$ was obtained through a numerical calculation. The number of subjects enrolled in the second stage was then calculated by Equation (6) with an upper bound equal to four times D.

In the results of the simulation study, the overall power was 80%, and the mean D^* was 1279. When the sample size re-estimation was performed with $c=1$, the overall power under the true hazard ratio was 86%, and the mean D^* was 1800. This means that the value of D^* when $c=1/2$ was lower than when $c=1$; this was despite the overall power being greater than 80%, and it is even lower than the D of 1692 when the hypothesized hazard ratio was equal to the true one of 0.85.

Table I. Estimates in interim analysis and pre-specified values relative to weighted hazard ratio with c.

Estimated or pre-specified value	Mean* (min, max)
Standard error of hazard ratio from observed data	1.099 (1.098, 1.102)
Standard error of hazard ratio in the historical study	1.08†
c	0.495 (0.495, 0.496)
Hazard ratio from observed data	0.853 (0.592, 1.200)
Hypothesized hazard ratio	0.80†
Weighted hazard ratio with c	0.825 (0.689, 0.978)

*Mean of estimates of 10 000 simulated studies, except for pre-specified values.
†Pre-specified value.

6. DISCUSSION

In the proposed method to estimate interim hazards, constant c is set in proportion to the degree of confidence in the hypothesized hazard and the observed data. We would recommend specifying the decision rule of the c at the planning stage, while it may be difficult to pre-specify the value of c itself. The information fraction of the interim analysis can be one possible factor to determine the value of c. The proposed method with $c=1/2$ could control excessive increase in sample size modified at the interim analysis, while a little reduction in the overall power was observed. However, if investigators are highly confident in the hypothesized hazard ratio, there is no need for the sample size re-estimation, and the fixed design should be employed.

The CHW method applied to the log-rank test was comparable to other methods in terms of the overall power and control of the type-I error rate. Since the CHW method uses α for the fixed design, it can easily be applied in actual clinical trials. However, this may cause critical problems from a regulatory view point if the sample size re-estimation allows the sample size to be reduced from the preplanned sample size. If the interim analysis does not result in an early termination for success but results in a modified number of events equal to zero or a very few, there may be serious doubt about leaving the final significance level as the preplanned α_2 in the CHW method. Cui *et al.* [5] does not suppose a decrease in the sample size at the interim analysis. If the CHW method is used, the sample size may not be reduced from the preplanned one at the interim analysis.

In the simulation discussed in this article, we set an upper bound on the additional number of events, d_2^*, in the second stage of the trial, and did not allowed a decrease in the sample size when modifying it. The upper bounds for d_2^* and n_2 are mandatory, because n_2 can be ten times as large as the preplanned size, even if the study does not terminate for futility. The upper bound should be determined so that it is at least less than the size needed to detect a clinical meaningful difference. The budget of the study and feasibility may further decrease this upper bound.

The lower bounds for d_2^* and n_2 are more complicated. When the sample size can be reduced at the interim analysis, the time of the interim analysis or the accrual duration should be planned so that the interim analysis is performed before the end of enrollment. In the results of a simulation, the percentage of such cases was low when the interim analysis was conducted at the information fraction of 1/2. As the information fraction became smaller than 1/2, the percentage of the completion of the enrollment was getting lower. However, the percentage of the early termination for success was also low, which made the mean sample size larger. Furthermore, as mentioned above, if the interim analysis results in a modified number of events equal to zero or a very few, the final significance level of α_2 reaches an *impasse* in the CHW method. Therefore, we think that it is practical for any decrease in sample size at the interim analysis to be caused only by an early termination for futility or success, while any modification involves an increase.

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厚生労働科学研究費補助金（肝炎等克服緊急対策研究事業）
分担研究報告書

データマイニング手法を用いた効果的なC型肝炎治療法に関する研究：ウイルス遺伝子および宿主IL28B遺伝子情報を統合したC型慢性肝炎治療のオーダーメイド化

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研究要旨： C型慢性肝炎治療におけるウイルス変異（HCV Core70/91およびISDR遺伝子変異）及び宿主SNP情報（IL28B及びITPA）について解析を加え、ウイルス・宿主因子を統合した治療効果予測の可能性について検討し、以下の結果を得た。1)Genotype1(G1)の著効率はIL28B major群に比し、hetero/minor群では有意に低率だったが、ISDR変異2以上の症例ではIL28B多型にかかわらず治療高感受性であった。2)Genotype2(G2)でもIL28B hetero/minor群でSVRは有意に低率で、とくにgenotype2bではRVRが得られにくいことが示された。3) ITPA hetero/minor群では貧血発症が有意に低頻度で、G1高ウイルス量症例以外ではSVRと相関した。従って、G2またはG1かつISDR変異2以上の症例ではIL28B多型の如何に関わらず良好な治療効果が得られるため、積極的に治療導入すべきであるが、G1かつISDR変異1以下、IL28B抵抗型では高率に無効となり、新規治療法の早期導入が待たれることが示された。

