In conclusion, among patients infected with HCV genotype 1b with the TT genotype for rs8099917, a rapid virologic response or a $\geq$3 $\log_{10}$ reduction in HCV RNA levels at 4 weeks after starting therapy, or a complete early virologic response indicate strongly that these patients will achieve a sustained virologic response as a final outcome for PEG-IFN and ribavirin combination therapy. Early viral dynamics retain the predictive value in this patient subpopulation. A reduction in HCV RNA levels at 4 weeks after starting therapy or the type of an early virologic response does not predict the likelihood that patients with the TG/GG genotype will achieve a sustained virologic response. In contrast, the lack of an early virologic response retains a strong predictive value for the failure to achieve a sustained virologic response regardless of *IL28B* polymorphisms, which remains useful as a factor to stop therapy.

REFERENCES

Abe H, Ochi H, Maekawa T, Hayes CN, Tsuge M, Miki D, Mitsui F, Hiraga N, Imamura M, Takahashi S, Ohishi W, Arihiro K, Kubo M, Nakamura Y, Chayama K. 2010. Common variation of *IL28B* affects gamma-GTP levels and inflammation of the liver in chronically infected hepatitis C virus patients. J Hepatol 53:439–443.

Berg T, Sarrazin C, Herrmann E, Hinrichsen H, Gerlach T, Zachoval R, Wiedenmann B, Hopf U, Zeuzem S. 2003. Prediction of treatment outcome in patients with chronic hepatitis C: Significance of baseline parameters and viral dynamics during therapy. Hepatology 37:600–609.

Berg T, von Wagner M, Nasser S, Sarrazin C, Heintges T, Gerlach T, Buggisch P, Goeser T, Rasenack J, Pape GR, Schmidt WE, Kallinowski B, Klinker H, Spengler U, Martus P, Alshuth U, Zeuzem S. 2006. Extended treatment duration for hepatitis C virus type 1: Comparing 48 versus 72 weeks of peginterferon-alfa-2a plus ribavirin. Gastroenterology 130:1086–1097.

Buti M, Sanchez-Avila F, Lurie Y, Stalgis C, Valdes A, Martell M, Esteban R. 2002. Viral kinetics in genotype 1 chronic hepatitis C patients during therapy with 2 different doses of peginterferon alfa-2b plus ribavirin. Hepatology 35:930–936.

Colucci G, Ferguson J, Harkleroad C, Lee S, Romo D, Soviero S, Thompson J, Velez M, Wang A, Miyahara Y, Young S, Sarrazin C. 2007. Improved COBAS TaqMan hepatitis C virus test (version 2.0) for use with the High Pure system: Enhanced genotype inclusivity and performance characteristics in a multisite study. J Clin Microbiol 45:3595–3600.

Davis GL, Wong JB, McHutchison JG, Manns MP, Harvey J, Albrecht J. 2003. Early virologic response to treatment with peginterferon alfa-2b plus ribavirin in patients with chronic hepatitis C. Hepatology 38:645–652.

de Segadas-Soares JA, Villela-Nogueira CA, Perez RM, Nabuco LC, Brandao-Mello CE, Coelho HSM. 2009. Is the rapid virologic response a positive predictive factor of sustained virologic response in all pretreatment status genotype 1 hepatitis C patients treated with peginterferon-α2b and ribavirin? J Clin Gastroenterol 43:362–366.

Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales FL Jr, Haussinger D, Diago M, Carosi G, Dhumeaux D, Craxi A, Lin A, Hoffman J, Yu J. 2002. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 345: 975–982.

Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, Heinzen EL, Qiu P, Bertelsen AH, Muir AJ, Sulkowski M, McHutchison JG, Goldstein DB. 2009. Genetic variation in *IL28B* predicts hepatitis C treatment-induced viral clearance. Nature 461:399–401.

Ghany MG, Strader DB, Thomas DL, Seeff LB. 2009. Diagnosis, management, and treatment of hepatitis C: An update. Hepatology 49:1335–1374.

Hayes NC, Kobayashi M, Akuta N, Suzuki F, Kumada H, Abe H, Miki D, Imamura M, Ochi H, Kamatani N, Nakamura Y, Chayama K. 2011. HCV substitutions and *IL28B* polymorphisms on outcome of peg-interferon plus ribavirin combination therapy. Gut 60:261–267.

