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Brief Report

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Implications of integrase inhibitors for HIV-infected transplantation recipients: Raltegravir and dolutegravir (S/GSK 1349572)

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Summary **In the modern era of highly active antiretroviral therapy (HAART), reluctance to perform transplantation (Tx) in HIV-infected individuals is no longer justified. Non-nucleoside reverse transcriptase inhibitors (NNRTIs) or protease inhibitors (PIs), the current first line regimens of HAART, are metabolized by the cytochrome P450 family (CYP3A4). Most NNRTIs induce CYP3A4, whereas PIs inhibit it. Calcinuerin inhibitors (CNIs), which are mandatory for Tx, need the same enzyme complex for their clearance. Therefore, a significant drug-drug interaction (DDI) is encountered between current HAART and CNIs. This results in extreme difficulty in adjusting the optimal dose of CNIs, for which the therapeutic range is narrow. Of interest, integrase inhibitors (INIs) – novel, potent anti-HIV drugs – are mainly metabolized by uridine diphosphate glucuronosyltransferase (UGT) 1A1 and do not induce or inhibit CYP3A4. DDI is presumably absent when NNTRIs or PIs are replaced by INIs. Raltegravir (RAL), a first generation INI, has been introduced into kidney and liver Tx. There is increasing evidence that rejection is well controlled without renal impairment due to CNI over-exposure while persistent, robust suppression of HIV is achieved. Global phase III clinical trials of dolutegravir (DTG), a second generation INI, are currently in progress. *In vitro* data has suggested that DTG may be less prone to resistance than RAL (referred to as having a higher genetic barrier). The time has come to extensively discuss the implications of INIs in Tx for HIV positive patients.**

Keywords: Liver transplantation, kidney transplantation, drug-drug interaction (DDI), highly active antiretroviral therapy (HAART)

In the modern era of highly active antiretroviral therapy (HAART), which consists of non-nucleoside reverse transcriptase inhibitors (NNRTIs) or boosted protease inhibitors (PIs), reluctance to perform transplantation (Tx) in HIV-infected individuals is no longer justified (*1*,*2*). No fewer than 20-30% of HIV-positive patients are co-infected by HCV, and end-stage renal disease caused by HIV-associated nephropathy develops in as many as 5-10% of HAART-treated patients (2,3). As a result, HIV-positive patients are much more likely to receive needed liver or kidney Txs than are the HIV-negative. Recently, though, integrase inhibitors (INIs) have emerged. They are novel, potent anti-HIV drugs characterized by a distinct metabolic pathway; they include raltegravir (RAL, Isentress, Merck & Co., Inc., New Jersey, USA) and dolutegravir (DTG, S/GSK1349572, ViiV Healthcare/Shionogi, Middlesex,UK/Osaka, Japan) (*4*,*5*). Currently, their implications in the setting of Tx for HIV positive patients are being actively discussed.

To maintain undetectable plasma HIV-RNA, the current first line regimens of HAART consist of a PI boosted by ritonavir or a non-nucleoside reverse transcriptase inhibitor (NNRTI) and two nucleoside reverse transcriptase inhibitors (NRTIs) as described above (*2*). PIs and NNRTIs require metabolism by the cytochrome P450 family (*2*). Ritonavir and the

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other PIs are potent inhibitors of CYP3A4, while most NNRTIs are its inducers (*2*). The same enzyme complex is responsible for the clearance of calcineurin inhibitors (CNIs); the mainstay of current immunosuppression (IS) – *e.g.* cyclosporine (Neoral, Novartis International AG, Basel, Switzerland) and tacrolimus (Prograf. Astellas Pharma Inc., Tokyo, Japan) (*2*). Thus, a significant drug-drug interaction (DDI) exists between current HAART and IS (*2*). In the presence of PIs, a reciprocal DDI can potentially lead to CNI over-exposure and renal toxicity. This results in difficulty in adjusting optimal dosage of CNIs characterized by a narrow therapeutic range, or the requirement of transient discontinuation of HAART during the peri-Tx period (*2,6*). However, interruption of HAART may result in the accumulation of resistance-associated mutations. Earlier re-introduction of HAART can positively affect anti-HCV therapy after liver Tx for HCV diseases (*6*). Such a complex scenario warrants development of HAART that exhibits no DDI with CNIs (Figure 1).

Integrase inhibitors (INIs) are mainly metabolized by uridine diphosphate glucuronosyltransferase (UGT) 1A1. These INIs are neither inducers nor inhibitors of CYP3A4 (*4,5*). Therefore, they presumably do not interact with CNIs (*2*). RAL, a first generation INI, has been introduced into HIV-positive Tx mainly due to its distinct metabolic pathway and remarkable efficacy. PI-sparing, RAL-based HAART does not require either dose adjustment of CNIs or discontinuation of HAART early post-Tx. There is increasing evidence that RAL is effective in preventing renal toxicity while maintaining potent and sustained antiretroviral properties (*7,8*) (Figure 1). It has been reported that the negative impacts of abacavir sulfate/lamivudine (NRTIs)(Epzicom, ViiV healthcare,Middlesex,UK) on the kidney are smaller than those of tenofovir disoproxil fumarate/emtricitabine (NRTIs) (Truvada, Gilead Science, Inc., California, UAS) (*2*). Some researchers advocate that HAART regimens – RAL, a key drug, used with Epzicom as a backbone therapy – would be the best option for HIV-positive kidney Tx (*2*) (Figure 1).

DTG is a second generation INI (*5*). Its global phase III trials are currently in progress. During a phase II trial with HAART-naïve subjects, its non-inferior efficacy was demonstrated to be not inferior to that of efavirenz (NNRTI) (Sustiva, Bristol-Myers Squibb Pharma Company, New York, USA) (5). *In vitro* studies have raised the curious possibility that DTG could have the potential of a higher genetic barrier to resistance than RAL, in the setting of INI-naïve patients (*9*) (Figure 1). No serious drug-induced adverse effects were observed throughout phase II trials, and its tolerability was excellent (*10*). A slight self-limiting serum creatinine (Cr) elevation was observed. An *in vitro* study strongly suggested that such a Cr elevation was merely a sequel to a non-pathologic decrease in Cr secretion *via* the proximal tube, but did not represent renal toxicity (*5*).

Ideal anti-retroviral therapy for HIV positive transplant

To provide sustained viral suppression while minimizing drug interactions and toxicity, a regimen should:

1) Be potent and have a high genetic barrier to resistance
2) Not induce or inhibit metabolism of calcineurin inhibitors (CNIs)
3) Not have overlapping toxicities with immunosuppression (IS)
4) Have minimal impact on graft function
5) Have excellent tolerability and ease of dosing

Figure 1. Ideal anti-retroviral therapy for HIV positive transplants. (Beatty G, personal communication, American Transplant Congress April 30-May 4, 2011, Philadelphia, PA, USA)

This accords well with a recent phase I study in which administration of DTG in healthy individuals did not negatively affect the glomerular filtration rate (GFR) (*11*).

Once-daily DTG proved satisfactorily efficacious in the phase II trial for naïve subjects cited above, while RAL must be administered *b.i.d.* (*4,5*). This advantage may act in favor of using DTG (Figure 1). In support, a switch to (*q.d.*) modified release tacrolimus (Advagraf, Astellas Pharma Inc., Tokyo, Japan) from conventional *b.i.d.* (Prograf) increased using by Tx patients (*12*). Of note, several cases of rabdomyolysis were observed in RAL-treated subjects. One must pay attention to concomitant use of RAL with other drugs (*e.g.* statins) that can cause rabdomyolysis (*13-17*). In addition, one can not entirely exclude the possibility that the concern for rabdomyolysis caused by concomitant use of RAL and statins may be further enhanced by CNIs due to disturbance of statin uptake into the liver by organic anion transporting polypeptides (*18*). On the other hand, one case of grade 4 CPK elevation was observed during phase II trials of DTG, but it was exercise-related, transient, and asymptomatic (*10*). Even so, a caution is still needed before sufficient cases are accumulated during phase III trials and at post-market phase.

More accumulated experience using RAL is needed to fully understand the implications of INIs in HIV-infected Tx recipients. Meanwhile, the efficacy, safety, and tolerability of DTG must be carefully analyzed in comparison to those of RAL, PIs and NNRTIs during the phase III studies. Finally, the authors propose to discuss the possibility of using DTG in Tx, taking its attractive *in vitro* and *in vivo* characteristics into account.

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Original Article

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Overcoming of P-glycoprotein-mediated multidrug resistance in K562/A02 cells using riccardin F and pakyonol, bisbibenzyl derivatives from liverworts

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Summary **Riccardin F and pakyonol, macrocyclic bisbibenzyls from *Plagiochasm intermedium*, have been confirmed to possess antifungic activities against *Candida albicans*. Herein, we evaluated their anti-tumor activity *in vitro* by employing K562 and K562/A02 cells, the well-known adriamycin (ADR)-induced multidrug resistance (MDR) tumor cell lines over-expressing P-glycoprotein (P-gp). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assays showed that riccardin F and pakyonol ranging from 0 to 6 μg/mL exhibited no inhibitory effects on the growth of the two cell lines. However, in the presence of 3 μg/mL riccardin F or pakyonol (non-cytotoxic concentration), the IC_{50} of ADR against K562/A02 cells decreased by 2.51- and 4.78-fold, respectively. Flow cytometry showed that riccardin F and pakyonol significantly enhanced the accumulation of ADR in K562/A02 cells. Furthermore, fluorescence intensity detection revealed that the two natural products remarkably increased the retention of rhodamine-123 in K562/A02 cells rather than in K562 cells, indicating that the major cause for riccardin F and pakyonol to reverse P-gp-mediated MDR in K562/A02 cells is probably due to the constrained transport activity of P-gp. This study explores the potential application of bisbibenzyl type compounds as modulators of P-gp-mediated MDR in tumor cells.**

Keywords: P-glycoprotein (P-gp), bisbibenzyl, riccardin F, pakyonol, K562/A02 cells, multidrug resistance (MDR)

1. Introduction

Multidrug resistance (MDR), a major obstacle to successful tumor chemotherapy in the clinic, refers to the resistance of cancer cells to multiple structurally and mechanically unrelated hydrophobic anticancer drugs (*1*). Over-expression of P-glycoprotein (P-gp), a 170 kDa transmembrane glycoprotein, is widely considered to be the cause of MDR under most circumstances. P-gp, an ATP-dependent drug transporter, unilaterally expels intracellular drugs out of cells, resulting in drug resistance. A large number of well known anti-cancer drugs such as paclitaxel and vincristine, substrates of P-gp, were limited by P-gp-mediated MDR in clinical application (*2*). To circumvent efficaciously drug resistance in tumor chemotherapy, it is essential to develop either novel anticancer agents to overcome P-gp-mediated MDR and/or inhibitors that specifically block the function of P-gp as drug transporter. A number of synthetic and natural substances, such as verapamil, dihydropyridine analogs, quinidine and cyclosporin A, have been confirmed for their abilities to overcome P-gp-mediated MDR by inhibiting the transport activity of P-gp (*3*). However, these compounds have failed in clinic applications due to their serious side effects and poor pharmacokinetics (*4*). Therefore, exploration of alternatives to current P-gp inhibitors with higher selectivity and stronger potency remains a major goal in this field.

Natural products with bioactivities and various structural properties are becoming the major source

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of novel agents with pharmacological interests (*5*). A considerable number of natural polyphenolic compounds, including curcuminoids, curcumin and eigallocatechin gallate (EGCG) have been revealed to modulate P-gp efflux activity (*6-8*). Bisbibenzyls, a class of plant metabolites produced exclusively in liverworts, is a family of phenolic compounds belonging to stilbenoids (*9*). They have attracted more attention for their wide-range of bioactivity and medicinal potency, including antibacterial, antifungic, antioxidative, muscle-relaxing and cytotoxic activities as well as inhibitory effects on 5-lipoxygenase, cyclooxygenase and calmodulin (*10*).

In the previous study, we demonstrated that plagiochin E, a macrocyclic bisbibenzyl compound isolated from *Marchantia polymorpha*, not only enhanced the sensitivity of resistant *Candida albicans* toward fluconazole but also reversed P-gp-mediated MDR in resistant tumor cells (*11,12*). Recently, we isolated riccardin F and pakyonol from *Plagiochasm intermedium*, and substantiated their antifungal activity against *C. albicans* (*13,14*). In the present study, we further evaluate their reversal efficacy on P-gp-mediated MDR by employing the K562/A02 cell line, which is known for over-expressing P-gp (*15*).

2. Materials and Methods

2.1. *Chemicals*

The tested macrocyclic bisbibenzyl compounds riccardin F and pakyonol (Figure 1) were isolated from *Plagiochasm intermedium*, collected in Shandong Province. Their structures were identified by interpretation of their mass spectrum (MS) and 1D- and 2D-nuclear magnetic resonance (NMR) data as well as by comparison with reported values . They were dissolved in dimethylsulfoxide (DMSO) and then diluted in RPMI-1640 medium for *in vitro* assays.

2.2. *Reagents*

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), Rhodamine-123, adriamycin (ADR) and DMSO were purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI-1640 medium and fetal bovine serum (FBS) were purchased from HyClone (Logan, UT, USA).

2.3. *Cell lines and cell culture*

The human myeloid leukemia cell line K562, and its MDR counterpart K562/A02, were obtained from the Department of Pharmacology, the Institute of Hematology of Chinese Academy of Medical Sciences (Tianjin, China). K562 and K562/A02 cells were

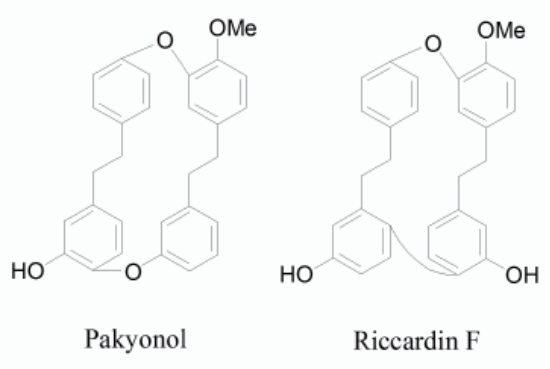

Figure 1. The chemical structures of riccardin F and pakyonol.

maintained in complete RPMI-1640 medium in the absence and presence of 1 μg/mL ADR at 37°C in a humidified atmosphere of 5% CO_2, respectively. Resistant cells were cultured for two weeks in drug-free medium prior to use in experiments (*16*).

2.4. *MTT assay*

Cytotoxicity of riccardin F and pakyonol toward K562 and K562/A02 cells was first measured by the MTT method. Briefly, K562/A02 cells (1~2 × 10^4 cells per well) were seeded in 96-well plates. After 24 h incubation, cells were treated with various concentrations of riccardin F and pakyonol for 48 h, respectively. Light absorbance of the solution was measured at 570 nm on a plate reader (TECAN, Grodig, Salzburg, Austria). Cell viability was assessed by MTT assay as reported (*17*).

The reversal effect of riccardin F and pakyonol was further investigated with a similar procedure. K562/A02 and K562 cells were treated for 48 h with varying concentrations of ADR in absence or presence of riccardin F or pakyonol at doses of minimum inhibitory effect on cell growth, respectively. The inhibitory rate of cell growth was obtained through previously described procedures (*18*), and IC_{50} values for ADR were calculated from plotted results using untreated cells as reference. The reversal fold (RF) values, as potency parameter of reversal, were calculated from dividing IC_{50} of ADR alone by IC_{50} of ADR in combination with riccardin F or pakyonol. Triplicate experiments with triplicate samples were performed. Control medium included an equivalent amount of DMSO (as solvent control), but the applied dosage of riccardin F or pakyonol did not exhibit modulation effects on cell growth or drug sensitivity. In all experiments, verapamil was used as a positive control.

2.5. *Intracellular ADR accumulation assay*

On the basis of ADR autofluorescence, effects of riccardin F and pakyonol on accumulation of ADR

inside K562 and K562/A02 cells were assessed by measuring the mean fluorescence intensity (MFI) associated with ADR (*19*). Briefly, the cells at 5×10^5 per well pretreated with 3 μg/mL of each sample for 1 h were incubated in medium containing 3 μg/mL ADR at 37°C for 90 min, respectively. MFI associated with ADR was measured using FACScan Caliber (Beckton Dickinson, USA) at 488 nm for emitted fluorescence and 615 nm for collected fluorescence. Data analysis was performed using CellQuest software.

2.6. *Intracellular rhodamine-123 accumulation assay*

Rhodamine-123 is used widely as the specific fluorescence indicator of P-gp. Therefore, regulation of the effect of riccardin F and pakyonol on drug-transport activity of P-gp was determined by measuring intracellular accumulation of rhodamine-123 in resistant cancer cells. K562 cells and K562/A02 cells in exponential growth at 5×10^5 per well were seeded in 6-well plates and pretreated with 3 μg/mL of each sample for 1 h, followed by incubating with 2.5 μg/mL of rhodamine-123 in culture medium at 37°C with 5% CO_2 for 90 min. The MFI associated with rhodamine-123 was measured with FACScan flow cytometry at 488 nm emitted fluorescence and 530 nm collected fluorescence (*20*). Data analysis was performed using CellQuest software.