A．研究目的

Type-IIIインターフェロン(IFN)であるIL28B遺伝子座の宿主遺伝子多型（SNP）はC型慢性肝炎に対するPeginterferon/Ribavirin (Peg IFN /Riba) 併用療法応答性に強く関連する(Nature Genetics 2009)。さらに、ITPA (inosine triphosphatase) のSNPがvariantの場合、ITPAが低活性型になりRBVによる貧血の発症頻度が著明に低下することが欧米人種での研究で見いだされた(Nature 2010)。またこれまで我々はC型肝炎ウイルス(HCV)の遺伝子構造がIFN感受性を規定していることを報告してきたが、IL28B遺伝子多型・ITPA遺伝子多型とHCV Core70/91およびISDR遺伝子変異について解析を加え、ウイルス・宿主因子を統合した治療効果予測の可能性について検討した。

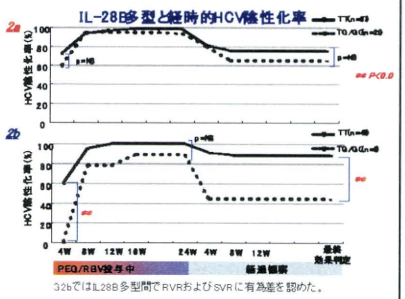
B．研究方法

2004年12月より本学および関連施設で行ったPEG-IFN/RBV併用療法の最終効果判定可能であった643例を対象に、Genotype1(G1)216例、Genotype2 (G2) 129例についてIL28B遺伝子座周辺のSNP解析を行い、ウイルス、宿主因子と併せ治療効果との関連を検討した。またITPA遺伝子座周辺のSNP解析は名古屋市立大学病院、山梨大学医学部附属病院、長崎医療センター、兵庫医科大学病院との共同研究として474例を対象に解析を行った。（本学倫理委承認）

C．研究結果

1)G1で治療効果と最も関連するSNPはIL28B exon上流のrs8099917であった($p=4.1\times10^{-15}$)。G1の著効率(SVR)はTT群52%に比し、TG+GG群では16%と有意に低率であり、67%が完全無効(NR)であった。

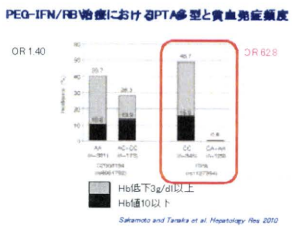
2) G1ではHCV-ISDRの変異数２以上の治療感受性ウイルスではIL28BSNPとSVRに相関がなかったが、ISDR変異1以下では治療の全経過で血中ウイルス消失率がTG+GG群で有意に低かった。



2bではIL28B多型間でRVRおよびSVRに有為差を認めた。

3) G2でもTT群81%に比し、TG+GG群では59%と有意に低率であった (p=0.05)。G2で治療効果と最も相関する因子はRVR (rapid viral response)であり、G2aではIL28BSNPとSVRに相関はなかった。一方でG2bの場合はTG+GG群では全例がRVRとならず、有意にSVR率が低かった。

4) Ribavirin製剤投与に伴う副作用として代表的な貧血の発症頻度と最も相関するのはITPA遺伝子座のrs1127354であった。CA+AA群では0.8%に対してCC群では48.7%と有意に貧血を発症した(Odds比62.8倍)。



5) G1高ウイルス量症例を除いて解析すると、ITPAのCA+AA群ではSVR96%に対して、CC群では70%と有意に低頻度だった (p=0.0066)。

厚生労働科学研究費補助金（肝炎緊急対策研究事業）
分担研究報告書

D．考察

生体内の防御機構とHCVウイルス蛋白の相互作用がウイルス排除あるいは持続感染に関与していることが報告されている。昨年IL28B宿主遺伝子多型がIFN治療応答性やHCV感染における自然経過に強く関連することが相次いで報告され(*Tanaka, Nature Genet.*2009, *Thomas, Nature 2009*)。本研究の結果、新たにG2でもIL28BとSVRに相関が有ることが示された(*Sakamoto, J Med Virol. in press*)。特にG2bの場合はIL28B TG+GG群では有意にSVR率が低かったため、治療期間の延長を模索する必要がある。ITPA多型も同様にG1低ウイルス量あるいはG2の治療の際にdrug adherenceを落とす一因となるため、治療期間の延長を模索する必要がある。一方、近年IL28B多型とISG発現に強い相関があるとの報告もなされ、両者のクロストークがIFN抵抗性に関与していることが示唆される。今後本研究をすすめることでHCVに限らず広汎なウイルス防御機構に対する理解を深め、IFN抵抗性を排除する新たな抗ウイルス分子標的療法創出にも応用可能となると期待される。