Huang C-F, Yang J-F, Huang J-F, Dai C-Y, Chiu C-F, Hou N-J, Hsieh M-Y, Lin Z-Y, Chen S-C, Hsieh M-Y, Wang L-Y, Chang W-Y, Chuang W-L, Yu M-L. 2010. Early identification of achieving a sustained virological response in chronic hepatitis C patients without a rapid virological response. J Gastroenterol Hepatol 25:758–765.

Kurosaki M, Tanaka Y, Nishida N, Sakamoto N, Enomoto N, Honda M, Sugiyama M, Matsuura K, Sugauchi F, Asahina Y, Nakagawa M, Watanabe M, Sakamoto M, Maekawa S, Sasaki A, Kaneko S, Ito K, Masaki N, Tokunaga K, Izumi N, Mizokami M. 2011. Pretreatment prediction of response to pegylated-interferon plus ribavirin for chronic hepatitis C using genetic polymorphism in *IL28B* and viral factors. J Hepatol 54:439–448.

Lee SS, Ferenci P. 2008. Optimizing outcomes in patients with hepatitis C virus genotype 1 or 4. Antiviral Ther 13:S9–S16.

Marcellin P, Rizzetto M. 2008. Response-guided therapy: Optimizing treatment now and in the future. Antiviral Ther 13:S1–S2.

Martinez-Bauer E, Crespo J, Romero-Gomez M, Moreno-Otero R, Sola R, Tesei N, Pons F, Forns X, Sanchez-Tapias JM. 2006. Development and validation of two models for early prediction of response to therapy in genotype 1 chronic hepatitis C. Hepatology 43:72–80.

Martinot-Peignoux M, Maylin S, Moucari R, Ripault M-P, Boyer N, Cardoso A-C, Giuily M, Castelnau C, Pouteau M, Stern C, Auperin A, Bedossa P, Asselah T, Marcellin P. 2009. Virological response at 4 weeks to predict outcome of hepatitis C treatment with pegylated interferon and ribavirin. Antivir Ther 14:501–511.

McCarthy JJ, Li JH, Thompson A, Suchindran S, Lao XQ, Patel K, Tillmann HL, Muir AJ, McHutchison JG. 2010. Replicated association between an *IL28B* gene variant and a sustained response to pegylated interferon and ribavirin. Gastroenterology 138:2307–2314.

Pearlman BL, Ehleben C, Saifee S. 2007. Treatment extension to 72 weeks of peginterferon and ribavirin in hepatitis C genotype 1-infected slow responders. Hepatology 46:1688–1694.

Pittaluga F, Allice T, Abate ML, Ciancio A, Cerutti F, Varetto S, Colucci G, Smedile A, Ghisetti V. 2008. Clinical evaluation of the COBAS Ampliprep/COBAS TaqMan for HCV RNA quantitation in comparison with the brancd-DNA assay. J Med Virol 80:254–260.

Poordad F, Reddy KR, Martin P. 2008. Rapid virologic response: A new milestone in the management of chronic hepatitis C. Clin Infect Dis 46:78–84.

Rauch A, Kutalik Z, Descombes P, Cai T, Di Iulio J, Mueller T, Bochud M, Battegay M, Bernasconi E, Borovicka J, Colombo S, Cerny A, Dufour JF, Furrer H, Günthard HF, Heim M, Hirschel B, Malinverni R, Moradpour D, Müllhaupt B, Witteck A, Beckmann JS, Berg T, Bergmann S, Negro F, Telenti A, Bochud PY. Swiss Hepatitis C Cohort Study; Swiss HIV Cohort Study. 2010. Genetic variation in *IL28B* is associated with chronic hepatitis C and treatment failure: A genome-wide association study. Gastroenterology 138:1338–1345.

Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML, Bassendine M, Spengler U, Dore GJ, Powell E, Riordan S, Sheridan D, Smedile A, Fragomeli V, Müller T, Bahlo M, Stewart GJ, Booth DR, George J. 2009. *IL28B* is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet 41:1100–1104.

Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, Nakagawa M, Korenaga M, Hino K, Hige S, Ito Y, Mita E, Tanaka E, Mochida S, Murawaki Y, Honda M, Sakai A, Hiasa Y, Nishiguchi S, Koike A, Sakaida I, Imamura M, Ito K, Yano K, Masaki N, Sugauchi F, Izumi N, Tokunaga K, Mizokami M. 2009. Genome-wide association of *IL28B* with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet 41:1105–1109.