2.7. *Data analysis*

Student's *t*-test was used to compare data and a *p*-value of 0.05 was taken as the minimal level of significance. All data are expressed as mean ± S.D.

3. Results

3.1. *Determination of non-cytotoxic concentration of the compounds to K562 and K562/A02 cells*

In the assay, we assessed inhibitory effects of riccardin F and pakyonol on growth of K562 and K562/A02 cells using the MTT colorimetric method. As shown in Figure 2, concentrations of riccardin F and pakyonol ranging from 6 to 48 μg/mL remarkably decreased the viability of the multidrug resistant cell line K562/A02 as well as the sensitive cell line K562, while treatments with 0 to 6 μg/mL exhibited no significant effects on cellgrowth. Therefore, riccardin F and pakyonol concentrations of 0-6 μg/mL were taken as the optimal doses for the reversal investigation of drug resistance of K562/A02.

3.2. *Effects on potency of ADR*

As shown in Table 1, treatments with riccardin F or pakyonol at 3 μg/mL (non-toxic dosage) significantly

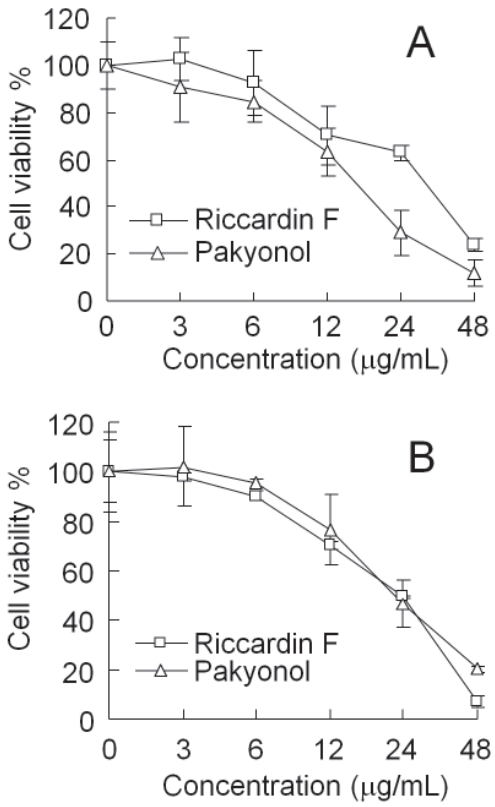

Figure 2. The effect of riccardin F and pakyonol on the proliferation of K562 and K562/A02 cells. Cells were treated with various doses (0, 3, 6, 12, 24, 48 μg/mL) of riccardin F and pakyonol for 48 h, respectively. Data are expressed as mean ± S.D. of three independent experiments. (**A**) K562 cells; (**B**) K562/A02 cells.

Table 1. The effect of combinations of the test compounds with ADR on growth of K562/A02 cells

Treatment	K562/A02 cells		K562 cells	
	IC_{50} (μg/mL)	RF	IC_{50} (μg/mL)	RF
ADR	5.78 ± 0.76	–	0.17 ± 0.056	–
ADR + riccardin F	2.34 ± 0.54*	2.51	0.16 ± 0.086	1.06
ADR + pakyonol	1.21 ± 0.41*	4.78	0.15 ± 0.077	1.13
ADR + verapamil	2.39 ± 0.37*	2.42	0.16 ± 0.061	1.06

Data are presented as mean ± S.D. from three independent experiments. * $p < 0.01$ *vs*. ADR alone. The RF of drug resistance, the IC_{50} of ADR alone divided by the IC_{50} of combination ADR with each compound tested, respectively, and cells.

decreased the IC_{50} value of ADR against K562/A02 cells in comparison to the treatment of ADR alone, while this fact was not observed in K562 cells. It suggested that the two natural products at non-toxic concentrations enhanced the sensitivity of the resistant K562/A02 cells to ADR. Moreover, the RF of pakyonol against K562/A02 cells was about two times higher than that of riccardin F.

3.3. *Modulation on intracellular ADR accumulation*

Reversal activities were evaluated by flow cytometry red fluorescence histograms (Figure 3). Figure 3A

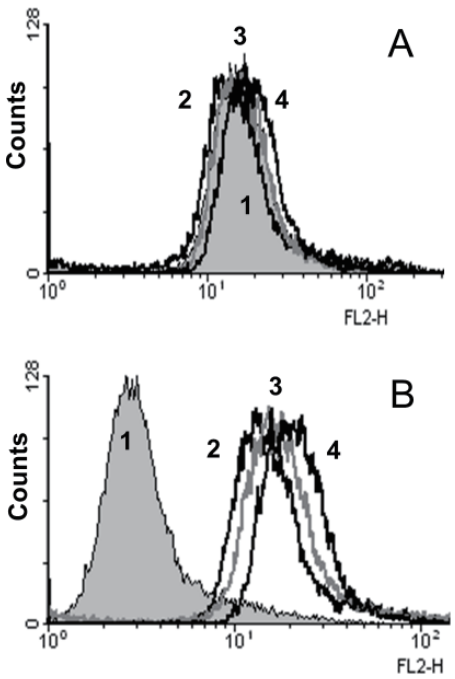

Figure 3. Effects of riccardin F and pakyonol on intracellular accumulation of ADR in K562 and K562/A02 cells, respectively. Cells were incubated with 3 μg/mL of ADR for 90 min following pretreatment with 3 μg/mL of riccardin F and pakyonol respectively for 60 min. The MFI (mean fluorescence intensity) was measured by flow cytometry. Data were obtained from three independent experiments. (**A**) K562 cells; (**B**) K562/A02 cells. (**1**) Control, (**2-4**) Treatmentcombinations of ADR with verapamil, riccardin F and pakyonol, respectively.

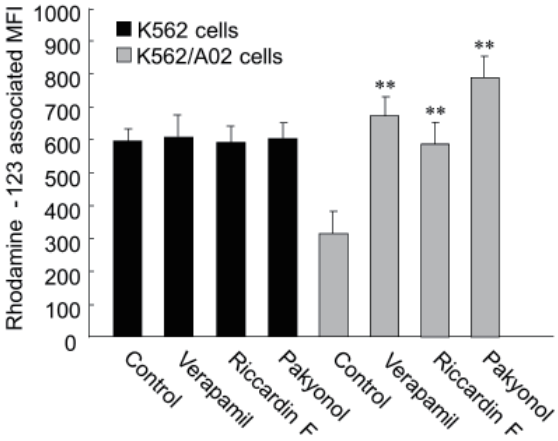

Figure 4. Effects of riccardin F and pakyonol on intracellular accumulation of rhodamine-123 in K562 and K562/A02 cells. Cells were incubated with 2.5 μg/mL of rhodamine-123 for 90 min following pretreatment with 3 μg/mL of riccardin F and pakyonol, respectively, for 60 min. The MFI was measured by flow cytometry. Data were obtained from three independent experiments and expressed as mean ± S.D., and ** $p < 0.01$ *vs.* K562/A02 cells control.

indicates that ADR accumulated in parent K562 cells efficiently, with a maximum drug accumulation plateau at 90 min incubation. Addition of riccardin F and pakyonol didn't influence ADR accumulation in K562 cells. In contrast, the accumulation level of ADR in K562/A02 cells was much lower, but it was increased by 3.8, 4.3 and 5.7-fold in the presence of verapamil, riccardin F and pakyonol, respectively. These results indicate that the synergistic effects of riccardin F or pakyonol with ADR against K562/A02 cells were related to the increased accumulation of ADR in resistant tumor cells.

3.4. *Regulation of rhodamine-123 accumulation*

Rhodamine-123, a fluorescence substrate for P-gp, is widely used to estimate the drug transportation capability of P-gp. To further investigate the mechanism of riccardin F and pakyonol to modulate the ADR accumulation level inside K562/A02 cells, the intracellular accumulation of rhodamine-123 was assessed by monitoring its fluorescence intensity. As shown in Figure 4, verapamil, riccardin F and pakyonol exhibited no influence on the rhodamine-123 accumulation ability of ADR-sensitive K562 cells. However, in K562/A02 cells, all the tested compounds increased the rhodamine-123 accumulation efficiency compared with the reference, indicating the desired reversal of MDR. Among the three compounds, pakyonol exhibited the highest reversal activity, followed by verapamil and riccardin F. Since rhodamine-123 is a substrate of P-gp, the successful reversal of MDR using novel pakyonol and riccardin F reveals that the role of these two compounds in recovery of ADR accumulation is correlated with inhibition of P-gp activity.

4. Discussion

Inhibiting activity of P-gp is an ideal way to overcome tumor MDR. Many natural phenolic compounds, such as curcuminoid and EGCG, have been reported to restrain P-gp activity through blocking its drug-transport function or down-regulating its expression (*6-8*). Bisbibenzyl derivatives including riccardin F and pakyonol are dimeric bibenzyls which are chemically composed of two lunularin moieties with diarylether and/or biphenyl linkages (*21*). This type of natural product has been confirmed to confer a selective feature on plants against competition from the other plants and microbial attacks. On the other hand, they boast a wealth of medicinally important activities. Such a class of natural products was validated to have antioxidative potential and radical scavenging activity (*21,22*). In this paper, we report reversal activities of riccardin F and pakyonol against P-gp-mediated MDR by employing K562/A02 cells for the first time. Riccardin F and pakyonol as potent reversal agents of MDR were supported by the following evidence: (*a*) riccardin F or pakyonol at 3 μg/mL (non-toxic dosage toward K562 and K562/A02 cells) significantly

enhanced cytotoxicity of ADR toward K562/A02 cells, while such a result was not observed in K562 cells; (*b*) increased ADR accumulation occurred in P-gp positive cells, but not in P-gp negative cells; and (*c*) increased intracellular rhodamine-123 stagnation in K562/A02 cells suggested that riccardin F and pakyonol inhibited the transport activity of P-gp. Such findings were not observed in ADR-sensitive K562 cells, which indicated that enhanced accumulation of ADR is correlated with the blocking of P-gp efflux activity.

As reported previously, the uptake of rhodamine-123, is the result of passive inward diffusion, while its efflux is known to be P-gp-dependent. Consequently, rhodamine-123 has been used extensively as an indicator for examing the efflux activity of P-gp in drug-resistant cell lines over-expressing P-gp (*23*,*24*). In P-gp activity assays, K562/A02 cells were pretreated with riccardin F and pakyonol, and intracellular rhodamine-123-associated MFI was increased by 90.3% and 151.7%, respectively. The increased accumulation of rhodamine-123 demonstrated that riccardin F and pakyonol could retrieve P-gp activity in P-gp-mediated resistant cancer cells. It was reported that a number of phenolic compounds such as flavones (hesperetin, limettin and 7-OH-coumarin) decreased P-gp efflux activity in human colon carcinoma Caco-2 cells through directly binding to unspecified sites of P-gp or down-regulating P-gp expression (*25*). In addition, methoxylated flavones, including tangeretin, hepatomethoxyflavone and nobiletin, have been demonstrated to show an inhibitory effect on P-gp-mediated delivery in the above-mentioned cancer cell line as well (*26*,*27*). Riccardin F and pakyonol structurally have a methoxyl at lunularin moieties. However, pakyonol possesses much stronger reversal activities than riccardin F since it might be easier to enter tumor cells due to fewer hydroxyl groups caused by lipophilic features. In the P-gp activity assay of this study, the time of exposure of cells to riccardin F and pakyonol was short (1 h), and enhanced accumulation of ADR or rhodamine-123 by riccardin F and pakyonol in K562/A02 cells is likely carried out by suppressing directly P-gp efflux activity.

In conclusion, the present results confirmed that riccardin F and pakyonol exhibited potentially *in vitro* inhibitory effects on P-gp function and the reversal of activity of P-gp-mediated MDR. This study exploited a class of bisbibenzyl compounds with inhibitory activities against P-gp-mediated MDR. The reversal effects and mechanisms of riccardin F and pakyonol in different resistant cancer cell lines and their *in vivo* pharmacokinetics are in progress.

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Original Article

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Effects of lipoprotein lipase gene variations, a high-carbohydrate low-fat diet, and gender on serum lipid profiles in healthy Chinese Han youth

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Summary **A high-carbohydrate low-fat (HC/LF) diet and lipoprotein lipase gene (*LPL*) Ser447Stop and *Hind* III polymorphisms have separately been found to be associated with triacylglycerol (TG) and high density lipoprotein cholesterol (HDL-C). This study sought to test the effects of *LPL* polymorphisms and an HC/LF diet on the serum lipid profile of Chinese with a lower incidence of coronary artery disease (CAD) consuming a diet with less fat and more carbohydrates. Fifty-six healthy subjects (22.89 ± 1.80 years) were given a control diet of 30.1% fat and 54.1% carbohydrates for 7 days, followed by an HC/LF diet of 13.8% fat and 70.1% carbohydrate for 6 days; there were no changes in the fatty acid composition or restrictions on total energy. Serum lipid profiles at baseline, before and after the HC/LF diet, and *LPL* polymorphisms were analyzed. After 6 days of the HC/LF diet, TG and the homeostasis model assessment of insulin resistance (HOMA-IR) index were found to increase only in females with S447S. No decrease in HDL-C was noted. In subjects with *Hind* III polymorphism, increased TG was found in all females but not in males. Increased HDL-C, together with apolipoprotein (apo) AI, was found in male H- carriers but not in males with H+/H+ and females. In conclusion, *LPL* Ser447Stop and *Hind* III polymorphisms modified the effects of an HC/LF diet on the serum lipid profiles of a young Chinese population in different ways. Effective strategies for dietary interventions targeted at younger populations should take into account the interplay between genetic polymorphisms, diet, and gender.**

Keywords: High-carbohydrate low-fat diet, lipoprotein lipase, SNP, serum lipids, glucose, insulin resistance

1. Introduction

Numerous studies have indicated that substitution of fat with carbohydrates as dietary energy is an effective way to reduce serum low density lipoprotein cholesterol (LDL-C) (*1*), one of the key factors for coronary artery disease (CAD). However, a high-carbohydrate low-fat (HC/LF) diet can lead to hypertriacylglycerolemia (HPTG) by elevating triglycerides (TG) and decreasing high density lipoprotein cholesterol (HDL-C) in serum, both of which are considered to be key risk factors for CAD (*1*,*2*). However, the mechanism of changes in the lipid profile after an HC/LF diet has yet to be fully elucidated. Genetic factors, and their interaction with diets, are believed to play important roles in the development of carbohydrate-induced HPTG and subsequently lead to CAD (*2*,*3*). Previous studies on carbohydrate-induced HPTG mainly focused on middle-aged or elderly subjects since CAD is often

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diagnosed after 45 years of age (*4*). Few efforts have been made to study younger populations, whose risk of CAD has risen steadily in recent decades (*5*).

Abundant evidence provided by epidemiologic studies has indicated that HPTG is rare and the incidence of CAD is much lower in rice-eating populations of the world and particularly in Chinese (*6,7*). There is ample documentation that the Chinese population has a diet with more carbohydrates and less fat, including less saturated, monounsaturated, and polyunsaturated fat (*8,9*). Additionally, the Chinese population has been found to have a better lipid profile, including less total cholesterol (TC), more HDL-C, and a higher apolipoprotein (apo) AI/B100 ratio (*10*). These findings highlight the importance of environmental, lifestyle, and potential genetic factors in modulating the lipid response to carbohydrate consumption.

Lipoprotein lipase (LPL) catalyzes the hydrolysis of TG in chylomicrons (CM) and very low density lipoprotein (VLDL) to promote cellular uptake of lipoproteins and is thus a key enzyme influencing serum TG. Several polymorphisms of the LPL gene (*LPL*) have been found to be linked to abnormalities of the serum lipid profile and also to the development of CAD (*11*). *LPL* Ser447Stop polymorphism results from a C to G transition at position 1595 in exon 9 that removes two amino acid residues at the C-terminal of LPL. The truncated LPL protein has enhanced LPL activity and has been found to be related to decreased TG and increased HDL-C (*12,13*). *LPL Hind* III polymorphism occurs in intron 8 with a T to G transition at position +495. The H+ allele has been reported to be associated with elevated TG, lower HDL-C, and an increased risk of CAD (*14,15*). However, little is known about the interaction between these two *LPL* polymorphisms and an HC/LF diet and their effects on the serum lipid profiles of young subjects and particularly Chinese.

Previous studies by the present authors have found that polymorphisms in the key enzymes and lipoproteins involved in lipid metabolism, such as *Taq*IB polymorphism of the cholesterol ester transfer protein (*CETP*) gene (*16*) and polymorphism of sterol regulatory element-binding protein (SREBP) genes (Zhang *et al.*, accepted by "Applied Physiology, Nutrition and Metabolism"), may modify the impact of an HC/LF diet on the lipid profile. Therefore, this study sought to test the hypothesis that *LPL* polymorphisms of *Hind* III and Ser447Stop affect changes in serum lipid levels in response to an HC/LF diet. Since *LPL* polymorphisms were found to be associated with insulin resistance (IR) (*17*), effects of the polymorphisms on the response of insulin and IR were also studied.