E．結論　G2またはG1かつISDR変異2以上の症例ではIL28B多型の如何に関わらず良好な治療効果が得られる。一方、G1かつISDR変異1以下、IL28B抵抗型では高率に無効となり、新規治療法の早期導入が待たれる。宿主・ウイルス因子による治療効果の事前予測は「治療の質」の向上に極めて重要である。（謝辞：田中靖人，御茶ノ水リバーカンファレンス参加施設）。

G．研究発表

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Cell culture and in vivo analyses of cytopathic hepatitis C virus mutants☆,☆☆

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ABSTRACT

HCV-JFH1 yields subclones that develop cytopathic plaques (Sekine-Osajima Y, et al., Virology 2008; 371:71). Here, we investigated viral amino acid substitutions in cytopathic mutant HCV-JFH1 clones and their characteristics in vitro and in vivo. The mutant viruses with individual C2441S, P2938S or R2985P signature substitutions, and with all three substitutions, showed significantly higher intracellular replication efficiencies and greater cytopathic effects than the parental JFH1 in vitro. The mutant HCV-inoculated mice showed significantly higher serum HCV RNA and higher level of expression of ER stress-related proteins in early period of infection. At 8 weeks post inoculation, these signature mutations had reverted to the wild type sequences. HCV-induced cytopathogenicity is associated with the level of intracellular viral replication and is determined by certain amino acid substitutions in HCV-NS5A and NS5B regions. The cytopathic HCV clones exhibit high replication competence in vivo but may be eliminated during the early stages of infection.

Introduction

Hepatitis C virus (HCV) is one of the most important pathogens causing liver-related morbidity and mortality (Alter, 1997). Antiviral therapeutic options against HCV have been limited to type I interferons and ribavirin and have yielded unsatisfactory responses (Fried et al., 2002). Given this situation, a precise understanding of the molecular mechanisms of interferon resistance has been a high priority of research in academia and industry.

Molecular analyses of the HCV life cycle, virus–host interactions, and mechanisms of liver cell damage by the virus are not understood completely, mainly because of the lack of cell culture systems. These problems have been overcome to some extent by the development of the HCV subgenomic replicon (Lohmann et al., 1999) and HCV cell culture systems (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). The HCV-JFH1 strain, which is a genotype 2a clone derived from a Japanese fulminant hepatitis patient and can replicate efficiently in Huh7 cells (Kato, 2001; Kato et al., 2003), has contributed to the establishment of the HCV cell culture system. Furthermore, the Huh7-derived cell lines, Huh-7.5 and Huh-7.5.1 cells, allow production of higher viral titers and have a greater permissivity for HCV (Koutsoudakis et al., 2007; Lindenbach et al., 2005; Zhong et al., 2005). The HCV-JFH1 cell culture system now allows us to study the complete HCV life cycle: virus–cell entry, translation, protein processing, RNA replication, virion assembly and virus release.

HCV belongs to the family *Flaviviridae*. One of the characteristics of the *Flaviviridae* is that they cause cytopathic effects (CPE). The viruses have positive strand RNA genomes of ~10 kilo-bases that encode polyproteins of ~3000 amino acids. These proteins are processed post-translationally by cellular and viral proteases into at least 10 mature proteins (Sakamoto and Watanabe, 2009). The viral non-structural proteins accumulate in the ER and direct genomic replication and viral protein synthesis (Bartenschlager and Lohmann, 2000; Jordan et al., 2002; Mottola et al., 2002). It has been recently

Abbreviations: HCV, hepatitis C virus; CPE, cytopathic effect; ER, endoplasmic reticulum; RdRp, RNA dependent RNA polymerase.

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reported that HCV-JFH1 transfected Huh-7.5.1 cells die when all of the cells are infected and intracellular HCV RNA reaches maximum levels (Zhong et al., 2006). These findings suggest HCV-induced cytopathogenicity. However, the mechanisms have not been well documented.