The French METAVIR Cooperative Study Group. 1994. Intraobserver and interobserver variations in liver biopsy interpretation in patients with chronic hepatitis C. Hepatology 20:15–20.

Thompson AJ, Muir AJ, Sulkowski MS, Ge D, Fellay J, Shianna KV, Urban T, Afdhal NH, Jacobson IM, Esteban R, Poordad F,

Lawitz EJ, McCone J, Shiffman ML, Galler GW, Lee WM, Reindollar R, King JW, Kwo PY, Ghalib RH, Freilich B, Nyberg LM, Zeuzem S, Poynard T, Vock DM, Pieper KS, Patel K, Tillmann HL, Noviello S, Koury K, Pedicone LD, Brass CA, Albrecht JK, Goldstein DB, McHutchison JG. 2010. Interleukin-28B polymorphism improves viral kinetics and is the strongest pretreatment predictor of sustained virologic response in genotype 1 hepatitis C virus. Gastroenterology 139: 120–129.

Toyoda H, Kumada T, Kiriyama S, Tanikawa M, Hisanaga Y, Kanamori A, Tada T, Arakawa T, Fujimori M, Niinomi T, Ando N, Yasuda S, Sakai K, Kimura J. 2011. High ability to predict the treatment outcome of peginterferon and ribavirin combination therapy based on the reduction in HCV RNA levels at 4 weeks after starting therapy and amino acid substitutions in hepatitis C virus in patients infected with HCV genotype 1b J Gastroenterol 46:501–509.

Yu JW, Wang GQ, Sun LJ, Li XG, Li SC. 2007. Predictive value of rapid virological response and early virological response on sustained virological response in HCV patients treated with pegylated interferon α-2a and ribavirin. J Gastroenterol Hepatol 22:832–836.

Zeuzem S, Herrmann E, Lee JH, Fricke J, Neumann AU, Modi M, Colucci G, Roth WK. 2001. Viral kinetics in patients with chronic hepatitis C treated with standard or peginterferon alpha2a. Gastroenterology 120:1438–1447.

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SHORT REPORT

The *FOXE1* and *NKX2-1* loci are associated with susceptibility to papillary thyroid carcinoma in the Japanese population

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ABSTRACT

Background *FOXE1* and *NKX2-1* are two known genetic risk factors for the predisposition to sporadic papillary thyroid carcinoma (PTC) in Europeans, but their association in other ethnicities is still unknown.

Objective We aim to examine the association of the two genes with Japanese sporadic PTC, which exhibits high $BRAF^{V600E}$ mutation rate.

Methods 507 Japanese sporadic PTC cases and 2766 controls were genotyped for rs965513 (*FOXE1*) and rs944289 (*NKX2-1*). PTC cases were also examined for their $BRAF^{V600E}$ mutational status.

Results The association of both rs965513 ($p=1.27\times10^{-4}$, OR=1.69, 95% CI 1.29 to 2.21) and rs944289 (p=0.0121, OR=1.21, 95% CI 1.04 to 1.39) with the risk of sporadic PTC was confirmed. Subgroup analysis based on the *BRAF* mutational status showed strong association of rs965513 with $BRAF^{V600E}$-positive cases ($p=2.26\times10^{-4}$, OR=1.72, 95% CI 1.29 to 2.29), but not with $BRAF^{V600E}$-negative cases (p=0.143, OR=1.52, 95% CI 0.87 to 2.65). However, there was no difference in the observed effect size between both subgroups. For rs944289, both subgroups showed marginal association (p=0.0585, OR=1.17, 95% CI 0.99 to 1.37 for $BRAF^{V600E}$-positive cases; p=0.0492, OR=1.35, 95% CI 1.00 to 1.81 for $BRAF^{V600E}$-negative cases).

Conclusions Both *FOXE1* and *NKX2-1* were associated with the increased risk of sporadic Japanese PTC. No clear associations were observed for either SNP with $BRAF^{V600E}$ status.

INTRODUCTION

Papillary thyroid carcinoma (PTC) is the most common malignant tumour in endocrine organs, and its only well-known environmental risk factor is exposure to ionising radiation. Genetic polymorphisms have been shown to contribute to individual susceptibility to PTC.[1] The identification and further assessment of the relevant genetic variations are important for understanding the potential mechanisms involved in thyroid carcinogenesis.