2. Methods

2.1. *Study population*

Volunteers were recruited *via* an advertisement seeking healthy young students at Sichuan University. Recruitment criteria included no history of metabolic disease, understanding of the procedures involved, and provision of written consent. Volunteers who were on any lipid-lowering drugs or hormones, who consumed alcohol or smoked, or whose physical activity or sleep times varied widely were excluded. Of 209 Chinese Han students recruited, 60 met the above criteria for inclusion in the study. Four subjects dropped out due to personal reasons while the remaining 56 subjects (27 males and 29 females) completed the study. The study protocol was approved by the Human Ethics Committee of Sichuan University.

2.2. *Diets*

This study consisted of 7 days of a control diet as a wash-out period, followed by 6 days with intervention in the form of an HC/LF diet. Each diet was designed to have constant ratios of carbohydrates, proteins, and fat in relation to total energy and the control and HC/LF diets had a similar composition of fatty acids (Table 1). All meals were prepared from foods regularly consumed by locals and were provided by the Department of Nutrition, West China Hospital, Sichuan University. There were no restrictions on total energy

Table 1. Daily intake and fatty acid concentrations during diet consumption

Items	Control diet	HC/LF diet
Protein (% of total energy intake)	15.8 ± 1.8	16.2 ± 1.6
Total fatty acids (% of total energy intake)	30.1 ± 3.6	13.8 ± 1.4
Saturated fatty acids	7.5 ± 0.9	3.6 ± 0.5
Monounsaturated fatty acids	16.1 ± 1.4	7.3 ± 0.8
Polyunsaturated fatty acids	6.4 ± 1.5	2.8 ± 0.3
Carbohydrates (% of total energy intake)	54.1 ± 2.4	70.1 ± 2.8
Fiber (g/d)	11.6 ± 2.3	15.4 ± 3.6
Fatty acid composition (% of total fatty acids by energy intake)		
Palmitic fatty acids (16:0)	15.9 ± 4.4	18.9 ± 5.8
Palmitoleic fatty acids (16:1)	2.1 ± 0.7	2.0 ± 0.4
Stearic fatty acids (18:0)	6.9 ± 1.3	7.4 ± 0.9
Oleic fatty acids (18:1)	30.7 ± 6.5	32.1 ± 3.7
Linoleic fatty acids (18:2)	13.2 ± 3.3	17.0 ± 5.1

in each meal. All subjects ate to satiation as usual. All subjects were informed not to consume anything else in addition to the prepared meal except water. A daily checklist was used to assess each subject's compliance with the study design.

2.3. *Blood collection and laboratory analysis*

Blood samples after 12 hours of fasting were collected on the morning of the first day of the study, the morning of the day the HC/LF diet started, and the morning of the day after the HC/LF diet concluded. Serum TG, TC, glucose, HDL-C, and LDL-C were analyzed using enzymatic methods described previously (*18*). Apo B100 and apo AI were measured by immunoturbidimetry with a Hitachi 7070 Analyzer and insulin concentration was determined by electrochemical luminescence with a Roche E170 Analyzer. The homeostasis model assessment of insulin resistance (HOMA-IR) index was calculated as (insulin × glucose)/22.5. The inter- and intra-assays coefficients of variation were less than 6%. Each variable of a given sample was measured three times, and the average value of the three measurements was used in statistical analysis.

2.4. *Genetic analysis*

Polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) analysis was used to screen for *LPL* Ser447Stop and *Hind* III polymorphisms using the primers and procedures described previously (*12,14*). The enzyme *Mnl* I was used to detect Ser447Stop polymorphism. Homozygotes of the mutation were designated X447X, homozygotes of the wild type were designated S447S, and heterozygotes were designated S447X. *Hind* III polymorphism was detected by digestion of PCR products with *Hind* III. Homozygotes of the mutation were designated H-/H-, homozygotes of the wild type were designated H+/H+, and heterozygotes were designated H+/H-. Because of the small sample size, S447X and X447X were combined and designated 447X carriers (447XC), and heterozygotes of H+/H- and homozygotes of H-/H- were pooled and designated H- carriers (H-C) for further analyses.

2.5. *Statistical analysis*

Gene-counting was used to calculate the frequencies of genotypes and alleles. SPSS (16.0) was used for statistical analysis. A χ^2 test was used to evaluate the gender difference in the frequency of *LPL* Ser447Stop and *Hind* III. Data were expressed as mean ± S.D. All variables were tested for normality before analyses. The value of TG was log-transformed to reduce skewness. One-way ANOVA was used to evaluate differences in variables among subjects of each gender with different genotypes and the least significant difference (LSD) was used for post-hoc multiple comparisons. A paired *t*-test was used to analyze differences in variables before and after the HC/LF diet. A value of $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. *Frequencies of LPL polymorphisms*

Genotype and allele frequencies of Ser447Stop and *Hind* III polymorphisms of *LPL* in the study population are shown in Table 2. Genotype distributions of the two polymorphisms were found to be in accordance with Hardy-Weinberg equilibrium expectations (p = 0.105 for Ser447Stop and p = 0.075 for *Hind* III). There were no differences in genotype frequencies of the two polymorphisms in males and females (p = 0.639 for Ser447Stop and p = 0.830 for *Hind* III).

3.2. *Effects of an HC/LF diet on lipid and glucose metabolism in subjects with different LPL Ser447Stop genotypes*

Table 3 shows lipid profiles in subjects with different *LPL* Ser447Stop genotypes at baseline, after the wash-

Table 2. Allele and genotype frequencies of *LPL* Ser447Stop and *Hind* III polymorphisms

	Total (n = 56) n (%)	Males (n = 27) n (%)	Females (n = 29) n (%)
Ser447Stop			
S447S	45 (80.4%)	21 (77.8%)	24 (82.8%)
S447X	9 (16.0%)	4 (14.8%)	5 (17.2%)
X447X	2 (3.6%)	2 (7.4%)	0
Hind III			
H+/H+	34 (60.7%)	16 (59.3%)	18 (62.1%)
H+/H-	16 (28.6%)	6 (22.2%)	10 (34.5%)
H-/H-	6 (10.7%)	5 (18.5%)	1 (3.4%)
Allele frequency			
S447	0.88	0.85	0.91
447X	0.12	0.15	0.09
H+	0.75	0.67	0.79
H-	0.25	0.33	0.21

out diet, and after the HC/LF diet. There were no statistically significant differences in variables from the baseline for subjects with the genotype S447S and genotype 447XC who were either male or female (data not shown); the same was true after the wash-out diet and after the HC/LF diet. However, males with the 447X allele had a significantly lower glucose level compared to the level before the HC/LF diet, but males with the S447S genotype did not have a lower glucose level after the HC/LF diet intervention. Females with S447S were found to have increased TG and insulin and a higher HOMA-IR as well as decreased LDL-C in comparison to values after the wash-out diet. Therefore, the increased TG and insulin and higher HOMA-IR level were presumably modified by the *LPL* Ser447Stop polymorphism in healthy females.

3.3. *Effects of an HC/LF diet on lipid and glucose metabolism in subjects with different LPL Hind III genotypes*

Table 4 shows lipid profiles at baseline, after the wash-out diet, and after the HC/LF diet for subjects with different *LPL Hind* III genotypes. There were no significant differences in variables at the baseline and after a 13-day diet intervention for subjects with H+/H+ and H-C who were either male or female (data not shown); the 13-day diet intervention consisted of 7 days of a wash-out diet followed by 6 days of the HC/LF diet. However, females in both two genotype subgroups were found to have increased TG compared to the level before the HC/LF diet, but males had no such increase. Notably, only male H-allele carriers were found to have increased HDL-C and elevated ApoAI after the HC/LF diet. Female H+/H+ homozygotes had increased insulin and a higher HOMA-IR index after the HC/LF diet intervention while female H- allele carriers had significantly decreased glucose. Therefore, HDL-C did not decrease in the young Chinese population on an HC/LF diet. In contrast, male carriers of *LPL Hind* III H- had increased HDL-C while on the HC/LF diet.

4. Discussion

Most previous studies on carbohydrate-induced HPTG focused on middle-aged or elderly subjects because CAD is often diagnosed after 45 years of age (*4*). Much less effort has been made study younger populations,

Table 3. BMI and serum biochemistry of subjects with different *LPL* Ser447Stop genotypes before and after an HC/LF diet

Variables	Males		Females	
	S447S (n = 21)	447XC (n = 6)	S447S (n = 24)	447XC (n = 5)
Frequency (%)	77.78	22.22	82.76	17.24
Age (years)	23.14 ± 2.06	22.33 ± 1.51	22.60 ± 1.83	22.60 ± 0.55
BMI (kg/m^2)				
Before HC/LF diet	22.03 ± 3.87	20.64 ± 5.18	20.13 ± 2.64	20.15 ± 2.29
After HC/LF diet	21.93 ± 3.83	20.45 ± 5.19	20.06 ± 2.63	19.94 ± 2.14
TG (mg/dL)[c]				
Before HC/LF diet	84.43 ± 41.35	71.30 ± 17.84	66.12 ± 15.29	63.32 ± 21.07
After HC/LF diet	92.75 ± 42.39	69.70 ± 15.56	79.48 ± 23.42[b]	78.03 ± 25.05
TC (mg/dL)				
Before HC/LF diet	134.26 ± 23.54	125.68 ± 15.62	138.77 ± 23.42	143.29 ± 19.08
After HC/LF diet	119.13 ± 19.02[b]	119.17 ± 13.17[a]	132.65 ± 18.58[a]	138.08 ± 28.13
LDL-C (mg/dL)				
Before HC/LF diet	68.38 ± 22.36	63.39 ± 13.63	69.61 ± 21.49	74.35 ± 13.91
After HC/LF diet	53.43 ± 16.22[b]	54.63 ± 14.47[a]	57.77 ± 14.49[b]	67.85 ± 22.71
HDL-C (mg/dL)				
Before HC/LF diet	48.03 ± 11.24	51.31 ± 7.05	58.80 ± 10.70	59.71 ± 9.54
After HC/LF diet	52.32 ± 10.81	58.60 ± 10.97	60.14 ± 9.90	58.87 ± 8.57
Glucose (mg/dL)				
Before HC/LF diet	79.43 ± 9.24	87.21 ± 6.54	78.94 ± 9.81	80.84 ± 7.71
After HC/LF diet	78.44 ± 6.30	77.24 ± 7.38[a]	77.72 ± 7.56	76.19 ± 5.88
Insulin (μU/mL)				
Before HC/LF diet	5.70 ± 5.11	4.13 ± 2.26	4.22 ± 2.78	6.22 ± 4.13
After HC/LF diet	5.93 ± 3.43	5.22 ± 2.99	6.02 ± 3.17[b]	6.02 ± 3.40
HOMA-IR				
Before HC/LF diet	1.15 ± 1.06	0.91 ± 0.53	0.84 ± 0.59	1.23 ± 0.74
After HC/LF diet	1.17 ± 0.75	1.02 ± 0.60	1.17 ± 0.65[b]	1.14 ± 0.66
ApoAI (mg/dL)				
Before HC/LF diet	166.95 ± 29.72	169.33 ± 15.58	191.75 ± 22.97	195.20 ± 21.38
After HC/LF diet	168.48 ± 26.66	177.50 ± 21.95[a]	195.88 ± 20.84	198.40 ± 29.11
ApoB100 (mg/dL)				
Before HC/LF diet	59.81 ± 21.70	51.67 ± 11.94	58.04 ± 16.51	64.40 ± 16.83
After HC/LF diet	58.43 ± 23.06	52.50 ± 13.16	57.63 ± 14.96	67.00 ± 23.73

[a] $p < 0.05$ when compared to subjects with the same genotype before the HC/LF diet; [b] $p < 0.01$ when compared to subjects with the same genotype before the HC/LF diet; [c] The value of TG was log-transformed for statistical analysis.

whose risk of CAD has risen steadily in recent decades (*5*). An HC/LF diet may have different effects in younger populations than in middle-aged or older populations; this is especially true for Chinese, who generally have a better lipid profile and consume a diet containing less fat and more carbohydrate (*8*). To the extent known, the present study is the first attempt to investigate the effects of an HC/LF diet on the serum lipid profiles of young, healthy Chinese subjects with different genotypes of *LPL* Ser447Stop and *Hind* III polymorphisms.

The time an HC/LF diet takes to induce HPTG depends on the composition of dietary carbohydrates as part of energy intake, the types of carbohydrates, the physical form of those carbohydrates in the diet, the fiber content in the diet, and even the ethnicity of the subjects (*1*). Previous studies sought to introduce HPTG with an HC/LF diet in 48 hours to several months (*1*). However, Ginsberg *et al.* noted that "during the high carbohydrate period, plasma TG was found to increase in most subjects for 5-7 days before establishing a new plateau (*19*)". In a recent study, Ji (*20*) used a 7-day interval to compare the postprandial lipemic response after a low-fat meal and a high-fat meal consumed by non-obese men with the -1131T>C polymorphism of the apo A5 gene. Holmback *et al.* (*21*) used a 6-day period for HC/LF diet adaptation. Since studies have shown that plasma TG rises and then plateaus after 5-7 days of an HC/LF diet (*22*,*23*), the present study used an intervention of 6 days with a regular diet for 7 days as a wash-out period.

An HC/LF diet (*1*,*2*) and *LPL* Ser447Stop (*12*,*13*) and *Hind* III (*14*,*15*) polymorphisms have been reported to be associated with variations in HDL-C and TG. The present study found no significant differences in HDL-C and TG as well as other serum lipid parameters in carriers and non-carriers of the *LPL* Ser447Stop or *Hind* III mutation after 7 days on a control diet as a wash-out period, followed by 6 days of an HC/LF diet (Tables 3 and 4). A number of factors that are known to be associated with the effects of *LPL* polymorphisms on the lipid profile may account for this finding, including matching for environmental and other genetic factors in carriers and non-carriers of the mutations (*12*,*24*) as well as the small study size. Since most of the genetic and environmental factors for each individual were constant in the 6-day HC/LF diet, a *t*-test was used to compare serum biochemical profiles before and after

Table 4. BMI and serum biochemistry of subjects with different *LPL Hind* III genotype s before and after an HC/LF diet

Variables	Males		Females	
	H+/H+ (*n* = 16)	H-C (*n* = 11)	H+/H+ (*n* = 18)	H-C (*n* = 11)
Frequency (%)	59.26	40.74	62.07	37.93
Age (years)	23.50 ± 2.03	22.18 ± 1.60	23.11 ± 1.97	22.36 ± 0.92
BMI (kg/m^2)				
Before HC/LF diet	21.29 ± 3.00	22.35 ± 5.47	20.33 ± 2.62	19.82 ± 2.51
After HC/LF diet	21.17 ± 2.95	22.23 ± 5.48	20.23 ± 2.67	19.71 ± 2.33
TG (mg/dL)[c]				
Before HC/LF diet	78.14 ± 27.82	86.42 ± 49.54	67.61 ± 16.61	62.41 ± 15.20
After HC/LF diet	87.31 ± 36.29	88.09 ± 44.58	81.52 ± 25.25[b]	75.48 ± 20.12[b]
TC (mg/dL)				
Before HC/LF diet	132.01 ± 16.22	132.84 ± 29.49	139.97 ± 23.35	138.86 ± 22.08
After HC/LF diet	118.83 ± 15.71[b]	119.58 ± 20.97[b]	132.62 ± 18.58[a]	135.18 ± 23.04
LDL-C (mg/dL)				
Before HC/LF diet	65.20 ± 18.50	70.27 ± 23.96	70.94 ± 20.62	69.59 ± 20.60
After HC/LF diet	51.66 ± 14.54[b]	56.67 ± 17.26[b]	58.31 ± 14.42[a]	61.47 ± 19.27[a]
HDL-C (mg/dL)				
Before HC/LF diet	50.97 ± 10.90	45.53 ± 9.22	58.95 ± 10.72	58.97 ± 10.23
After HC/LF diet	54.55 ± 10.65	52.50 ± 11.76[b]	60.43 ± 10.14	59.08 ± 8.93
Glucose (mg/dL)				
Before HC/LF diet	79.64 ± 10.32	83.37 ± 7.17	78.00 ± 10.49	81.35 ± 7.21
After HC/LF diet	78.27 ± 5.30	78.02 ± 8.09	77.46 ± 8.42	77.45 ± 5.07[a]
Insulin (μU/mL)				
Before HC/LF diet	4.99 ± 3.57	5.88 ± 3.40	3.94 ± 2.34	5.59 ± 5.35
After HC/LF diet	5.28 ± 2.47	6.50 ± 4.16	6.03 ± 3.39[b]	6.00 ± 2.87
HOMA-IR				
Before HC/LF diet	1.00 ± 0.71	1.23 ± 1.29	0.78 ± 0.51	1.13 ± 1.13
After HC/LF diet	1.03 ± 0.54	1.29 ± 0.92	1.16 ± 0.68[b]	1.16 ± 0.59
ApoAI (mg/dL)				
Before HC/LF diet	173.00 ± 28.46	159.45 ± 23.57	191.61 ± 24.11	193.55 ± 20.26
After HC/LF diet	173.81 ± 24.06	165.64 ± 28.09[b]	195.33 ± 20.09	197.91 ± 25.50
ApoB100 (mg/dL)				
Before HC/LF diet	56.25 ± 18.11	60.55 ± 23.24	58.00 ± 17.02	61.00 ± 16.08
After HC/LF diet	54.75 ± 19.49	60.55 ± 23.98	57.78 ± 15.92	61.64 ± 18.32

[a] $p < 0.05$ when compared to subjects with the same genotype before the HC/LF diet; [b] $p < 0.01$ when compared to subjects with the same genotype before the HC/LF diet; [c] The value of TG was log-transformed for statistical analysis.

the HC/LF diet. Results revealed that *LPL* Ser447Stop and *Hind* III polymorphisms have different effects on serum glucose, insulin, and the lipid profile after an HC/LF diet (Tables 3 and 4). The 447X allele offers females significant protection from elevated levels of TG and insulin and a higher HOMA-IR index induced by the HC/LF diet but does not offer males that protection; this may be what protects females from HC/LF diet-induced IR.