In a previous study, we investigated the cellular effects of HCV infection and replication using the HCV-JFH1 cell culture system and we reported that HCV-JFH1 transfected and infected cells show substantial CPE that are characterized by massive apoptotic cell death with expression of several ER stress-induced proteins. Taking advantage of the CPE, we developed a plaque assay for HCV in cell culture and isolated subclones of HCV that showed enhanced replication and cytopathogenicity (Sekine-Osajima et al., 2008). We have demonstrated that these viral characters were determined by mutations at certain positions in the structural and nonstructural regions of the HCV genome, especially the NS5A and NS5B regions.

In this study, we investigated the mechanisms and viral nucleotide sequences involved in HCV-induced cytopathic effects using HCV-JFH1 cell culture and a newly developed cytopathic plaque-forming assay. We demonstrated that introduction of NS5A and NS5B mutations into the JFH1 clone resulted in a higher replication efficiency, although introduction of these mutations into the JFH1 subgenomic replicon has no effect on viral replication. These mutations do not affect virion entry or release of viral particles but regulate virus replication, and high levels of virus replication result in cytopathogenicity.

Results

Development of cytopathic plaques by HCV infection of Huh-7.5.1 cells

A plaque assay was performed to investigate the morphological CPE following HCV-JFH1 infection (see Materials and methods). Culture supernatants from JFH1-transfected cells were diluted serially and inoculated onto uninfected Huh-7.5.1 cells. The cells were subsequently cultured in medium containing agarose. On 9 days after the inoculation, viable cells were stained and plaques were visualized (Fig. 1A). HCV-inoculated cell cultures developed plaques as unstained areas, accompanied by rounded cells in the periphery (Fig. 1B). The formation of cytopathic plaques was not observed in a parental Huh7 cell line (data not shown). Those results were consistent with our previous study (Sekine-Osajima et al., 2008).

Introduction of mutations in the NS5A and NS5B regions of the JFH1 clone augmented its cytopathic effects

Among the amino acid substitutions that developed in the plaque-derived HCV-JFH1 strains, 6 of the 9 amino acid changes appeared redundantly among 5 independently isolated plaques, and clustered in the C terminal part of the NS5A and NS5B regions. To investigate the phenotype of each amino acid substitution, we constructed mutant JFH1

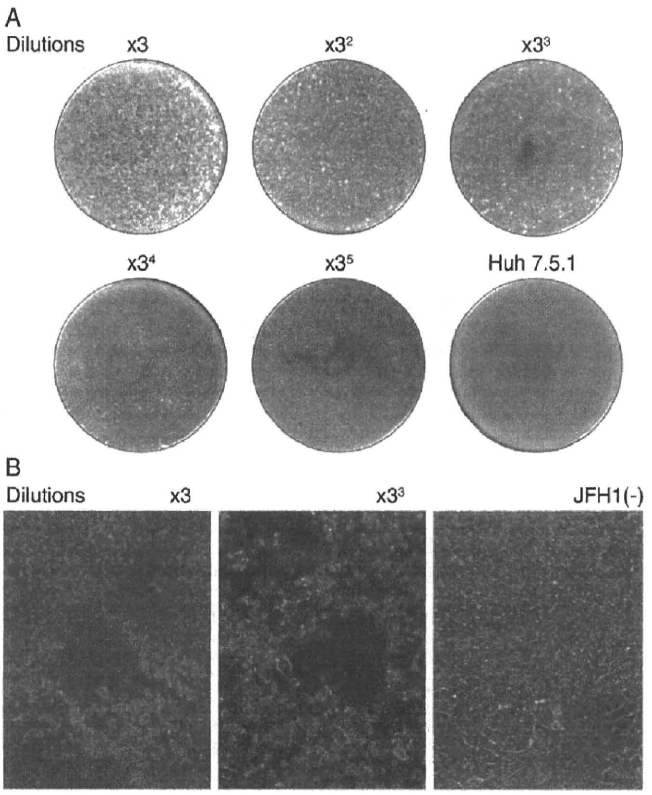

Fig. 1. The cytopathic effects of HCV-JFH1 *in vitro*. A. Plaque assay. Huh-7.5.1 cells were seeded in collagen-coated 60mm-diameter plates at density of 4×10^5 cells per plates and were incubated at 37 °C under 5.0% CO_2 (as described above). After overnight incubation, HCV-infected culture supernatants were serially diluted in a final volume of 2 ml per plates and transferred onto the cell monolayers. After ~5 h of incubation, the inocula were removed and the infected cells were overlaid with 8 ml of culture medium containing 0.8% methyl-cellulose and incubated under normal conditions. After 7 days culture, formation of cytopathic plaque was visualized by staining with 0.08% crystal violet solution. B. The cytopathic plaques were observed by phase-contrast microscopy at day 7 after HCV-JFH1 infection.