Recently, two genome-wide association (GWA) studies on thyroid cancer have been achieved. The first study dealt with sporadic thyroid cancer in Icelandic population using 192 and 37 196 cases and controls, respectively, followed by a replication study in individuals of European descent.[2] The *FOXE1* (*TTF2*) gene on 9q22.33 ($p=1.7\times10^{-27}$, odds ratio (OR)=1.75, 95% CI 1.59 to 1.94 for rs965513) and *NKX2-1* (*TTF1*) on 14q13.3 ($p=2.0\times10^{-9}$, OR=1.37, 95% CI 1.24 to 1.52 for rs944289) showed the strongest association signals. Both genes encode thyroid-specific transcription factors and appear to contribute to an increased risk of both PTC and follicular thyroid carcinoma. The second GWA study focused on radiation-related PTC using 667 young patients exposed to radioiodine fallouts during childhood and 1275 age-matched control subjects residing in the radio-contaminated regions of Belarus at the time of the Chernobyl accident.[3] In this study, the *FOXE1* gene showed strong association with radiation-related PTC ($p=4.8\times10^{-12}$, OR=1.65, 95% CI 1.43 to 1.91 for rs965513), whereas no association was found with *NKX2-1* (p=0.17, OR=1.13, 95% CI 0.95 to 1.36 for rs944289). These results demonstrate that *FOXE1* is a major genetic determinant of predisposition to thyroid carcinoma regardless of aetiology and age. In contrast, *NKX2-1* may be associated only with adult sporadic PTC. Furthermore, the involvement of these genes in thyroid carcinogenesis is yet to be examined in non-European populations.

Thyroid carcinoma in the Japanese population shows distinct characteristics: higher incidence of PTC than in European populations, most PTCs are low-risk tumours with classic papillary morphology, and higher $BRAF^{V600E}$-positive rate (~80%) than in European populations (~50%).[4–8] Similar findings have also been observed in the Korean population.[9] These characteristics are presumably due to high iodine intake, since distribution of thyroid carcinoma type seems to be related to the intake of iodine: more aggressive follicular and anaplastic carcinomas in iodine-deficient areas and more papillary carcinomas in iodine-rich areas.[10 11] A recent study demonstrated significant association between the prevalence of $BRAF^{V600E}$ mutation and high iodine intake.[12] The $BRAF^{V600E}$ mutation is the most prevalent genetic alteration in adult sporadic PTC and is related to aggressive clinicopathological characteristics including extrathyroidal invasion, lymph node

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metastasis, advanced tumour stages and poorer prognosis.[8] This mutation is also observed in micropapillary carcinomas and may thus be an early event in thyroid carcinogenesis.

In this study, we aimed to examine the association of the formerly identified genetic loci, namely, *FOXE1* and *NKX2-1*, with PTC in a Japanese case-control series. In addition, we have evaluated if the two genes are associated with the presence of the $BRAF^{V600E}$ mutation in PTC.

METHODS

Subjects

A total of 509 patients with sporadic PTC (mean (SD) age 51.3 (16.0) years, range 13–87 years; 84.4% women) were recruited from Kuma Hospital (Kobe, Japan). Histological diagnosis was performed by a thyroid pathologist (MH). Two thousand seven hundred and sixty-six Japanese individuals were collected as population controls at Kyoto University. All patients and controls have no history of radiation exposure. The protocol was approved by the ethics committees of Nagasaki University, Kuma Hospital and Kyoto University.

DNA extraction and *BRAF* status screening

DNA from PTC subjects was extracted from formalin-fixed paraffin-embedded primary tumour tissues using QIAamp DNA mini kit (QIAGEN, Tokyo, Japan) according to the manufacturer's protocol. DNAs with sufficient quality for genotyping and sequencing were obtained from 507 of 509 PTC specimens. *BRAF* status was screened by direct DNA sequencing. Primer sequences used for PCR and sequencing were BRAFi14F, 5′-ACATACTTATTGACTCTAAGAGGAAAGATGAA-3′, and BRAFi15R, 5′-GATTTTTGTGAATACTGGGAACTATGA-3′. PCR products were treated with ExoSAP-IT PCR clean-up reagent (GE Healthcare Japan, Tokyo, Japan), and sequencing was performed with Big Dye Terminator sequencing kit version 3.1 (Life Technologies, Foster City, California, USA) on an ABI3100 automated sequencer (Life Technologies). We prepared five negative controls (without tissue section) per 96 samples to ensure contamination-free amplifications.