Recent studies showed that the response of serum lipids to an HC/LF diet may be gender-specific (*25*). An HC/LF diet was reported to be associated with lower HDL-C in males and higher TG in females. The response of TG to the HC/LF diet in this study was basically the same as that reported previously (*1*,*2*). However, there was no significant decrease in HDL-C after the HC/LF diet for this young Chinese population. When *LPL* polymorphism is taken into account, the interaction of the H- allele and HC/LF diet has a significant effect on HDL-C in that HDL-C is elevated after the HC/LF diet. Such an increase in HDL-C after an HC/LF diet has not been reported before. This may result from the interaction between the allele and the diet. Alternatively, the increased HDL-C may reflect environmental and other genetic differences that modify the outcome of interactions between the *LPL Hind* III allele and the diet in this young Chinese population with a high basal HDL-C. The association between 447X and decreased TG was found to be stronger in males than in females in the Danish general population (*13*). In contrast, there was a significant association between the 447X allele and decreased TG in females but not in males in the Singaporean population (*12*). The present study found that the LPL 447X allele provided only females with significant protection from increased TG induced by an HC/LF diet. This finding is consistent with findings for Asian populations.

LPL is reported to be a gene related to IR (*17*). *Hind* III polymorphism has been found to be associated with steady-state plasma glucose concentrations in nondiabetic males with CAD (*26*). Normoglycemic subjects with H+/H+ were found to be more likely to have increased fasting insulin than subjects with H+/H- (*27*). The present study found significantly increased insulin and a higher HOMA-IR index after the HC/LF diet was consumed by female wild-type homozygotes but not by female carriers of *LPL* Ser447Stop and *Hind* III polymorphisms. This result further supports the hypothesis that *LPL* is one of the genes underlying IR. Further studies are needed to confirm the gender specificity of *LPL* Ser447Stop and *Hind* III polymorphisms and to elucidate the mechanisms responsible for the protection from HC/LF diet-induced elevation of insulin and HOMA-IR in these young females.

The effects of particular nutrients, such as the fatty acid composition of the diets, were not tested in this study. However, the purpose of this study was to examine interaction between the ratios of carbohydrate and fatty acids, instead of the fatty acid composition of the diets, and *LPL* polymorphisms and the effect of that interaction on lipid profiles. The ratios of total fatty acids, saturated fatty acids, monounsaturated fatty acids, and polyunsaturated fatty acids to total energy intake were changed accordingly for the control diet and HC/LF diet in this study (Table 1). The percentage of palmitic, palmitoleic, stearic, oleic, and linoleic fatty acids in relation to total fatty acids as energy intake remained constant for the control diet and HC/LF diet in this study (Table 1). In fact, observations pertaining to the different effects of the fatty acid composition may not be valid for diets with extremely low levels of total fat (*28*). More importantly, isolating particular nutrients such as different fatty acids is not possible in everyday life, and especially in developing countries such as China. The present design focusing on diet patterns is much more practical than focusing on particular nutrients in a diet.

In conclusion, *LPL* Ser447Stop and *Hind* III polymorphisms modify the effects of an HC/LF diet on the serum lipid profile, serum levels of insulin, and insulin sensitivity in different ways in a young Chinese population. First, the mutated allele of *LPL Hind* III polymorphism is associated with increased HDL-C and apo AI in males consuming an HC/LF diet. Second, the mutated allele of *LPL* Ser447Stop polymorphism provides only females with significant protection from increased TG induced by an HC/LF diet, although the mutated alleles of both Ser447Stop and *Hind* III polymorphisms provide females with significant protection from increased insulin and a higher HOMA-IR index induced by an HC/LF diet. These findings provide new insight into the interaction of gender, *LPL*, and an HC/LF diet and their effects on risk factors for CAD. These findings are especially relevant to a young Chinese population with a low incidence of CAD consuming a diet with less fat and more carbohydrates. Once confirmed by studies with a large sample size, the current findings may help with the formulation of personalized strategies for diet-based prevention of CAD in a country with a quarter of the world's population.

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Association of *p53 codon 248* (*exon7*) with urinary bladder cancer risk in the North Indian population

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Abstract **p53 is the most frequently mutated gene in all forms of human cancer. It responds to diverse stresses including UVR-induced DNA damage and regulates many downstream genes to initiate cell-cycle arrest, DNA repair or apoptosis. p53 gene variants at *codon 11*, *Pro47Ser* and *codon 248* (*exon 7*) were evaluated for bladder cancer (BC) risk in North Indians. In the present study, the above encoding regions in p53 genes were analyzed in a hospital based study in 200 BC and 200 healthy controls age and gender matched and of similar ethnicity. The genotyping was assessed by the polymerase chain reaction restriction fragment length polymorphism technique and statistically evaluated using SPSS software ver. 15.0. A significant association was found with *p53 codon 248* polymorphism and BC risk whereas *p53 codon 11* and *p53 Pro47Ser* polymorphism showed no association with BC risk. The individuals carrying the heterozygous genotype (Arg/Trp-Arg/Gln) in the *p53 codon 248* polymorphism showed high BC risk ($p < 0.001$). Combinations with heterozygous and variant genotypes also showed a high risk for BC ($p < 0.001$). The minor allele (Trp/Gln) carriers of the *p53 codon 248* demonstrated a 1.7-fold risk for BC. Furthermore, haplotype analysis revealed that the Glu-Pro-Trp/Gln haplotype is associated with a 1.9-fold risk for BC. A protective role was observed with tumor stage/grade of BC patients with *p53 codon 248* ($p = 0.003$; OR = 0.32). Thus, it is evident from our study that of all the 3 single nucleotide polymorphisms evaluated, only *p53 codon 248* (*exon7*) gene polymorphism has an implication for risk in BC in the North Indian population.**

Keywords: Bladder cancer, p53, gene polymorphism, BCG, haplotype

1. Introduction

Bladder cancer (BC) is one of the most common forms of urogenital cancer. Globally the prevalence of BC is estimated at over 1 million and is steadily increasing (*1*). Despite its low incidence as compared to western countries, it still continues to be a major problem in India. The occurrence rate of BC is three times more common in men than in women (*2*). More than 90% of all newly diagnosed BC patients are transitional cell carcinomas.

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p53 is the most frequently mutated gene in all forms of human cancer (*3*), it is a gatekeeper or guardian of the cell division and plays a critical role in cell cycle control and apoptosis (*4*). It is located on chromosome 17p13. Several other functions of p53 in tumor suppression have been discovered that are independent of its ability to transactivate gene expression. These include direct effects on survival proteins in the mitochondria (*5,6*). The ability of p53 to eliminate excess, damaged or infected cells by apoptosis is vital for the proper regulation of cell proliferation in multi-cellular organisms. The latter activity is crucial for tumor suppression and therefore it is reasonable to analyze the p53 status in human cancer.

Somatic mutations in p53 are found in more than 50% of human cancers (*7,8*). Alterations in the p53

gene are the most frequently documented somatic alterations in human BC, as well as other tumor types, and detection of this altered gene holds prognostic significance.

Several studies have reported on association of p53 polymorphisms with increased risk for various cancers such as esophageal (*9*), gastric (*10*), lung carcinoma (*11*) and colorectal cancer (*12*), respectively. Although the possibility that polymorphic variants in p53 might contribute to cancer susceptibility has been extensively investigated, the issue remains highly complicated. As the genetic nature of BC is complex, individual polymorphisms are likely to have a modest effect on risk. Mounting evidence indicates that, besides environmental factors, genetic components and gene-gene and gene-environment interactions also play important roles in BC development (*13,14*). Considering the complexity of bladder carcinogenesis, single-factor studies may not have sufficient power to detect small genetic effects on cancer risk (*15*). It is also plausible that examining several polymorphisms within biologically relevant pathways may reveal subgroups of individuals who are at a significantly elevated risk for this disease.

Hence the rationale of our study was to investigate the effect of 3 single nucleotide polymorphisms (SNPs) in loss of heterozygosity (LOH) sites of p53 in *codon 11, Pro47Ser* and *codon 248* (*exon 7*) for BC risk. These mutations affect normal protein functions and *codon 248* was characterized to acquire novel oncogenic gain of function and contribute to tumor malignancy and chemoresistance (*16*). Further, modulation by lifestyle habits such as smoking in a hospital based case-control study in the North Indian population was also determined.

2. Materials and Methods

2.1. *Study subjects*

BC patients for the study were acquired from an on-going case-control study of BC, which started patient recruitment in 2008. All patients (200) (mean age 58.5 years; 175 men and 25 women) were incident cases of histologically confirmed invasive or superficial BC recruited from the Urology Department at Sanjay Gandhi Postgraduate Institute of Medical Sciences, from May 2008 to June 2010. BC patients with a previous history of other cancer, cancer metastasized to the bladder from another origin, or previous radiotherapy treatments were excluded. Healthy and genetically unrelated individuals visiting the hospital for a routine checkup or health awareness camps and hospital employees were recruited as controls (n = 200). All the controls were age and sex matched (56.8 years, and M/F ratio 179/21) with similar ethnicity and had no evidence of malignancy or chronic disease.

2.2. *Epidemiology data collection*

An epidemiologic questionnaire was designed for study participants to collect data on demographic characteristics such as smoking, occupation, and other lifestyle factors involved. Informed consent was obtained from all subjects when interviewing for demographic details and a blood sample collection. The Ethical Review Board of the Institute approved the study. The response rate from the interview was 95% for the subjects. Individuals who smoked once a day for more than 5 years were defined as smokers. The individuals who had never smoked in their lifetime were regarded as non-smokers. At the conclusion of the interview, a 5 mL blood sample was drawn into coded vials.

2.3. *Clinical data collection*

The demographic and clinical characteristics of the patients are presented in Table 1. The clinical information about tumor size, number, stage and tumor grade, intravesical therapy and dates of recurrence, chemotherapy, radical cystectomy and pathological findings at cystectomy were provided by the uro-oncologist in our department. The classification of tumor stages were from the American Joint Committee on Cancer's TNM staging system (*17*). Of the 200 total

Table 1. Demographical details of BC patients and healthy controls

Variable	Cases (n = 200) n (%)	Controls (n = 200) n (%)	p value*
Sex			
Female	25 (12.5)	21 (10.5)	0.531
Male	175 (87.5)	179 (89.5)	
Age (years)			
Mean age ± S.D.	58.5 ± 12.4	56. 8 ± 10.8	0.117
Smoking**			
Non-smokers	47 (30.1)	155 (77.5)	< 0.001
Smokers	109 (69.9)	45 (22.5)	
Tumor number**			
Single	115 (60.8)	–	–
Multiple	74 (39.2)	–	
Tumor size (cm)**			
< 1	35 (24.3)	–	–
1-3	73 (50.7)	–	
> 3	36 (25.0)	–	
Stage			
Ta	64 (32.0)	–	–
T1	85 (42.5)	–	
T2	51 (25.5)	–	
Grade			
G1	67 (33.5)	–	–
G2	43 (21.5)	–	
G3	90 (45.0)	–	
Intravesical therapy			
Non-treated	71 (47.7)	–	–
BCG Induction (BCG i + m)	78 (52.3)	–	
Event			
Recurrence	65 (43.9)	–	–
Non-recurrence	84 (56.1)	–	

* Student's t-test was used to determine the p value; ** The sum could not add up to the total due to some missing values.

patients enrolled in the study, 149/200 (74.5%) patients had non-muscle invasive BC (NMIBC) while the rest 51/200 (25.5%) had muscle invasive BC (MIBC). Patients with NMIBC at high risk (high grade, multiple, and large tumors) were treated with intravesical *Bacillus* Calmette-Guerin (BCG) (n = 78). The patients with NMI cancer of low risk (low grade and single small tumor) were kept on cystoscopic surveillance and considered as non-BCG patients. Subsequently, all the patients were examined by cystoscopy every 3 months in the first and second years and later at six monthly intervals as long as there was no tumor recurrence. BCG treatment consisted of 6 weekly instillation induction BCG (n = 78). Since the number of patients receiving maintenance BCG was too low, we did not categorize the patients according to BCG regime for statistical analysis. The end point of the study included tumor recurrence, defined as a newly found bladder tumor following a previous negative follow-up cystoscopy, or end of the study time. Patients with invasive BC (n = 51) were treated with radical cystectomy with or without adjuvant chemotherapy, which included cisplatin, and gemcitabine followed by periodical cystoscopy. A blood sample was collected in EDTA from all subjects for genotyping at the time of enrollment and stored at −70ºC.

2.4. *Genotyping*

Genomic DNA was extracted from peripheral blood lymphocytes by the salting out method (*18*). Polymorphisms in p53 (*codon 11, Pro47Ser* and *codon 248*) genes were analyzed using the polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) method. Details of the primers and PCR conditions for *p53 codon 11* and *codon 248* have been previously described (*19*) and for *p53 Pro47Ser* (*20*). Genotyping was done on 10% polyacrylamide gel using molecular weight markers and visualized after staining with ethidium bromide. Positive and negative controls were used in each genotyping assay, and 10% of the samples were randomly selected and run in duplicate with 100% agreement. The results were reproducible with no discrepancies in genotyping.

2.5. *Statistical analysis*

The study power was calculated using Quanto software, version 1.0 (available from: *http://hydra.usc.edu/gxe*) with input of the following variables: case-control study design, significance level (alpha) > 0.05 (Two sided), model of inheritance was log additive, allele frequency was 0.28, and the genetic effect for odds ratio (OR) was 1.5 or greater. The present study achieved 80% of the statistical power. The goodness-of-fit chi square test was used to analyze any deviation from the Hardy-Weinberg equilibrium in controls. A binary logistic regression model was used to estimate the risk as the OR at the 95% confidence interval (CI). Haplotypes of each individual consisting of SNP in p53 was constructed, and the maximal likelihood haplotype frequencies were estimated using the expectation-maximization algorithm using the Arlequin program, version 2000. Bonferroni's correction was applied in the case of multiple comparisons using the formula $P_c = p \times n$ (P_c represents corrected value where n is the number of comparisons performed). The statistical analysis was done using the Statistical Package for Social Sciences software, version 15.0 (SPSS, Chicago, IL), and $p < 0.05$ was considered statistically significant.

3. Results

3.1. *Characteristics of subject*

No significant age difference between the cases (58.5 ± 12.4 years) and the controls (56.8 ± 10.8 years; p = 0.117) was observed. The cases had a significantly higher percentage of smokers (69.9%) than the controls (22.5%) (p = 0.001) (Table 1).

3.2. *Genotypic frequency of p53 gene polymorphisms and BC risk*

The genotype and allele frequencies of p53 gene polymorphism in healthy individuals (controls) and BC patients are presented in Table 2. The genotype frequencies of controls were in Hardy Weinberg Equilibrium (HWE) except for *p53 codon 11*. We found a significant association between the *p53 codon 248* with BC risk, whereas *p53 codon 11* and *p53Pro47Ser* showed no association with BC. In *p53 codon 248* the heterozygous genotype was at higher risk of BC ($p < 0.001$; OR = 2.69; 95% CI, 1.58-4.59). While combining the heterozygous and variant genotypes a high risk for BC ($p < 0.001$; OR = 2.23; 95% CI, 1.42-3.49) was observed. This effect was also evident in the case of Trp/Gln allele carrier (p = 0.004; OR = 1.78; 95% CI, 1.20-2.62).