clones in which we introduced separately one amino acid substitution in NS5A and five substitutions in NS5B (Fig. 2A) and transfected the mutant HCV RNAs into Huh-7.5.1 cells. To compare the electroporation efficiencies of viral RNAs, Huh-7.5.1 cells were harvested 8 h after transfection and the levels of intracellular core antigen were measured. There was no difference in the efficiencies of electroporation (Fig. 2D). The substitutions G2964D, H3004Q, and S3005N did not lead to cytopathic effects but three mutant subclones (C2441S, P2938S and R2985P) produced much more cell death compared to the wild type JFH1 (Fig. 2B). To assess the quantitative cytopathic effect seen in host cells for each of the mutants, we also performed MTS assay at 6 days post transfection. It showed that Huh-7.5.1 cells transfected with the triple mutants (C2441S, R2938S, or R2985P) induced apparently much more cytopathic effect compared to the parental JFH1 and other mutant clones, although the three mutant clones encoding the substitutions C2441S, P2938S, or R2985P did not show significant difference but

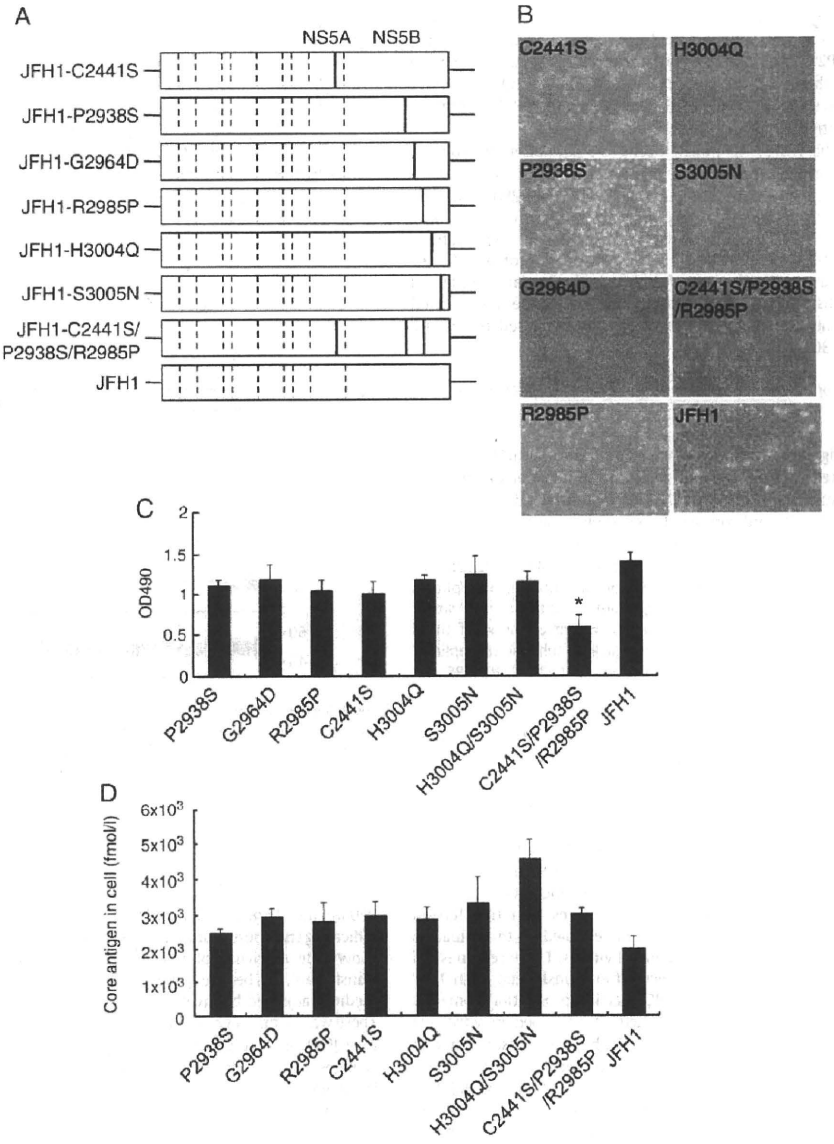

Fig. 2. Introduction of mutations into the NS5A and NS5B regions of JFH1. A. The mutations identified in the cytopathic plaque were introduced individually into the parental JFH1. Each JFH1 mutant was transfected into Huh-7.5.1 cells by electroporation. B. Huh-7.5.1 cells transfected with JFH1-mutants were observed by phase-contrast microscopy at day10 after transfection. C. MTS assay was performed to assess the quantitative cytopathic effect seen in Huh-7.5.1 cells for each of the mutants 6 days post transfection. Asterisks indicate p-values of less than 0.05 as compared with JFH1. D. Huh-7.5.1 cells were harvested at 8 h after transfection and the levels of intracellular core antigen were measured.

showed tendency to introduce more cytopathic effect than the parental JFH1 and the mutant clones encoding the substitutions G2964D, H3004Q, and S3005N (Fig. 2C).

Introduction of NS5A and NS5B mutations into the JFH1 clone led to a greater replication efficiency

To compare the expression levels of each mutant subclone, each HCV RNA was transfected and core antigen was detected subsequently in the culture medium. Similar to Fig. 2B, HCV clones with individual substitutions G2964D, H3004Q and S3005N produced significantly less core antigen or did not replicate at all. In contrast, the C2441S, P2938S and R2985P mutants produced significantly more core antigen than the wild type JFH1. In addition, an HCV clone with all 3 adaptive substitutions (C2441S, P2938S and R2985P) produced more core antigen than any other clone (Fig. 3A).

Next, we harvested the infected cells at 5 days after electroporation and performed western blotting. As shown in Fig. 3B, the three clones encoding the substitutions C2441S, P2938S, or R2985P, and the clone with all three mutations, expressed far more core protein than the parental JFH1, although the clones encoding the substitutions G2964D, H3004Q and S3005N did not express core protein. We also transferred culture media from the mutant clones onto uninfected Huh-7.5.1 cells and performed western blotting and the cells infected with the same mutant subclones as Fig. 3B expressed more core protein (Fig. 3C).