Genotyping

Genotyping was performed for PTC cases using the ABI TaqMan SNP assays (Life Technologies) in accordance with the manufacturer's guidelines. A predesigned and functionally tested probe for rs965513 (C_1593670_20) and rs944289 (C_1444137_10) were used. The conditions were denaturation at 95°C for 10 min followed by 50 cycles of 92°C for 15 s and 60°C for 1 min for both probes.

Table 1 Specification of the DNA collections recruited for the study

Sample	Number	Mean age (SD)	Age range	% Male
Cases	507	51.3 (16.0)	13–87	15.6
$BRAF^{V600E}$ (+)	388	52.4 (15.5)	17–87	16.1
$BRAF^{V600E}$ (−)	104	48.1 (17.2)	13–81	13.6
Not available	15	–	–	–
Controls	2766	50.1 (15.4)	20–79	36.9

As control subjects, genotypes of rs965513 and rs944289 were extracted from the genome scan results using Illumina Human610-Quad BeadChip of 2766 healthy Japanese individuals (mean (SD) age 50.1 (15.4) years, range 20–79 years; 63.1% women).

Statistical analysis

A case-control association in each study was examined using trend exact test to compare genotypic distributions between cases and controls.[13] A subtype analysis was performed based on the *BRAF* mutational status (*BRAF* mutant versus control, and *BRAF* wild-type versus control), and the homogeneity of the ORs between the two studies was examined with the Breslow–Day test.[14] For each case-control study, p values and ORs adjusted for age and sex were calculated using multiple logistic regression analyses. Haplotype frequency was calculated using haplo.stats R package, and linkage disequilibrium (LD) between SNPs was estimated with gap package.[15]

RESULTS

DNA samples extracted from 507 PTC tissues were genotyped by TaqMan assay for rs965513 and rs944289 (table 1). Genotyping success rates for cases were 479/507 (94.5%) and 467/507 (92.1%) for rs965513 and rs944289, respectively. As for controls, the genotyping results that could be extracted from the genome scan data were 2764/2766 (99.9%) and 2766/2766 (100%) for rs965513 and rs944289, respectively. The genotype distributions of the two SNPs conformed to Hardy–Weinberg equilibrium both in cases and in controls. A case-control association was examined using trend exact test to compare the genotypic distributions. Significant associations were obtained for both SNPs (rs965513: $p=1.27\times10^{-4}$, OR=1.69, 95% CI 1.29 to 2.21; rs944289: p=0.0121, OR=1.21, 95% CI 1.04 to 1.39) (table 2). Our results confirmed the previously reported risk alleles, namely, allele A for rs965513 and allele T for rs944289.

We next screened for *BRAF* mutation in PTC tissues by DNA sequencing. Of 507 samples, 492 (97.0%) were successfully

Table 2 Association results for rs965513 and rs944289 in Japanese PTC

SNP (ref/var)*	Study group	Genotyped samples Case†	Genotyped samples Cont	Allele frequency Case	Allele frequency Cont	HWE exact p value‡ Case	HWE exact p value‡ Cont	p Value§	OR (95% CI)¶	Power**
rs965513 (A*/G)	All cases	479	2764	0.090	0.057	0.255	0.721	1.27×10^{-4}	1.69 (1.29 to 2.21)	0.76
	$BRAF^{V600E}$ (+)	381	2764	0.092	0.057	0.113	0.721	2.26×10^{-4}	1.72 (1.29 to 2.29)	0.69
	$BRAF^{V600E}$ (−)	95	2764	0.079	0.057	1.000	0.721	0.143	1.52 (0.87 to 2.65)	0.31
rs944289 (C/T*)	All cases	467	2766	0.466	0.411	0.306	0.695	0.0121	1.21 (1.04 to 1.39)	0.60
	$BRAF^{V600E}$ (+)	373	2766	0.458	0.411	0.118	0.695	0.0585	1.17 (0.99 to 1.37)	0.52
	$BRAF^{V600E}$ (−)	93	2766	0.489	0.411	0.411	0.695	0.0492	1.35 (1.00 to 1.81)	0.19

*The reference (ref) and variant (var) alleles refer to NCBI Build 36.3, and the risk allele is indicated by an asterisk.
†*BRAF* mutational statuses were not available in three samples of 479 cases successfully typed for rs965513 and in one sample of 467 cases successfully typed for rs944289.
‡The exact p values for Hardy–Weinberg equilibrium (HWE) are shown.
§The p values using trend exact test adjusted for age and sex are shown.
¶ORs are calculated for the risk allele with a 95% CI.
**Statistical power is calculated using power function in Hmisc package (http://cran.r-project.org/web/packages/Hmisc/index.html) of R.
PTC, papillary thyroid carcinoma.