3.3. *Association of p53 genotypes with tumor stage/grade*

The patients with a similar stage but with different grades respond to treatment differently. Hence, we stratified the patients into three sub-groups according to stage/grade [TaG1 (low risk NMIBC), $TaG_{2,3}$+$T1G_{1-3}$ (high risk NMIBC) and T2+ (muscle invasive)]. TaG_1 was taken as a reference. Significant association was observed in the case of *p53 codon 248* combining with heterozygous and variant (Arg/Trp, Arg/Gln) + (Trp/Trp, Gln/Gln) genotypes (p = 0.003; OR = 0.32; 95% CI, 0.15-0.69) when TaG1 and $TaG_{2,3}$ + $T1G_{1-3}$ tumor stage/grade was considered for calculation. The association between group TaG1 and T2+ was not significant in any of the three polymorphic sites of

Table 2. *p53 codon 11*, *p53 Pro 47 Ser* and *p53 codon 248* gene polymorphisms in BC polymorphisms and susceptibility to BC

Genotype	Controls (n = 200) n (%)	Cases (n = 200) n (%)	p value	Age-gender-smoking adjusted OR (95% CI)[a]
p53 codon 11				
Glu/Glu	193 (96.5)	198 (99.0)	–[b]	
Glu /Gln, Glu/Lys,	7 (3.5)	2 (1.00)	0.281	0.31 (0.04-2.61)
Gln/Gln, Lys /Lys	0 (0.0)	0 (0.0)	NC[c]	NC
Glu	393 (98.3)	398 (99.5)	–	
Gln/Lys	7 (1.8)	2 (0.5)	0.116	0.28 (0.06-1.37)
p53 Pro 47 Ser				
Pro/Pro	176 (88.0)	181 (90.5)	–	
Pro/Ser	7 (3.5)	12 (6.0)	0.355	1.66 (0.57-4.86)
Ser/Ser	17 (8.5)	7 (3.5)	0.247	0.55 (0.20-1.51)
Pro/Ser + Ser/Ser	24 (12.0)	19 (9.5)	0.421	0.77 (0.41-1.46)
Pro allele	359 (89.8)	374 (93.5)	–	
Ser allele	41 (10.3)	26 (6.5)	0.058	0.61 (0.37-1.02)
p53 codon 248				
Arg/Arg	159 (79.5)	127 (63.5)	–	
Arg/Trp, Arg/Gln	34 (17.0)	68 (34.0)	< 0.001	2.69 (1.58-4.59)
Trp/Trp, Gln/Gln	7 (3.5)	5 (2.5)	0.929	1.06 (0.30-3.7)
(Arg/Trp, Arg/Gln) + (Trp/Trp, Gln/Gln)	41 (20.5)	73 (36.5)	< 0.001	2.23 (1.42-3.49)
Arg	352 (88.0)	322 (80.5)	–	
Trp/Gln	48 (12.0)	78 (19.5)	0.004	1.78 (1.20-2.62)

[a] Adjusted for Age, gender and smoking habits; [b] Reference; [c] Not calculated (NC).

Table 3. Influence of *p53 codon 11*, *p53 Pro 47 Ser* and *p53 codon 248* gene polymorphisms on the Tumor-stage/grade in BC patients

Genotypes	TaG1 (a) n (%)	TaG_{2-3} + $T1G_{1-3}$ (b) n (%)	T2+ (c)[a] n (%)	p value (a - b)	OR (95% CI)[b]
p53 codon 11					
Glu/Glu	36 (97.3)	111 (99.1)	51 (0.0)	–[c]	
Glu/Gln,Glu/Lys	1 (2.7)	1 (0.9)	0 (0.0)	0.430	0.32 (0.20-5.32)
Gln/Gln, Lys /Lys	0 (0.0)	0 (0.0)	0 (0.0)	NC[d]	NC[d]
p53 Pro47Ser					
Pro/Pro	35 (94.6)	104 (92.9)	42 (82.4)	–	
Pro/Ser	2 (5.4)	3 (2.7)	7 (13.7)	0.464	0.51 (0.08-3.15)
Ser/Ser	0 (0.0)	5 (4.5)	2 (3.9)	NC	NC
Pro/Ser + Ser/Ser	2 (5.4)	8 (7.2)	9 (17.6)	0.160	0.45 (0.15-1.37)
p53 codon 248					
Arg/Arg	25 (67.6)	71 (3.4)	31 (60.8)	–	
Arg/Trp, Arg/Gln	12 (32.4)	37 (33.0)	19 (37.3)	0.839	1.09 (0.49-2.40)
Trp/Trp, Gln/Gln	0 (0.0)	4 (3.6)	1 (2.0)	NC	NC
Arg/Trp, Arg/Gln) + (Trp/Trp, Gln/Gln)	12 (32.4)	41 (36.6)	20 (39.3)	0.003	0.32 (0.15-0.69)

[a] The association between groups a and c was not significant (data not shown); [b] Adjusted for Age, Gender and Smoking habits; [c] Reference; [d] Not calculated (NC).

the codon (data not shown). However, the other two polymorphic sites of *p53 codon 11* and *p53 Pro47Ser* showed no significant association with tumor stage/grade (Table 3).

3.4. *Association of p53 genotypes with smoking*

We evaluated the gene smoking interaction to study the modulation of BC risk with respect to p53 gene polymorphisms. The patients were grouped as non-smokers and smokers. However, no significant association was observed for any of the three polymorphic sites of p53 with BC risk (data not shown).

3.5. *Association of p53 haplotypes with BC risk*

The ability of haplotypes to further substantiate the detection of association over the single locus analysis incited us to analyze haplotypes and their association with BC susceptibility in p53. Seven haplotype combinations were possible from haplotype analysis of p53. In the case of p53, Glu-Pro-Arg was taken as reference. The haplotype results demonstrated that Glu-Pro-Trp/Gln was associated with a two fold (OR = 1.91; 95% CI, 1.27-2.87; p = 0.002) increased risk in BC patients. After applying the Bonferroni correction the p value remained

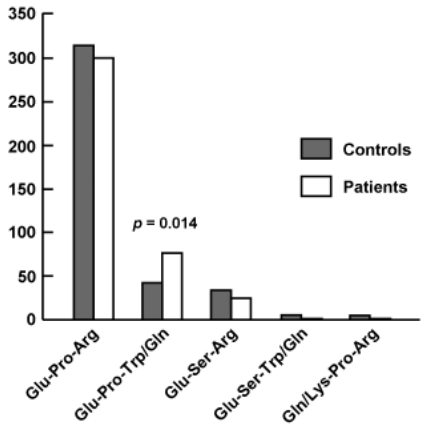

Figure 1. Haplotype analysis of p53 gene polymorphisms and BC risk.

significant ($p = 0.014$) for BC risk (Figure 1).

3.6. *Modulation of genotype variants and outcome after BCG immunotherapy*

To analyze the association of p53 gene polymorphisms and risk of recurrence in NMIBC patients, further analysis was restricted to NMIBC patients only ($n = 148$). The median follow-up of NMIBC patients was 14 months. We analyzed the association of genotypes and risk of recurrence after BCG immunotherapy. We grouped patients into BCG treated ($n = 78$) and non-treated ($n = 71$) because these patients with low grade tumors did not require BCG immunotherapy. None of the polymorphisms of p53 genes were associated with risk of recurrence free survival (data not shown).

4. Discussion

p53 is a nuclear protein that is essential for cell-cycle control, DNA repair and induction of apoptosis from many stresses. The strongest and undisputed fact about p53 is the high frequency of p53 alterations in human cancer and that mutant p53 proteins constitute a complex family of several hundred proteins with heterogeneous properties. Alterations of the p53 gene are some of the most frequently documented somatic alterations in human BC, as well as other tumor types, and detection of this altered gene holds prognostic significance. Several studies, including our own, have clearly shown that inactivation of the p53 pathway is prevalent in this disease. Several studies (*21,22*) and even including our own, have clearly shown that the p53 pathway plays an important role in this disease.

Most cancer-related mutations of p53 are clustered in the four hot spots (*23*) *codon 175*, *248*, *273*, and *281/282*. To the best of our knowledge, this is the first study reporting an increased risk of BC among individuals with the *p53 codon 248* heterozygous genotype, showing a 2.69-fold risk. The combination of heterozygous and variant genotypes demonstrated a 2.23-fold risk for BC susceptibility. In the case of Trp/Gln allele carrier, we found a significant association with BC predisposition having a 1.78-fold risk. However, there was no significant association found between *p53 codon 248* and endometriosis (*19*). There is no other study reported of this SNP in other cancers, though some mutational studies have been stated as in the case of ovarian cancer (*24*) UL-3A cells that contained two point mutations, in codon *248 of exon 7* of the *p53* gene.

In the case of *p53 codon 11*, we found no significant association with BC predisposition; and this result supports the results showing no-significant association between *p53 codon 11* and endometriosis (*20*). However mutation in *p53 codon 11* in gastric adenocarcinomas has been reported earlier by direct DNA sequencing analysis (*25*). For the *p53 Pro47Ser* SNP, we observed Pro/Ser frequency to be 3.5% in controls as compared to 6.0% in cases. The heterozygous Pro/Ser genotype has been reported for the first time in the Indian population. However, we did not find significant association of BC risk with this p53 gene variant that complemented the observation in colorectal cancer in the Kashmiri population (*20*). A significant association was observed between the mutant Ser/Ser genotype and lung cancer (*26*). The effectiveness of mutant Ser/Ser could be due to a decreased capacity for inducing apoptosis (*27*).

Recent studies have demonstrated that haplotypes analysis may be more affirmative in predicting disease association compared with an analysis of a single polymorphism (*28*). Because an individual polymorphism is likely to confer modest effects to the risk of BC, we examined the effects of multiple p53 polymorphisms by constructing haplotype analyses for all three SNPs. A risk of 1.91-fold was observed in case of haplotypes Glu-Pro-Trp/Gln for BC. Other haplotypes did not show any association in connection with BC risk.

We analyzed the association of p53 polymorphisms with risk of recurrence in NMIBC patients. The NMIBC patients were categorized on the basis of BCG treatment in the BCG group and no BCG group. According to age, gender, and smoking adjusted multivariate Cox regression hazards model, no statistically significant association was observed for any SNP of p53 taken in the present study. Thus, there is no role of BCG immunotherapy in the case of this polymorphism. We also studied the gene smoking interaction for modulation of BC risk but did not find any association with any of three gene variants of the p53 gene SNPs in the present study. A significant association was observed in the case of *p53 codon 248* combining heterozygous and variant genotype with a p value of 0.003 when TaG1 and $TaG_{2,3}$+$T1G_{1-3}$ tumor stage/grade was considered for calculation. However the other two polymorphic sites of *p53 codon 11* and *p53Pro47Ser* showed no significant association with tumor stage/grade. The genetic variations that might be

stabilized among the population are those that cause no pivotal effect under normal conditions. However, under special circumstances, the variations may predispose the carriers to diseases. Therefore, analysis combined with the genetic variations and the other internal and external factors may lead to a better understanding of the role of genetic makeup in cancer pathogenesis.

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Evaluation of usefulness of 3D views for clinical photography

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Summary **This is the first report investigating the usefulness of a 3D viewing technique (parallel viewing and cross-eyed viewing) for presenting clinical photography. Using the technique, we can grasp 3D structure of various lesions (*e.g.* tumors, wounds) or surgical procedures *(e.g.* lymph node dissection, flap) much more easily even without any cost and optical aids compared to 2D photos. Most recently 3D cameras started to be commercially available, but they may not be useful for presentation in scientific papers or poster sessions. To create a stereogram, two different pictures were taken from the right and left eye views using a digital camera. Then, the two pictures were placed next to one another. Using 9 stereograms, we performed a questionnaire-based survey. Our survey revealed 57.7% of the doctors/students had acquired the 3D viewing technique and an additional 15.4% could learn parallel viewing with 10 minutes training. Among the subjects capable of 3D views, 73.7% used the parallel view technique whereas only 26.3% chose the cross-eyed view. There was no significant difference in the results of the questionnaire about the efficiency and usefulness of 3D views between parallel view users and cross-eyed users. Almost all subjects (94.7%) answered that the technique is useful. Lesions with multiple undulations are a good application. 3D views, especially parallel viewing, are likely to be common and easy enough to consider for practical use in doctors/students. The wide use of the technique may revolutionize presentation of clinical pictures in meetings, educational lectures, or manuscripts.**

Keywords: Dermatology, plastic surgery, tumor

1. Introduction

3D or stereoscopy is technology capable of making the illusion of depth in an image and showing three-dimensional visual information. Until recently, convincing practical use of stereoscopy could only be seen in small fields such as simulators and visualization devices for a long time. However, 2010 was the year for 3D movies, and the introduction of 3D television to the public consumer market, which has been compared to the transition from black-and-white to color TV, and may be based on the success of some 3D movies in cinemas (*1*). In the near future, the application of 3D technology will be extended in various fields, especially in entertainment and education.

On the other hand, in the medical field, stereoscopy hovers at a level of limited use. For example, in the fields of dermatology and plastic surgery, it is important to grasp the 3D structure of the skin but sometimes difficult to obtain 3D information from 2D pictures. Our aim is to apply 3D views to decrease discrepancies in the grasp of 3D structure between actual clinical presentation and 2D clinical photos; *e.g.* at the exhibition of various lesions or operative procedures in scientific papers and poster sessions. In this study, we tried to evaluate the usefulness of 3D stereoscopy for clinical photography and found the best way to give 3D information to clinical photos using a questionnaire-based survey.

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2. Materials and Methods

2.1. *Creating stereograms for parallel viewing or cross-eyed viewing*

There are two methods for viewing a stereogram without glasses/headgear and any cost: parallel view format and cross-eyed view format. The former technique requires that the eyes take a relatively parallel angle. For cross-eyed viewing, the viewer has to cross his eyes (both eyes turn inward toward the nose). As shown in Figure 1A, to create a stereogram, two pictures (stereo pair) were taken from a slightly different angle – one from the right eye view and another from the left eye view. Then, the two different pictures were placed next to one another (Figure 1B). For parallel viewing, the picture that was taken from the right side was placed on the right, and the picture taken from the left side was placed on the left. For cross-eye viewing the two pictures were swapped around.

2.2. *Subjects*

Several stereograms with a questionnaire form were sent to 23 doctors and 3 medical students (18 males and 8 females; age range 23-51 years) randomly chosen from the Department of Dermatology and Plastic Surgery, Kumamoto University.

2.3. *Training for stereoscopy*

If the respondents to the survey have acquired neither parallel viewing nor cross-eyed viewing, we asked them to practice for less than 10 minutes based on the following procedure:

1) Parallel viewing
a) Bring your face close to the picture and get relaxed.
b) Try to look and focus at a distance behind the picture. Four blurred pictures (or 'black spots' for the aid of stereoscopy; see Figures 2A-2I) should be seen.
c) Pull the face back slowly away from the picture (20-50 cm), until the two middle pictures (or black spots) overlap together. Gradually, eyes will focus on the overlapped pictures as well as black spots and the brain will combine them into a 3D image.

2) Cross-eyed viewing
a) Keep distance of 20-50 cm from the picture and get relaxed.
b) To indicate to your eyes where to focus, bring your forefinger to the middle between you and the picture. Focus both your eyes on it.
c) While focusing on the finger, four blurred pictures (or black spots) should be seen at the same time.
d) Keep the focus and move your finger slowly between you and the picture back and forth until the two middle pictures (or black spots) overlap.
e) When you find the correct focal position and perceive the blurred 3D image, modify the focus slightly until a sharp and clear image is obtained.
f) Shift your attention from your finger to the pictures slowly.

2.4. *Questionnaire design*

The respondents to the survey capable of stereoscopy were asked to decide to use only one method (parallel viewing or cross-eyed viewing) and were requested to see 9 stereograms (Figures 2A-2I). After that, they were also asked to fill in a simple questionnaire as described below.

The questionnaire was designed with questions concerning age, sex, ease of obtaining sense of depth from each stereogram, evaluation of usefulness of stereograms for clinical use to grasp 3D structures, and visual fatigue caused by the stereoscopy.

Q1) On which stereograms could you perform stereoscopy easily? (Select all that apply)
Q2) Which stereograms gave a different impression about depth of lesion from the 2D picture? (Select all that apply and describe the reason).
Q3) Do you think stereoscopy is useful to grasp 3D structure of lesions?
Q4) On how many stereograms could you perform stereoscopy without visual fatigue?

2.5. *Statistical analysis*

All questions were analyzed and expressed as percentage of completed questionnaires. Statistical analysis was carried out with a Mann-Whitney *U*-test for comparison of medians and Fisher' exact probability test for analysis of frequency. p values less than 0.05 were considered significant.