Introduction of NS5A and NS5B mutations into the JFH1 subgenomic replicon

To investigate the primary phase of replication of JFH1 mutants, we constructed JFH1 subgenomic replicons by introducing individually the six mutations in NS5A and NS5B. We transfected each replicon RNA into Huh7 cells and compared their replication levels according to the luciferase activities. Consistently with the mutant viruses, the subgenomic replicon encoding the changes C2441S, P2938S or R2985P, which produced higher amounts of core antigen, did replicate at higher levels than the other subgenomic replicons with single mutation, G2964D, H3004Q and S3005N. However, none of these mutants replicated at higher than the parental JFH1 subgenomic replicon. Furthermore, replicon with triple mutations of C2241S, P2938S and R2985P did not replicate (Fig. 4).

Introduction of NS5A and NS5B mutations into the JFH1 clone had no effect on the production of infectious virions

We sought to investigate the effects of the NS5A and NS5B mutations on virus replication and virion secretion independent of re-infection and spread of the viruses produced. Therefore, we used the S29-subclone of Huh7 cells, which cannot be infected by HCV because of a defect in CD81 expression but does support viral genomic replication and releases infectious HCV particles after transfection (Russell et al., 2008). The Huh7-S29 cells enabled us to evaluate a single cycle of infection and production of virions. Those cell lines did not show apparent cytopathic effects after transfection with HCV RNAs (data not shown). To analyze HCV particle production from cells transfected with the viral genomic RNAs transcribed in vitro, we harvested culture media and cells at 72 h post transfection and measured the core antigen levels in culture media and intracellular HCV RNA by real-time RT-PCR. The C2441S, P2938S, and R2985P mutants produced significantly greater amounts of core antigen in the culture medium than the wild type JFH1. The HCV clone carrying all three mutations produced the greatest amount of core antigen (Fig. 5A, top). Consistent with the core antigen levels in the culture media, intracellular HCV RNA levels were also higher in the cells transfected with the mutated genomes encoding separately C2441S, P2938S, and R2985P, and that with all three mutations (Fig. 5A, middle), indicating that these mutations affected virus replication. Fig. 5A bottom shows the efficiency of infectious viral particle release from each transfectant, this being expressed as the core antigen level in the culture medium adjusted by dividing by the levels of intracellular HCV RNA. There was no difference in the efficiency of release of virions by the wild type JFH1 and the genomes carrying the C2441S, P2938S or R2985P changes. These results indicated that these three mutations in NS5A and NS5B did not affect virion entry or viral particle release but did regulate virus replication, and a high level of viral replication induces cytopathogenicity. Similarly, as shown in Fig. 3B, the three clones with C2441S, P2938S or R2985P, or all three mutations expressed much higher levels of core protein than the parental JFH1, while clones with G2964D, H3004Q or S3005N mutations did not express detectable amounts of core protein (Fig. 5B).