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genotyped, of which 388 were found to carry a heterozygous $BRAF^{V600E}$ mutation and 104 were negative for the mutation (table 1). Subgroup analysis based on the *BRAF* mutational status showed a strong association for rs965513 between 381 $BRAF^{V600E}$-positive cases and 2766 controls ($p=2.26\times10^{-4}$, OR=1.72, 95% CI 1.29 to 2.29) (table 2). On the other hand, no statistically significant association was found between 95 $BRAF^{V600E}$-negative cases and controls (p=0.143, OR=1.52, 95% CI 0.87 to 2.65), although there was no reversal of the risk allele, and a similar trend was observed (table 2). There was no difference in the observed effect size between the $BRAF^{V600E}$-positive and $BRAF^{V600E}$-negative groups (p=0.615).

For rs944289, both analyses showed marginal association (p=0.0585, OR=1.17, 95% CI 0.99 to 1.37 for $BRAF^{V600E}$-positive cases; p=0.0492, OR=1.35, 95% CI 1.00 to 1.81 for $BRAF^{V600E}$-negative cases) (table 2). Again, there was no reversal of the risk allele, and there was no difference in the observed effect size between the $BRAF^{V600E}$-positive and $BRAF^{V600E}$-negative groups (p=0.455).

DISCUSSION

Here, we report for the first time an association between the *FOXE1* gene and PTC in the Japanese population by genotyping rs965513 located 57 kb upstream to the *FOXE1* gene. The association of the *FOXE1* gene has been previously demonstrated by a GWA study for sporadic PTC[2] as well as for radiation-related PTC[3] in European populations. Furthermore, a recent study using an SNP panel of 97 genes related to thyroid cell differentiation and proliferation identified rs1867277, a causal SNP within the *FOXE1* 5′ UTR, functioning as a genetic risk factor associated with susceptibility to PTC.[16] The sequence containing the risk allele was demonstrated to recruit the USF1/USF2 transcription factors that in turn increased *FOXE1* transcriptional activity. Indeed, animal model experiments have shown that mice lacking the *FOXE1* locus exhibit neonatal hypothyroidism that shows similarity to thyroid dysgenesis in humans.[17] We additionally genotyped rs1867277 in 64 randomly selected cases in our series to estimate LD with rs965513. There was no strong evidence for LD between these two SNPs (D′=0.23), suggesting that the functional significance of rs965513 may be different from that of rs1867277.

The association between *NKX2-1* at chromosome 14q13.3 and sporadic PTC was also successfully reproduced in the Japanese population. However, the association was weaker for *NKX2-1* compared to *FOXE1* in our study in concordance with the results of the Icelandic study. Although rs944289 lies in a 249 kb LD region with no known genes, transcription units or predicted exons, *NKX2-1* is one of the closest genes residing in this region. NKX2-1 is another thyroid-specific transcription factor, which together with *FOXE1*, is expressed from early stages of thyroid morphogenesis and plays a major role in the development of the thyroid gland. Knockout mice lacking the *NKX2-1* gene die at birth because they lack normal thyroid and lungs, demonstrating the essential role of the gene in embryonic differentiation of these organs.[18] Interestingly, rs944289 was strongly associated with sporadic PTC in the Icelandic population[2] as well as in our Japanese series, but not in the Belarusian radiation-related PTC, suggesting that this variant may be associated only with sporadic PTC.

The strong association of rs965513 with Japanese PTC was also found between $BRAF^{V600E}$-positive cases and controls ($p=2.26\times10^{-4}$, OR=1.72, 95% CI 1.29 to 2.29) but not between $BRAF^{V600E}$-negative cases and controls (p=0.143, OR=1.52, 95% CI 0.87 to 2.65). However, the effect size is similar between the two groups (p=0.615), and the statistical power is relatively low (0.31) in the latter analysis, suggesting that the lack of significance is due to the lower minor allele frequency of the SNP in the Japanese population (0.090 in cases, 0.057 in controls) than in Europeans (0.462–0.490 in cases, 0.352–0.367 in controls)[2 3] and/or the much smaller number of $BRAF^{V600E}$-negative cases (*n*=95). On the other hand, a marginal association was observed for rs944289 in both subgroups (p=0.0585, OR=1.17, 95% CI 0.99 to 1.37 for $BRAF^{V600E}$-positive subgroup; p=0.0492, OR=1.35, 95% CI 1.00 to 1.81 for $BRAF^{V600E}$-negative subgroup). Again, the effect size is similar between the two groups (p=0.455). However, for both SNPs, the number of $BRAF^{V600E}$-negative cases needs to be increased to draw significant conclusions in the subtype analyses, especially for rs965513 for which the minor allele frequency in the Japanese population is so much lower compared to Europeans.