3. Results

3.1. *Stereo images*

We considered two methods are suitable for viewing a stereogram in a scientific paper or poster presentation: parallel view and cross-eyed view formats, because they do not need any costs or optical aids for stereoscopy. We prepared 9 pictures (Figures 2A-2I) with different features. Figure 2A is the picture of tense blister of a burn representing a raised lesion, whereas Figure 2B, a post-operative scar of dermatofibrosarcoma protuberans represents a depressed lesion. Figure 2C displays a depressed geographic skin ulcer, and stereoscopy can reveal the lesion has elevated edges caused by epithelization. Figure 2D also shows keloid with both raised and depressed areas, which is obvious

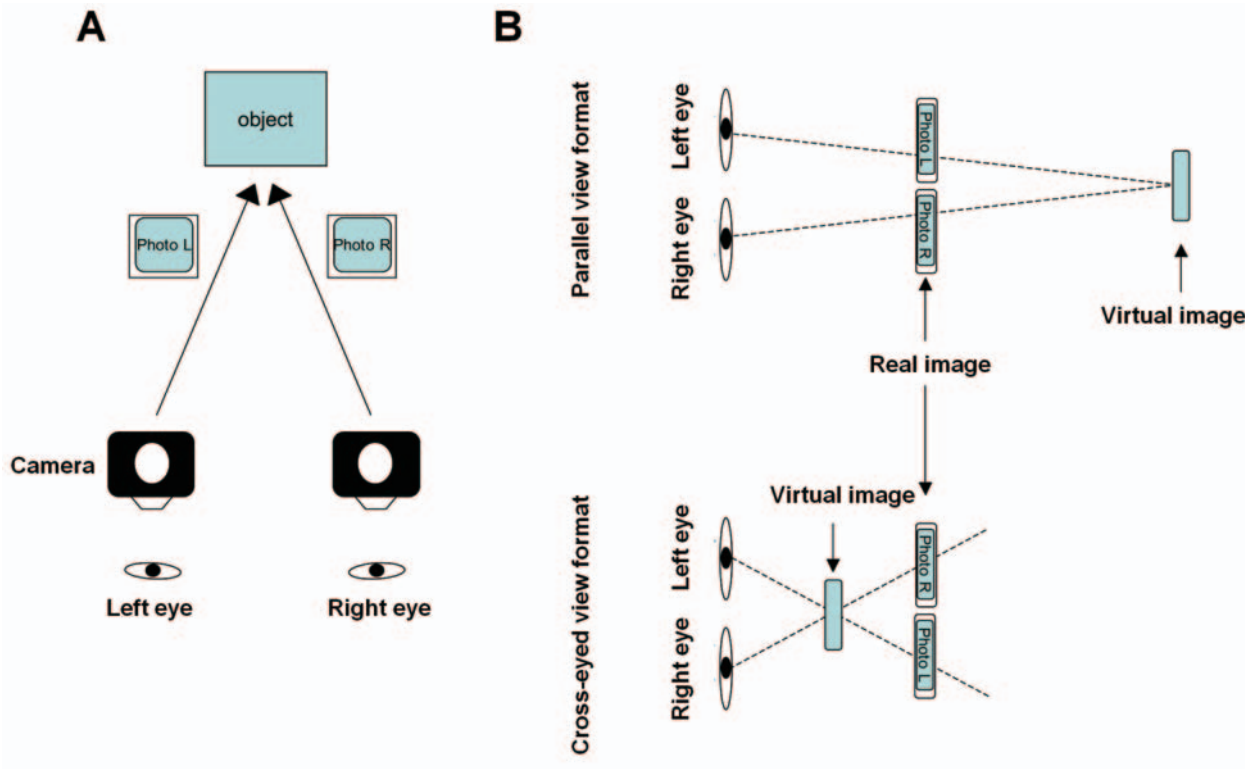

Figure 1. (A) Two pictures for stereograms. Two pictures (stereo pair) were taken from a slightly different angle - one from the left eye view (photo L) and another from the right eye view (photo R). **(B) Creating stereograms.** For parallel viewing, the left eye is forced to look only at the left picture, and the right eye is forced to look at the right one. The picture taken from the left side (photo L) was placed on the left, and the picture taken from the right side (photo R) was placed on the right. Virtual image is perceived behind the real images. For cross-eyed viewing, the left eye should look at the right eye-intended picture, and the right eye at the left. The picture taken from the left side (photo L) was placed on the right, and the picture taken from the right side (photo R) was placed on the left. Virtual image is perceived in front of the real images.

by 3D stereoscopy but not on the 2D photo. Figure 2E shows two raised lesions (nevus and seborrheic keratosis) with different heights, which is clearer by stereoscopy. Figures 2F-2I are pictures of 3D structures of operative procedures, which are sometimes difficult to understand; Figure 2F is the picture of an axillary lymph node dissection with considerable depth in the surgical field. Figure 2G shows complicated vessel structure in an inguinal lymph node dissection. Figure 2H is resection of dermatitis papillaris capillitii of the scalp and the grasp of 3D structure is disturbed by the hair. And Figure 2I is a picture of an operation of necrotizing fasciitis of the buttock, which has complicated muscle structure.

3.2. *Evaluation of usefulness of 3D stereoscopy*

Among the 26 respondents to the survey, 15 (57.7%) had already acquired stereoscopy; 5 subjects (19.2%) had acquired both parallel views and cross-eyed views, 5 (19.2%) had only parallel views, and 5 (19.2%) had only cross-eyed views. Interestingly, an additional 4 respondents (15.4%) could learn parallel views, not the cross-eyed view method, using training for less than 10 minutes. There is no statistically significant difference in age and sex between subjects capable of stereoscopy and those not.

The 19 subjects capable of stereoscopy were asked to decide to use only one method and were requested to see 9 stereograms (Figures 2A-2I). Among the 19 subjects, 14 chose parallel views and only 5 chose cross-eyed views; All 5 subjects capable of both parallel views and cross-eyed views chose the former technique. Subjects who decided to use parallel views selected Figure 2E (10/14 = 71.4%) and Figure 2C (9/14 = 64.3%) as the answer for Q1 about the ease of obtaining a 3D image from each stereogram (Table 1). They also chose Figure 2C (6/14 = 42.9%) and Figure 2E (4/14 = 28.6%) as well as Figure 2D (3/14 = 21.4%) as the answer for Q2 about the usefulness of each stereogram; The reasons subjects described were 'epithelization of the edge of ulcer was more clearly observed by stereoscopy' for Figure 2C, 'the depressed area (see Figure 2D-iii) was not obvious in the 2D picture' for Figure 2D, and 'he difference of height between two skin tumors (see Figure 2E-iii) were recognized better in the stereogram' for Figure 2E.

On the other hand, 5 cross-eyed view users selected Figure 2D (4/5 = 80.0%) and Figure 2E (4/5 = 80.0%) for Q1. Also, Figure 2E (3/5 = 60%) and Figure 2D (1/5 = 20%) were chosen for Q2. Taken together, there was no significant difference in the results of questionnaire

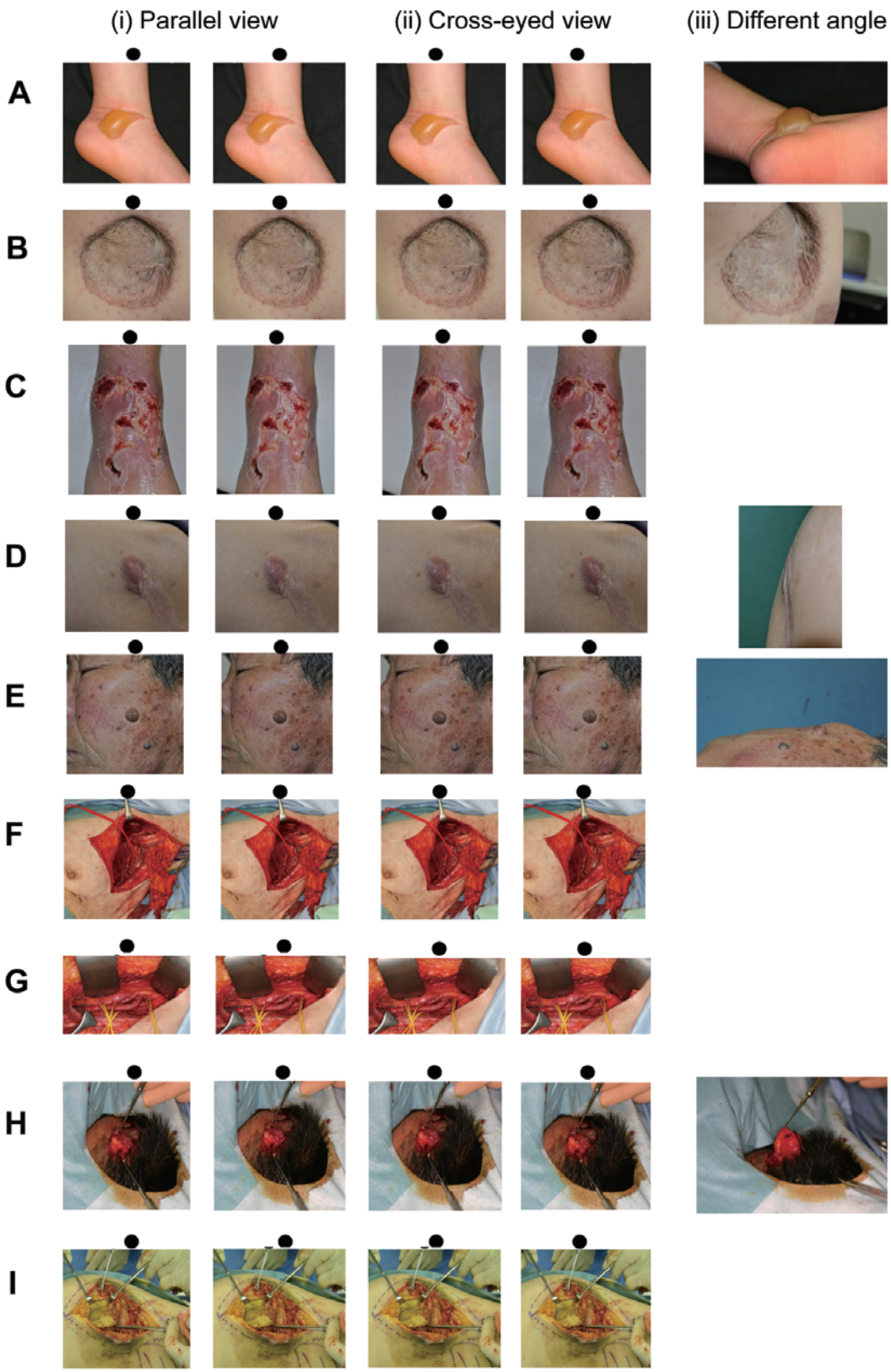

Figure 2. (i) for parallel view, (ii) for cross-eyed view, (iii) picture from different angle to show the height/depth of lesion. Black spots above the stereo pair were an aid for stereoscopy. (A) Blister of left ankle. The picture represents a raised lesion. **(B) Post-operative scar of dermatofibrosarcoma protuberans on the back.** The picture represents a depressed lesion. **(C) Leg ulcer.** Depressed lesion of skin ulcer with elevated edges caused by epithelization. **(D) Keloid on right shoulder.** The lesion has both raised and depressed areas. **(E) Nevus (left) and seborrheic keratosis (right) of the face.** The two tumors have different heights. **(F) Operative procedure of left axillary lymph node dissection.** This picture is difficult to grasp 3D structure on the 2D picture because of considerable depth in the surgical field. Thoracodorsal artery and vein were marked by red tape. **(G) Operative procedure of left inguinal lymph node dissection.** Arteries and veins were indicated by yellow tape. **(H) Operative procedure of resection of dermatitis papillaris capillitii on scalp.** The grasp of 3D structure is disturbed by the hair. **(I) Operative procedure of necrotizing fasciitis of buttock.** The area has complicated muscle structure. Yellowish tissue at the center is necrotic muscle.

Table 1. Results of the questionnaire survey

Items	Parallel view users (n = 14)	Cross-eyed view users (n = 5)	Total (n = 19)
Q1			
Figure 2A	7 (50.0%)	2 (40.0%)	9 (47.4%)
Figure 2B	1 (7.1%)	1 (20.0%)	2 (10.5%)
Figure 2C	9 (64.3%)	2 (40.0%)	11 (57.9%)
Figure 2D	5 (35.7%)	4 (80.0%)	9 (47.4%)
Figure 2E	10 (71.4%)	4 (80.0%)	14 (73.7%)
Figure 2F	1 (7.1%)	0 (0.0%)	1 (5.3%)
Figure 2G	0 (0.0%)	0 (0.0%)	0 (0.0%)
Figure 2H	4 (28.6%)	0 (0.0%)	4 (21.1%)
Figure 2I	2 (14.3%)	1 (20.0%)	3 (15.8%)
Q2			
Figure 2A	1 (7.1%)	0 (0.0%)	1 (5.3%)
Figure 2B	0 (0.0%)	0 (0.0%)	0 (0.0%)
Figure 2C	6 (42.9%)	0 (0.0%)	6 (31.6%)
Figure 2D	3 (21.4%)	1 (20.0%)	4 (21.1%)
Figure 2E	4 (28.6%)	3 (60.0%)	7 (36.8%)
Figure 2F	1 (7.1%)	0 (0.0%)	1 (5.3%)
Figure 2G	0 (0.0%)	0 (0.0%)	0 (0.0%)
Figure 2H	1 (7.1%)	0 (0.0%)	1 (5.3%)
Figure 2I	2 (14.3%)	0 (0.0%)	2 (10.5%)
Q3	13 (92.9%)	5 (100.0%)	18 (94.7%)
Q4 (No. of stereograms)	4.8 ± 1.4	5.0 ± 2.3	4.9 ± 1.6

Unless indicated, values are number of patients which selected each figure as the answer for each question.

between parallel view users and cross-eyed view users. Almost all of the 19 subjects capable of stereoscopy (18/19 = 94.7%) pointed out at least one stereogram as the answer for Q2, and also answered that 3D stereoscopy is useful (Q3). In 14 subjects who used parallel views, 7 (50.0%) did not feel visual fatigue, but the other 7 felt after 2-5 stereoscopy (Q4). In 5 subjects who used cross-eyed views, 1 felt fatigue after seeing 1 stereogram, and another did after 5 stereoscopies. The mean number of stereograms subjects could perform stereoscopy without visual fatigue was 4.8 ± 1.4 (mean ± S.D.) in parallel view users and 5.0 ± 2.3 in cross-eyed view users, and there was no statistically significant difference.

4. Discussion

Several systems have been used to bring the illusion of depth to pictures or movies. The anaglyph format, historically the earliest method used in various media, is made up of two different color layers for each eye. Glasses used consisted of two lenses with different colors (such as red and cyan), and the two layers are merged by the visual cortex of the brain to produce the stereoscopic 3D effect. Although the anaglyph format is much easier than the parallel view and cross-eyed view formats, it usually has problems in reproduction of color. The polarization format, especially circular polarization, is often used in the 3D movie system, but it needs polarized 3D glasses/headgear and a silver screen to maintain polarization. Frame sequential 3D or side by side 3D technology also needs active shutter glasses and is not likely to be suitable for figure presentation in scientific papers or poster sessions. On the other hand, most of us have to train our eyes to see the illusion by parallel viewing or cross-eyed viewing. In addition, finally, the persons with visual impairments affecting one or both eyes may not be able to perceive the sense of depth in spite of training, which is a common problem among most types of 3D technology. Moreover, these methods only can be used when the photos are printed in books, papers, or posters which are able to be seen at a near distance, but are not available for presentation from stage for a large audience. However, we regarded these methods as the most practical because of their low cost and the needlessness for any external aids for viewing including glasses/headgear. Our survey revealed 57.7% of the subjects had acquired stereoscopy and an additional 15.4% could learn parallel viewing with 10 minutes training, whereas the remaining 26.9% could not perform stereoscopy in the end. To increase the saturation level of stereoscopy in doctors/students and to consider practical uses, longer training of more than 10 minutes, establishment of training methodology, and preparation of easy and simple examples to learn stereoscopy may be needed. To note, by seeing each one of the two pictures for a stereogram, someone who cannot get a 3D image ultimately in spite of such training still can enjoy clinical photos by usual picture presentations. Thus, there may be no demerits utilizing stereograms for clinical presentations.

One of our interests was which method of parallel or cross-eyed viewing is better to be utilized for figure presentation of various lesions and surgical procedures. The percentages of subjects who had originally

acquired parallel viewing was the same as those who acquired cross-eyed viewing (38.4%), but additional subjects could learn parallel viewing, not cross-eyed viewing, easily by training, and the number of subjects who decided to use parallel views was greater than those who used cross-eyed views (53.8% *vs.* 19.2%). Furthermore, although an advantage of cross-eyed viewing is that a much larger and wider stereo pair can be merged together, virtual images by cross-eyed viewing was perceived in front of the real images but smaller than that of parallel views; our brain expects closer objects should be perceived larger. When the closer object is actually the same size as a distant one, the visual cortex decides it should be smaller. Considering that there was no significant difference in the results of the questionnaire between subjects with parallel views and cross-eyed views, taken together, parallel views may be preferable to present stereograms. Especially, based on the reasons subjects suggested, lesions with multiple undulations (Figures 2C, 2D and 2E), not simple and single raised or depressed lesions (Figures 2A or 2B), may be good applications for 3D stereoscopy.

Our questionnaire revealed that 94.7% of subjects capable of stereoscopy felt stereograms are useful. Most recently, autostereoscopic 3D television which does not need special headgear/glasses on the part of the viewer, or 3D cameras started to be commercially available. Of course, they will take a long time to be applied for academic use and may not be useful for clinical presentations in scientific papers or poster sessions. However, because 3D technology will generally and increasingly be paid attention to by such new products, parallel viewing or cross-eyed viewing may also be more acceptable and can be introduced easier than before.