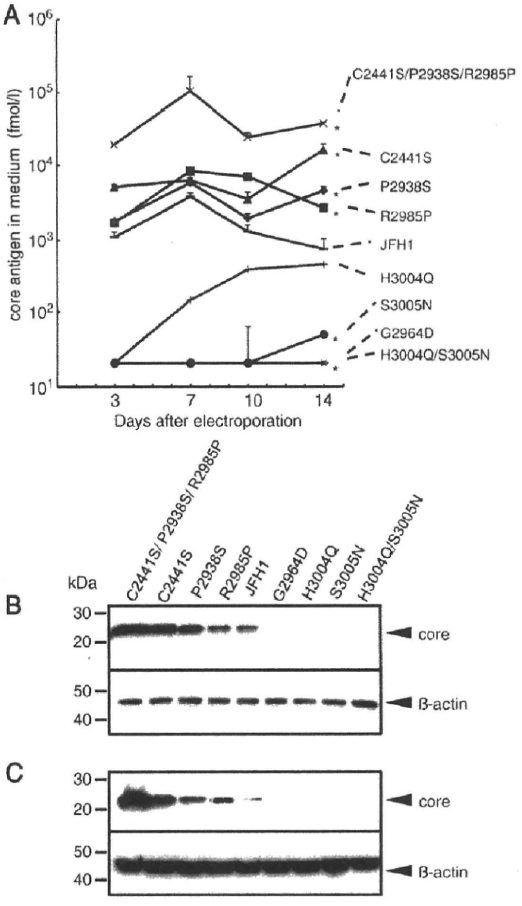

Fig. 3. Replication competences of HCV subclones with NS5A and NS5B mutations. A. Levels of core antigen in the culture medium. The culture media from transfected cells were collected on the days indicated and the levels of core antigen were measured. Asterisks indicate p-values of less than 0.05 as compared with JFH1. B. Huh-7.5.1 cells transfected with JFH1 mutants were harvested at 5 days after transfection and western blotting was performed. C. The culture media from Huh-7.5.1 cells transfected with JFH1 mutants were transferred onto uninfected Huh-7.5.1 cells. The cells were harvested at 3 days after infection. Western blotting was performed using anti-core and anti-beta-actin. kDa: kilo dalton.

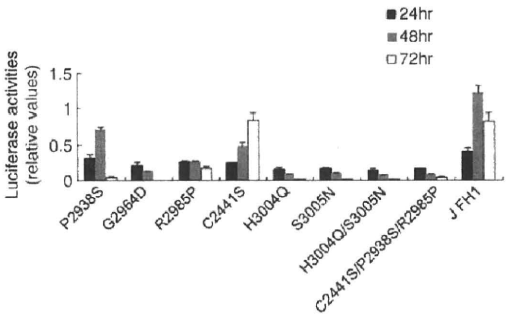

Fig. 4. Luciferase assay of the cytopathic JFH1-subgenomic replicon. Mutations were introduced into 2a-Fec subgenomic replicon and transcribed RNA for each replicon was transfected into Huh7 cells by electroporation. The cells were harvested at 24 h, 48 h and 72 h after electroporation and were used for Luciferase assay. Values are relative values to those of 8 h.

Mutations of NS5A and NS5B are associated with replication competence at earlier stages in vivo

We next used human hepatocyte chimeric mice to investigate the infectivity of the triple mutant of NS5A and NS5B. We confirmed the mouse liver chimerism greater than 70% by immunohistochemical analysis (data not shown). Culture media of the parental JFH1 and the mutant subclone with three mutations (C2441S, P2938S, and R2985P), were collected following transfection of Huh-7.5.1 cells, concentrated, and inoculated intravenously into human hepatocyte chimeric mice. We confirmed that the three mutations in NS5A and NS5B were conserved in the virus genome sequence of cell culture supernatants that were used for inoculation (data not shown). Two mice were inoculated with JFH1 and three were inoculated with the mutant virus. HCV RNA and human albumin in the sera of the mice were detected sequentially.