In conclusion, our study successfully confirms the association of both rs965513 and rs944289 with sporadic PTC in the Japanese population. Conceivably, *FOXE1* is likely to be the most important genetic determinant of susceptibility to PTC regardless of ethnicity. There was no clear difference in genetic impact for either of the SNPs with $BRAF^{V600E}$ status.

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Competing interests None.

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Contributors Conceived and designed the experiments: MM, MT, NM, FM, SY; performed experiments: MM, MT, AB; analyzed the data: MT, NM, TK, VS, RY, FM; contributed materials/analysis tools: EN, MH, TR, KS, KM, KT, AM; wrote the paper: MM, MT, NM, VS, FM.

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REFERENCES

1. **Adjadj E,** Schlumberger M, de Vathaire F. Germ-line DNA polymorphisms and susceptibility to differentiated thyroid cancer. *Lancet Oncol* 2009;**10**:181–90.
2. **Gudmundsson J,** Sulem P, Gudbjartsson DF, Jonasson JG, Sigurdsson A, Bergthorsson JT, He H, Blondal T, Geller F, Jakobsdottir M, Magnusdottir DN, Matthiasdottir S, Stacey SN, Skarphedinsson OB, Helgadottir H, Li W, Nagy R, Aguillo E, Faure E, Prats E, Saez B, Martinez M, Eyjolfsson GI, Bjornsdottir US, Holm H, Kristjansson K, Frigge ML, Kristvinsson H, Gulcher JR, Jonsson T, Rafnar T, Hjartarsson H, Mayordomo JI, de la Chapelle A, Hrafnkelsson J, Thorsteinsdottir U, Kong A, Stefansson K. Common variants on 9q22.33 and 14q13.3 predispose to thyroid cancer in European populations. *Nat Genet* 2009;**41**:460–4.
3. **Takahashi M,** Saenko VA, Rogounovitch TI, Kawaguchi T, Drozd VM, Takigawa-Imamura H, Akulevich NM, Ratanajaraya C, Mitsutake N, Takamura N, Danilova LI, Lushchik ML, Demidchik YE, Heath S, Yamada R, Lathrop M, Matsuda F, Yamashita S. The FOXE1 locus is a major genetic determinant for radiation-related thyroid carcinoma in Chernobyl. *Hum Mol Genet* 2010;**19**:2516–23.
4. **Kumagai A,** Namba H, Akanov Z, Saenko VA, Meirmanov S, Ohtsuru A, Yano H, Maeda S, Anami M, Hayashi T, Ito M, Sagandikova S, Eleubaeva Z, Mussinov D, Espenbetova M, Yamashita S. Clinical implications of pre-operative rapid BRAF analysis for papillary thyroid cancer. *Endocr J* 2007;**54**:399–405.
5. **Nakayama H,** Yoshida A, Nakamura Y, Hayashi H, Miyagi Y, Wada N, Rino Y, Masuda M, Imada T. Clinical significance of BRAF (V600E) mutation and Ki-67 labeling index in papillary thyroid carcinomas. *Anticancer Res* 2007;**27**:3645–9.
6. **Watanabe R,** Hayashi Y, Sassa M, Kikumori T, Imai T, Kiuchi T, Murata Y. Possible involvement of BRAFV600E in altered gene expression in papillary thyroid cancer. *Endocr J* 2009;**56**:407–14.
7. **Zuo H,** Nakamura Y, Yasuoka H, Zhang P, Nakamura M, Mori I, Miyauchi A, Kakudo K. Lack of association between BRAF V600E mutation and mitogen-activated protein kinase activation in papillary thyroid carcinoma. *Pathol Int* 2007;**57**:12–20.
8. **Xing M.** BRAF mutation in papillary thyroid cancer: pathogenic role, molecular bases, and clinical implications. *Endocr Rev* 2007;**28**:742–62.
9. **Kim SW,** Lee JI, Kim JW, Ki CS, Oh YL, Choi YL, Shin JH, Kim HK, Jang HW, Chung JH. BRAFV600E mutation analysis in fine-needle aspiration cytology specimens for evaluation of thyroid nodule: a large series in a BRAFV600E-prevalent population. *J Clin Endocrinol Metab* 2010;**95**:3693–700.