Basically, stereoscopy is not thought to be harmful and does not cause visual disorders (*2-6*), and is even utilized for treatment of binocular disorders by orthoptists or vision therapists (*1*). However, as another common problem of all 3D techniques, long time 3D view may cause visual discomfort and visual fatigue; the subjects in our study felt visual fatigue after an average of 4.9 stereoscopy views. Thus, to avoid visual fatigue, the number of stereograms in one presentation should be less than 4. Furthermore, poor quality of stereograms may further such visual discomfort and fatigue. Given that the stereograms selected as the answer for Q1 and Q2 were similar, the quality of each stereogram may also affect both understanding of 3D structure and visual comfort significantly. For a successful and comfortable introduction of increased numbers of stereograms, quality of stereograms must be improved. Larger studies are needed in the future.

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Original Article

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Oral valganciclovir *versus* intravenous ganciclovir as preemptive treatment for cytomegalovirus infection after living donor liver transplantation: A randomized trial

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Summary **It is unclear whether valganciclovir (VGCV) is effective compared with intravenous ganciclovir (GCV) for preemptive therapy of cytomegalovirus (CMV) infection in living donor liver transplantation (LDLT). A randomized trial was conducted to compare the efficacy of oral VGCV with intravenous GCV for preemptive treatment of CMV infection after LDLT. Patients who developed CMV infection within 6 months after LDLT at Tokyo University Hospital were randomly assigned to the VGCV or GCV group and received either oral VGCV 900 mg/day or intravenous GCV 5 mg/kg twice daily, respectively. The primary endpoint was the treatment success rate. Secondary endpoints were recurrence of CMV infection within 1 year after finishing the treatment, and safety and tolerability of the treatment. Twenty-two patients with CMV infection after LDLT fulfilled the inclusion criteria and were randomly assigned to the oral VGCV group (*n* = 11) or the intravenous GCV group (*n* = 11). Treatment success rates were 82% (9 of 11) and 91% (10 of 11) in the VGCV and GCV groups, respectively. One patient in the VGCV group developed recurrence, whereas no patients in the GCV group developed recurrence. All the patients completed the treatment protocol, and no patients in either group dropped out of the study. In conclusion, oral VGCV and intravenous GCV are safe, feasible options for preemptive treatment of CMV infection after LDLT.**

Keywords: Cytomegalovirus, preemptive treatment, living donor liver transplantation, valganciclovir, ganciclovir

1. Introduction

Cytomegalovirus (CMV) is one of the most common infectious complications after living donor liver transplantation (LDLT). Three etiologies of CMV infection in LDLT recipients are proposed; viral transmission *via* the donor graft, viral transmission *via* transfusion from a sero-positive donor, and reactivation of dormant CMV in the recipients (*1*). Approximately 23% to 85% recipients after liver transplantation develop CMV infection, and 15% to 40% (*1-3*) of them develop CMV-related disease, such as interstitial pneumonia, hepatitis, and enteritis. CMV infection is also reported to be the cause of other infectious complications, such as acute rejection, poor survival rate, increased graft loss, increased length of hospital stay, and high cost. Therefore, the establishment of optimal strategies, including the most effective antiviral agents and the most effective administration route for preventing CMV infection or disease after LDLT is in high demand.

Two strategies are currently acceptable for the prevention of CMV-related morbidity or mortality after liver transplantation; universal prophylaxis (*4-6*) and preemptive treatment (*7-9*). It is controversial whether either strategy is superior to the other, because both strategies have limitations: universal prophylaxis is

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associated with the risk of late-onset CMV disease (*10*) and ganciclovir (GCV) resistance, and preemptive treatment requires frequent monitoring of CMV by sensitive methods such as polymerase chain reaction or CMV pp65 antigenemia.

We selected the preemptive treatment strategy to prevent CMV-related complications from 1996 to November 2006 using intravenous GCV. Although intravenous GCV has been the gold standard for treating CMV infection and disease in liver transplant recipients, it is somewhat inconvenient for patients because it requires frequent hospitalization and long-term intravenous catheter access (*11*).

Valganciclovir (VGCV), which has an oral bioavailability of 60%, was recently shown to be effective for the treatment of CMV infection in solid organ recipients (*12*). If treatment with oral VGCV as an alternative to intravenous GCV is equally effective in treating CMV infection after liver transplantation, it might benefit patients, with regard to both convenience and cost.

Although earlier studies reported the efficacy of VGCV for the treatment of CMV infection in liver transplant recipients, these studies were not randomized controlled trials. In addition, although VGCV was clinically effective and well tolerated in a multicenter trial of high-risk solid organ transplant recipients, a subgroup analysis in this trial revealed that tissue-invasive CMV disease occurred more frequently in liver transplant recipients on VGCV *versus* oral GCV (*13*). Therefore, we conducted a prospective, randomized, open-label, single center trial for a head-to-head comparison of VGCV with GCV for preemptive treatment of CMV infection after LDLT.

2. Patients and Methods

2.1. *Study design*

This study was conducted in accordance with tenets of the Declaration of Helsinki. The study compared the efficiency of VGCV with GCV for preemptive treatment of CMV infection after LDLT. Eligibility criteria included age 20 and over, LDLT recipients who were able to receive an oral drug at the onset of CMV infection, acceptable bone marrow function profile (platelet count $\geq 5 \times 10^4$/mL, hematocrit level $\geq 18\%$, and neutrophil $\geq 10^3$/mL), and adequate renal function. Exclusion criteria were history of CMV infection before LDLT, the presence of CMV disease, severe diarrhea, malabsorption state, and other postoperative complications. Patients with a history of drug allergy to GCV, VGCV, acyclovir, or valacyclovir were also excluded. The study protocol was approved by the institutional review board of Tokyo University Hospital. The protocol was explained to eligible patients, and written informed consent was obtained from all patients before enrollment.

Enrolled patients were stratified according to postoperative day at onset of CMV infection (≥ 30 *versus* < 30), preoperative model for end-stage liver diseases score (≥ 15 *versus* < 15), CMV pp65 antigen-positive cell counts/50,000 white blood cells at the diagnosis of CMV infection (≥ 10 *versus* < 10), and the presence or absence of the history of acute rejection after LDLT. Patients were randomly assigned to either the VGCV or GCV group.

This study was registered as UMIN ID: C000000295.

2.2. *Immunosuppression and CMV surveillance*

Our immunosuppressive regimens (methylprednisolone plus tacrolimus) after LDLT are described elsewhere (*14*). No CMV-specific prophylaxis was administered. After LDLT, recipients in both groups underwent surveillance for CMV infection using the CMV pp65 antigenemia assay, which was measured at the Mitsubishi Kagaku Bio-Clinical Laboratory (Mitsubishi Kagaku Bio-Clinical Laboratories, Inc., Tokyo, Japan). CMV pp65 antigenemia was routinely measured twice a week for 1 month, once a week for the next 3 months, and twice a month thereafter until 6 months after LDLT. When CMV disease was clinically suspected, the CMV pp65-antigenemia assay was checked accordingly.

2.3. *Definition of CMV infection and disease*

CMV infection was defined as positive results of the CMV pp65 antigenemia assay, which was defined as $\geq$ 5 antigen-positive cells/50,000 white blood cells. CMV disease was defined by the involvement of visceral and end-organs with the presence of compatible symptoms and signs as well as positive CMV pp65 antigenemia assay results or isolation of CMV in biopsy specimens. Deterioration of CMV infection was defined as follows: when CMV pp65 antigen-positive cell counts/50,000 white blood cells at 2 weeks after enrollment were elevated to more than 50 or more than three times, or when CMV pp65 antigenemia assay results remained positive for 3 weeks after enrollment. Recurrence of CMV infection was diagnosed when CMV pp65 antigenemia assay became negative and then was again positive.

2.4. *Preemptive treatment of CMV infection*

In the VGCV and GCV groups, patients received oral VGCV at 900 mg/day or intravenous GCV at 5.0 mg/kg every 12 hours as induction therapy for up to 1 week after CMV pp65 antigenemia turned negative, respectively. Thereafter, oral VGCV 900 mg/day was administered as maintenance treatment in both groups for an additional week. VGCV and GCV doses during the induction period were adjusted based on the

individual renal function calculated by Cockcroft-Gault creatinine clearance, as described previously (*12*).

2.5. *Outcome measures*

The primary endpoint was the treatment success of CMV infection after LDLT, which was defined in each group as a negative CMV pp65 antigenemia assay result within 2 weeks after enrollment, which was sustained for 1 month. Secondary endpoints included recurrence rate of CMV infection for 1 year after LDLT, and the safety and tolerability of the treatment.

2.6. *Statistical analysis*

All analyses were performed on an intention-to-treat basis. Statistical analysis was performed using computer software JMP 5.1 (SAS Inc., Cary, NC). Continuous data were analyzed by one-way analysis of variance and *t*-tests. *p* values of less than 0.05 and 0.01 were considered statistically significant for *t* tests and analysis of variance, respectively. The categorical data between the two groups were compared using Fisher's exact test or Mann-Whitney *U*-test. Confidence intervals (95% CIs) were calculated for differences in proportions for categorical data. Recurrence-free and overall survival curves were analyzed by the Kaplan-Meier method. Recurrence rates and survival rates were compared between the groups by the log-rank test and one-tailed and two-tailed analyses were used to analyze the primary and secondary endpoints, respectively.

3. Results

Between December 2005 and December 2008, 75 recipients underwent LDLT and were followed up for 1 year after LDLT. Of the 75 recipients, 34 (45%) developed CMV infection during the study period. Of these 34, 12 were excluded because of the presence of severe postoperative complications (n = 6) and unavailable informed consent (n = 6). Accordingly, the remaining 22 recipients were enrolled in the study. The recipients were randomly assigned to either the VGCV (n = 11) or GCV (n = 11) group for preemptive treatment of CMV infection, and were followed up for 1 year after LDLT (Figure 1).

3.1. *Baseline characteristic*

All baseline characteristics were similar in both groups (Table 1). The 22 recipients comprised 15 men and 7 women with a median age of 53 and 51 (range, 21-64) years, respectively. The median model for end-stage liver disease score was 16 (range: 7-27). The indications included virus-related cirrhosis with or without hepatocellular carcinoma (n = 14), cholestatic disease (n = 3), fulminant hepatic failure (n = 2), and cryptogenic liver cirrhosis (n = 3). Patients developed CMV infection at a mean of 31 ± 13.8 and 30 ± 5.6 days after LDLT in the VGCV and in GCV groups, respectively. CMV pp65 antigen-positive cells/50,000 white blood cells at the onset of CMV infection were 7.1 ± 4.6 in the VGCV group and 9.2 ± 6.6 in the GCV group. None of the patients in either group had a history of acute rejection at the onset of CMV infection.

3.2. *Primary and secondary endpoints*

Preemptive VGCV and GCV treatment of CMV infection after LDLT was successful in 9 of 11 (82%) patients in the VGCV group and in 10 of 11 (91%) patients in the GCV group (hazard ratio, 0.70; 95% CI, 0.15 to 2.24). Mean time until the results of the CMV pp65 antigenemia assay were negative after the initiation of treatment was 9.4 ± 4.9 days in the VGCV group and 8.2 ± 5.1 days in the GCV group (p = 0.59). CMV recurrence within 1 month after starting treatment was detected in 1 of 11 (9%) patients in the VGCV group and in 0 of 11 patients in the GCV group. The patient developed recurrence of CMV infection at 23 days after finishing the CMV treatment.

The time-course of pp65-antigenemia is depicted in Figure 2. During the first 30 days after LDLT, there was no difference in the recurrence-free survival rates between treatment groups (Figure 3; p = 0.55). As for CMV detection in the blood after completion of treatment, the median interval between discontinuation of study drug and retreatment of CMV infection was 96 ± 58 days (range: 55-137) for patients who received VGCV and 75 ± 37 days (range: 23-124) for patients who received GCV. At Day 180, clinical success was achieved in 6 of 11 VGCV-treated patients (55%) and in 9 of 11 (82%) GCV treated patients; by Day 365 clinical success was achieved in the same 55% and 82%, respectively. None of the patients in either group developed CMV disease. In both groups, the overall 1-year survival rate after LDLT was 100%. The 1- and 3-year patient survival rates with CMV infection were 96% and 96%, *versus* 95% and 95% without CMV in December 2009.

3.3. *Allograft rejection*

Acute rejection was detected in 1 of 11 patients (9%) in the VGCV group at 2 weeks after CMV infection. None of the patients in either group developed chronic rejection during the study period.

3.4. *Safety and tolerability of preemptive treatment*

None of the patients in either group dropped out of the study after initiation of the CMV treatment. Only 1 of 11 patients (9%) in the GCV group developed severe neutropenia, in whom the neutrophil counts decreased

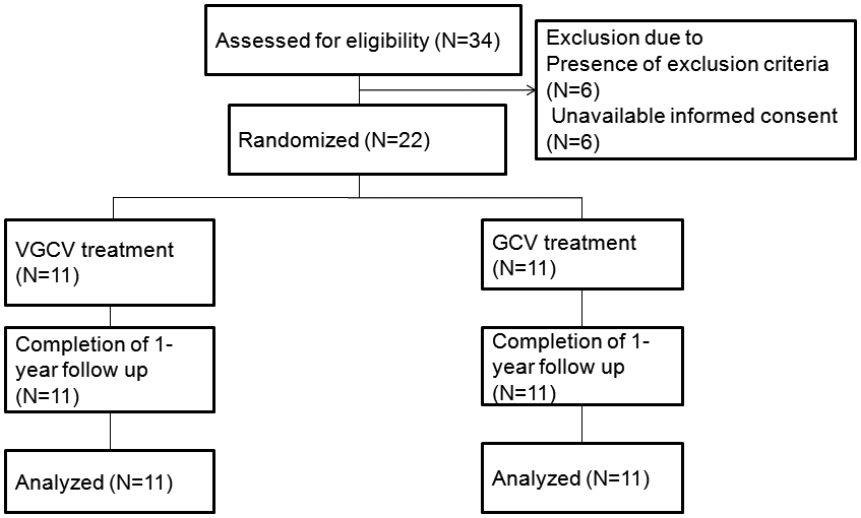

Figure 1. Study design.

Table 1. Baseline characteristics of patients in the two groups

Variable	VGCV group (n = 11)	GCV group (n = 11)	p-Value
Age (years)	51.1 ± 9.6	53 ± 11.8	0.70
Sex			
Men	6	9	0.36
Women	5	2	
MELD score	16 ± 7	17 ± 5	
≥ 15	6	7	0.67
< 15	5	4	
Primary liver disease			
Viral hepatitis	11	10	
Cryptogenic cirrhosis	2	0	
Fulminant hepatitis	0	2	
PBC	0	3	
Others	1	1	
Onset of CMV infection (days after LDLT)	30 ± 6	31 ± 14	0.83
≥ 30	5	4	
< 30	6	7	
CMV pp65 antigenemia			
≥ 10	6	6	1.00
< 10	5	5	
Acute rejection			
Present	3	2	0.10
Absent	8	9	
HLA-A,B,DR mismatch	3.5 ± 1.2	2.9 ± 1.1	0.22
Donor age	43 ± 11	39 ± 13	0.44

Some values are expressed as Mean ± Standard Error of the Means. (Abbreviations: LDLT, living donor liver transplantation; MELD, model for end-stage liver diseases; PBC, primary biliary cirrhosis; HLA, human leukocyte antigen.)

from 2.0×10^9/L to $< 0.5 \times 10^9$/L during treatment. Deterioration of renal function was observed in 1 of 11 (9%) patients in the VGCV group and in 2 of 11 (18%) patients in the GCV group. All of these adverse events were treated conservatively.

4. Discussion

To our knowledge, this study is the first randomized controlled trial comparing the effectiveness of oral VGCV with intravenous GCV for preemptive treatment of CMV infection after LDLT. Kalil *et al*. (*13*) reported that VGCV was not the preferred option as a first-line agent for CMV preemptive or universal prophylaxis in solid organ transplant recipients. In contrast, the success rate of preemptive treatment of CMV infection in our series, defined by the results of the CMV pp65 antigenemia assay, was similar in the VGCV and GCV groups (82% and 91%, respectively). In addition, none of the patients in either group developed CMV disease. This finding was acceptable compared with our previous reports, in which 5 of 75 (7%) LDLT recipients developed CMV disease during intravenous preemptive GCV treatment against CMV infection after LDLT. A

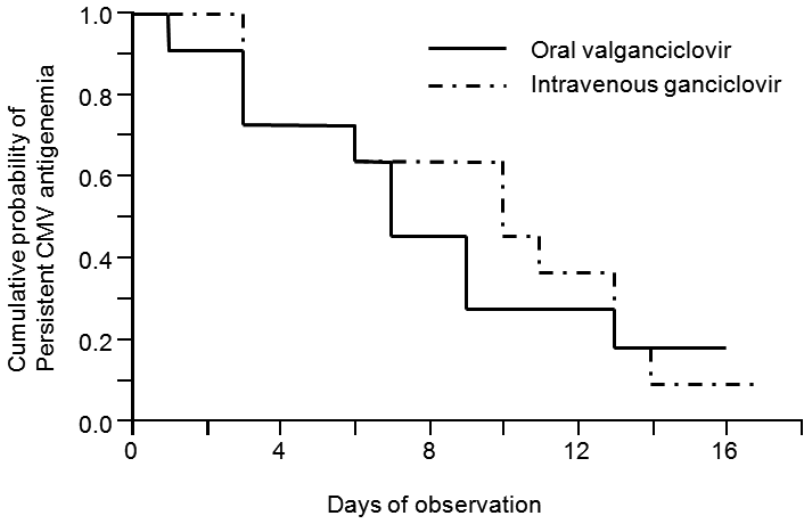

Figure 2. Kaplan-Meier curves showing cumulative probability of persistent CMV antigenemia (cutoff level, 5 copies/1 × 10^5 peripheral blood leukocytes) in patients treated with either oral valganciclovir or intravenous ganciclovir. There were no differences between groups. Straight lines denote the valganciclovir treatment arm and dotted lines represent the intravenous ganciclovir treatment arm.