We repeated the same exam twice and confirmed consistency of the results. In the early phase post inoculation, the concentration of HCV RNA in serum was significantly higher in mice inoculated with the culture medium from the mutant subclone (Fig. 6A), suggesting that the mutations in NS5A and NS5B (C2441S, P2938S, and R2985P) are associated with virus replication in vivo. However, there was no difference in the level of HCV RNA in later period. The disparity of viral production at early time point could be influenced by the disparate numbers of infectious virus between the 2 initial inoculums. However, the sharp elevation of serum HCV RNA at day 5 after dropping at day 3 indicates that the mutants (C2441S, P2938S plus R2985P) are more replication competent at early stages in vivo. Serum levels of human albumin remained constant throughout the observed periods and showed no significant differences between wild and mutant-infected mice (Fig. 6B).

We also investigated expression of ER stress-related proteins, the glucose regulated protein 78 (GRP78) and C/EBP homologous protein (CHOP), in liver of chimeric mice infected with JFH1 or the mutant in the early phase post inoculation. Human hepatocyte chimeric mice were inoculated in the same way as described above, and we verified that the level of virus titer in serum of each mouse was same as presented in Fig. 6A (data not shown). We sacrificed one each mouse that was infected with wild type or mutant JFH1 at 5 day of infection and investigated hepatic expression of GRP78 and CHOP. Liver histology showed no sign of inflammation or cytopathic cell death. However, as shown in Fig. 7, the expression level of both GRP78 and CHOP was higher in mice inoculated with the mutant viruses than the parental JFH1. There was no apparent difference in percents of hepatic chimerism between each mouse. These finding suggested that ER stress-related proteins were upregulated in the liver of HCV-infected

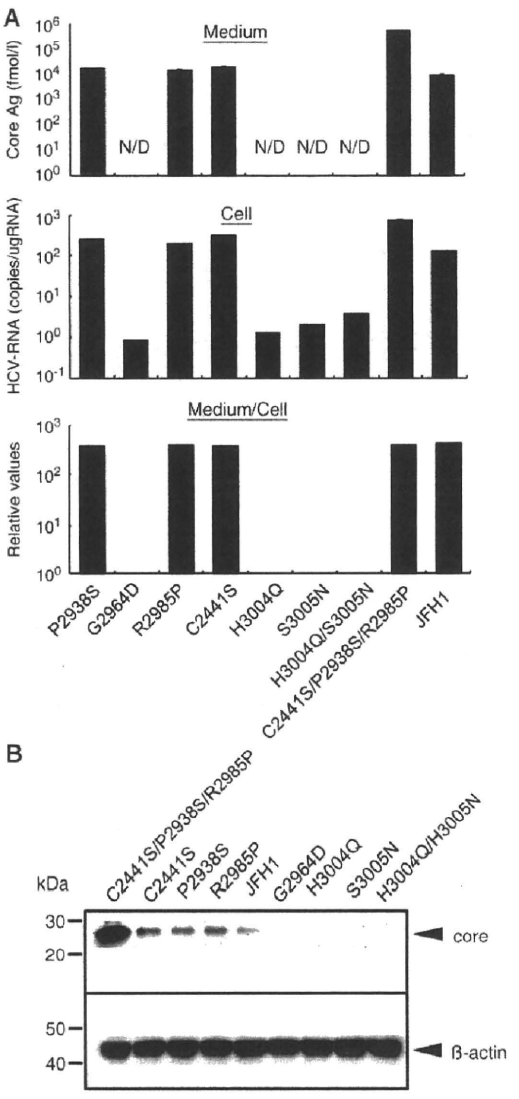

Fig. 5. Analysis of viral replication and production of viral particles using a single-cycle assay. A. Levels of core antigen in the culture media 3 days after transfection of JFH1 mutants into CD81-deficient Huh7-S29 cells (top). Levels of intracellular HCV RNA were quantified by real-time RT-PCR 3 days after transfection of JFH1 mutants into Huh7-S29 cells (middle). To determine the efficiency of infectious viral particle release from Huh7-S29 cells transfected with JFH1 mutants, the levels of core antigen in the culture media were adjusted by dividing by the levels of intracellular HCV RNA (bottom). Core Ag: Core antigen, N/D: not detectable. B. Huh7-S29 cells were harvested at 3 days after transfection of JFH1 mutants and western blotting was performed using anti-core and anti-beta-actin. kDa: kilo dalton.

mouse and that these responses were more strongly induced in the liver of mutant-infected mouse.

Highly adapted cytopathic mutations reverted to wild type in vivo

Finally, we analyzed the serum viral sequence at the specified time points. On days 1 and 5, the HCV genomic sequences of the mice