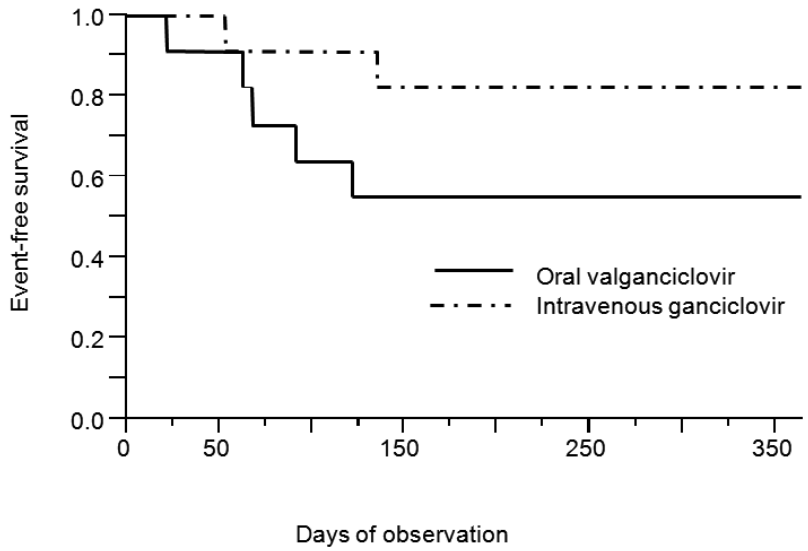

Figure 3. Kaplan-Meier estimates of event-free survival within the first year after living donor liver transplantation in both study groups. Events were defined as the recurrence of CMV antigenemia positive. There was no significant difference between patients treated with oral valganciclovir (straight line; $n = 11$) and patients treated with intravenous ganciclovir (dotted line; $n = 11$) ($p = 0.57$).

negative CMV pp65 antigenemia assay was obtained in 82% and 91% within 2 weeks, and in 91% and 100% within 1 month after the initiation of the treatment with VGCV and GCV, respectively. Although we observed a trend toward a higher proportion of recurrent CMV infection during the first year after LDLT in the VGCV group compared with GCV group (32% *versus* 18%), this difference was not statistically significant.

Both oral VGCV and intravenous GCV use for preemptive treatment of CMV infection after LDLT were well tolerated. There was no difference in the adverse event profiles between the two groups, which were comparable with those of previous studies (*13,15-17*). Patients treated with preemptive VGCV did not experience an increased incidence of adverse effects, such as leucopenia, neutropenia, and impaired renal function, compared with patients in the GCV group. Furthermore, the rate of discontinuation of the treatment due to these adverse effects in the present study was lower than that in previous reports, although the reason for this difference is unknown.

Our study has several limitations. First, the study is limited by the small sample size. To evaluate a hypothesized success rate of preemptive GCV treatment for CMV infection after LDLT of 65% with a one-tailed type I error of 5% and a statistical power of 80% would require 50 patients in each group in order to show that VGCV is not inferior compared to GCV.

Second, our preoperative examination did not include serologic CMV antibody status of either the recipients or donors; therefore we had no information on high-risk recipients with recipient-negative and donor-positive CMV serostatus. With regard to this point, however, CMV is endemic in Japan (81.7% of adults were CMV IgG positive (*18*), which suggests that almost all of the recipients in the present study were likely CMV IgG-positive (*19*). Third, this study was a not a multi-center, double-blinded study. Therefore, caution is required in applying the results of the present study to recipients with different immunosuppressive protocols or characteristics, which might lead to different outcomes. Further randomized controlled trials are necessary to establish optimal treatment strategies for CMV infection after LDLT.

In conclusion, both oral VGCV and intravenous GCV are safe, feasible options for preemptive treatment of CMV infection after LDLT.

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Case Report

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A case of Fournier's gangrene after liver transplantation: Treated by hyperbaric oxygen therapy

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Summary **Fournier's gangrene (FG) is known as a rapidly progressing necrotizing fasciitis arising from genitourinary and colorectal infections. Misdiagnoses have occurred often because the initial presentation varies and is unclear. We report a case of FG in a 59-year-old man who had undergone a living donor liver transplant. He was in the maintenance phase of immunosuppressant treatment. FG occurred rapidly without symptoms and required prompt and aggressive debridement. Computed tomography demonstrated a small air density in his left testis. Treatment with hyperbaric oxygen therapy followed by intra-operative Gram's staining navigated debridement was additionally performed with general systematic anti-biological therapy and successfully cured the patient. Extra caution should be paid to patients who are maintained on immunosuppressants. Earlier detection and intervention will reduce the rate of mortality to a minimum.**

Keywords: Fournier's gangrene, hyperbaric oxygen therapy, living-donor liver transplantation

1. Introduction

A combination regimen comprising steroids and FK506 was the standard protocol to improve graft survival in the context of this infectious disease (*1*). Fournier's gangrene (FG) is known to be a sudden-onset and rapidly progressing necrotizing fasciitis arising from genitourinary and colorectal infections. Misdiagnoses have occurred often because the initial presentation varies and is unclear (*2-4*).

2. Case report

A 59-year-old man underwent living donor liver transplantation (LDLT) because of HBV cirrhosis. He had a history of left epididymitis that had first been identified one year before LDLT and was treated with oral antibiotics. The postoperative course was uneventful without acute rejection or infection. Two months after the LDLT, the patient was re-admitted because of a continuing low-grade fever. He was in the maintenance phase of immunosuppressant treatment and was maintained with FK506 (8 mg/day) and prednisone (10 mg/day) without exceeding the recommended trough level. At admission, laboratory data revealed a slightly elevated white blood cell count of 12,000/mm^3, a C-reactive protein level of 5.2 mg/dL (normal < 0.3) and a temporary increase in his blood sugar level to 288 mg/dL.

On the 5th hospital day, the patient complained of continuous dull pain in the left lower abdomen with rebound tenderness. Thereafter, a sudden-onset systemic reaction due to sepsis occurred. The patient's blood pressure dropped to 68/46 mmHg while his body temperature rose to 39.8^{o}C. The laboratory data demonstrated severe infection with a white blood cell count of 800/mm^3, a C-reactive protein level of 12.27 mg/dL and a blood sugar level of 116 mg/dL. Computed tomography showed a small air density in his left testis (Figure 1A). A balloon-like induration with retract-pain was found in the left scrotum without redness of the skin. He was diagnosed with FG and was immediately subjected to wide debridement with broad-band antibiotics.

Two days after the operation, his laboratory data and complaints showed improvement, but micro air

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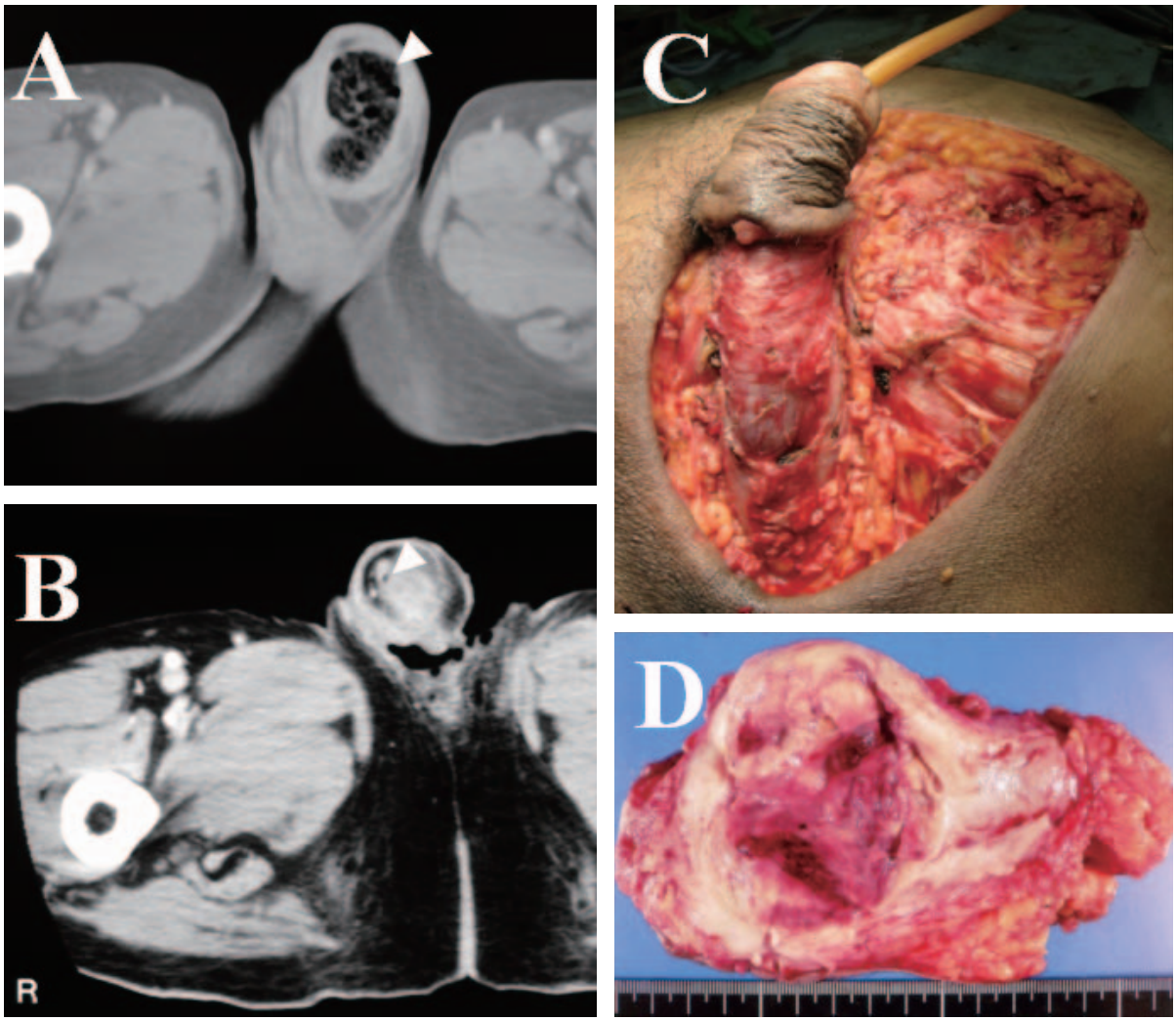

Figure 1. The left testis swelled like a balloon because of the gas-producing bacterial infection. (A) A pelvic CT scan was used to visualize the inner air in the left testis. **(B)** Micro air was found in the right testis during follow-up CT, which was performed 2 days after the operation. **(C)** The second debridement was navigated using intra-operative Gram's staining, which showed no bacterium or neutrophilic infiltration in the excised tissue. **(D)** Marked necrotizing tissue with gas-infiltration was found in the extracted testis.

was found in the remnant testis on follow-up CT (Figure 1B). We immediately performed re-debridement and excised the right testis along with perineum tissue. At first, the debridement was performed until the healthy tissue margin was macroscopically identified. Next, the margin of the resected tissue was stumped to the glass and checked for bacterial phagocytosis by neutrophilic leukocytes using Gram's staining. This process was continued until all of the bacterium and neutrophilic infiltration assays in the excised tissues yielded negative results (Figure 1C).

After the operation, marked necrotizing tissue containing gas was found in the extracted testis (Figure 1D). The bacterial organisms cultured from blood were E. coli and Streptococcus species. Additionally, hyperbaric oxygen therapy (HBO) was performed daily starting 2 days after the operation for two weeks. No recrudescence occurred during this treatment, and split-thickness skin-grafting was performed followed by HBO after one month. The patient successfully recovered within two months after the initial operation (Figure 2).

3. Discussion

Extra caution should be used for patients who are maintained by an immunosuppressant. Earlier detection and intervention for FG will reduce the rate of mortality to a minimum. In the present case, the patient's epididymitis was treated with oral antibiotics one year before LDLT. The occult infection in his left epididymis may have been one of the causes of FG. However, no sign of infection was found during the preoperative screenings of LDLT.

FG is no longer a rare disease in urologic-related transplantation patients, but it is rarely reported in liver transplantation patients. Patients who are treated with immunosuppressants should be recognized as most noteworthy (*2*,*3*). A delay in the treatment of FG has a significant impact on the prognosis. The initial

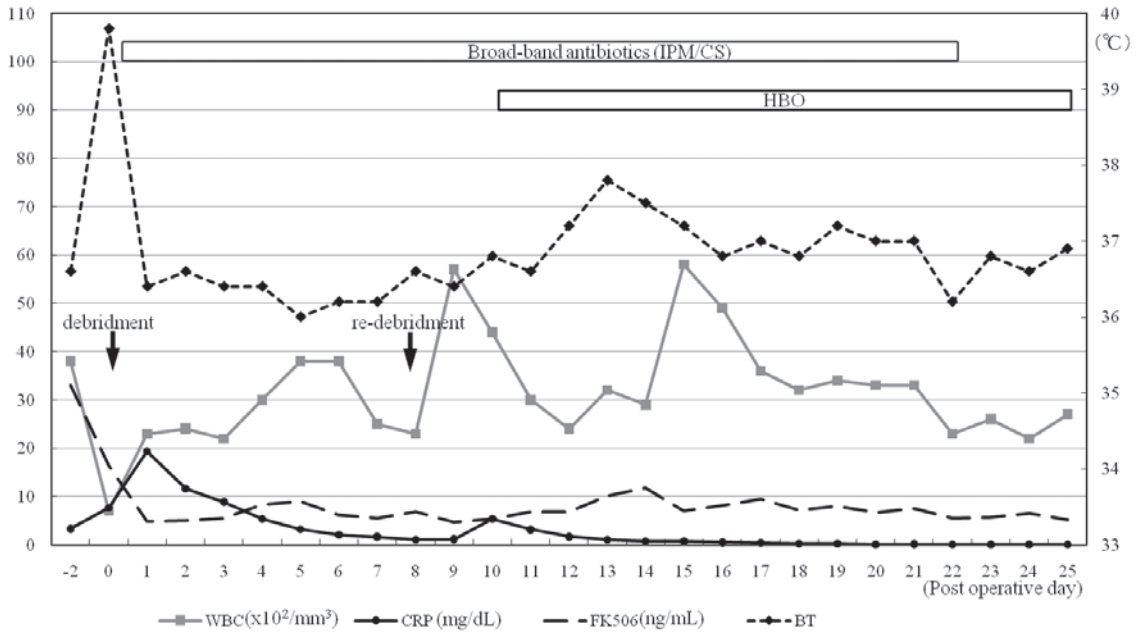

Figure 2. The treatment course after admission. The trends of laboratory data and treatments were expressed.

symptoms are little and non-specific, but local infection spreads rapidly at the rate of 1 inch per hour (*3*). The mortality rate is approximately 15-20% in non-diabetic patients, whereas it increases to twice that in diabetic patients (*2-4*).

A prompt and aggressive surgical approach with optimal antibiotic therapy is the only way to cure this disease. Computed tomography is the best modality for diagnosing FG and for monitoring the progress of infection (*1*). We did not hesitate to perform early and repeated debridement. However, the borderline of debridement was uncertain because the demarcation between the healthy tissue and the necrotizing tissue was invisible. In the present case, the debridement was guided by intra-operative Gram's staining, which continued until all of the bacterium and neutrophilic infiltration in the excised tissue was negative. This method may help determine the borderlines for debridement in future cases.

HBO in FG has a positive effect on infection control and wound healing. HBO may help decrease the number of debridements required (*4,5*) HBO increases tissue oxygen tension to a high level that in turn inhibits and kills anaerobic bacteria while suppressing aerobic bacteria proliferation (*5*). Thus, HBO may be suitable for post-LDLT patients as a supplemental protocol.

FG occurs commonly in urologic-related transplantation, whereas to our knowledge, there has been no report of a case in post-liver transplantation patients. The radiological findings and episode of this case were so unusual that we were immediately able to make the diagnosis and recommend a suitable treatment. Earlier recognition and intervention will provide opportunities to improve outcomes.

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