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10. **Feldt-Rasmussen U.** Iodine and cancer. *Thyroid* 2001;**11**:483—6.
11. **Williams ED,** Abrosimov A, Bogdanova T, Demidchik EP, Ito M, LiVolsi V, Lushnikov E, Rosai J, Tronko MD, Tsyb AF, Vowler SL, Thomas GA. Morphologic characteristics of Chernobyl-related childhood papillary thyroid carcinomas are independent of radiation exposure but vary with iodine intake. *Thyroid* 2008;**18**:847—52.
12. **Guan H,** Ji M, Bao R, Yu H, Wang Y, Hou P, Zhang Y, Shan Z, Teng W, Xing M. Association of high iodine intake with the T1799A BRAF mutation in papillary thyroid cancer. *J Clin Endocrinol Metab* 2009;**94**:1612—17.
13. **Yamada R,** Okada Y. An optimal dose-effect mode trend test for SNP genotype tables. *Genet Epidemiol* 2009;**33**:114—27.
14. **Breslow NE,** Day NE. Statistical methods in cancer research. Volume I—The analysis of case-control studies. *IARC Sci Publ* 1980;**32**:5—338.
15. **Zhao H,** Nettleton D, Dekkers JC. Evaluation of linkage disequilibrium measures between multi-allelic markers as predictors of linkage disequilibrium between single nucleotide polymorphisms. *Genet Res* 2007;**89**:1—6.
16. **Landa I,** Ruiz-Llorente S, Montero-Conde C, Inglada-Perez L, Schiavi F, Leskela S, Pita G, Milne R, Maravall J, Ramos I, Andia V, Rodriguez-Poyo P, Jara-Albarran A, Meoro A, del Peso C, Arribas L, Iglesias P, Caballero J, Serrano J, Pico A, Pomares F, Gimenez G, Lopez-Mondejar P, Castello R, Merante-Boschin I, Pelizzo MR, Mauricio D, Opocher G, Rodriguez-Antona C, Gonzalez-Neira A, Matias-Guiu X, Santisteban P, Robledo M. The variant rs1867277 in FOXE1 gene confers thyroid cancer susceptibility through the recruitment of USF1/USF2 transcription factors. *PLoS Genet* 2009;**5**:e1000637.
17. **De Felice M,** Ovitt C, Biffali E, Rodriguez-Mallon A, Arra C, Anastassiadis K, Macchia PE, Mattei MG, Mariano A, Scholer H, Macchia V, Di Lauro R. A mouse model for hereditary thyroid dysgenesis and cleft palate. *Nat Genet* 1998;**19**:395—8.
18. **Kimura S,** Hara Y, Pineau T, Fernandez-Salguero P, Fox CH, Ward JM, Gonzalez FJ. The T/ebp null mouse: thyroid-specific enhancer-binding protein is essential for the organogenesis of the thyroid, lung, ventral forebrain, and pituitary. *Genes Dev* 1996;**10**:60—9.

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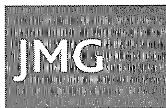

The *FOXE1* and *NKX2-1* loci are associated with susceptibility to papillary thyroid carcinoma in the Japanese population

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ORIGINAL ARTICLE

A polymorphism of the *POLG2* gene is genetically associated with the invasiveness of urinary bladder cancer in Japanese males

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Urinary bladder cancer (UBC) is a common cancer with male predominance. Pathologically it is classified into two distinct tumor entities related to the risk of patients. The low-grade tumors with relatively well-differentiated tumor histology (G1 and G2) at stage Ta are non-invasive and pose a minimal risk, whereas high-grade tumors (G2 and G3) with stages T1 to T4 are aggressive with invasion, and therefore, pose a serious risk for the patients. DNA repair and metabolic process genes may have major roles in cancer progression and development. To identify genes associated with invasiveness of UBC, we have extensively genotyped 802 single nucleotide polymorphisms in 114 genes related to DNA repair mechanisms and metabolic processes. A genetic association study was performed between non-invasive (G1 and G2 with Ta) and invasive (G2 and G3 with T1 to T4) groups of Japanese UBC patients. We found that rs17650301 in *POLG2* showed marked difference in genotype distribution between the two groups in males (P=6.93×10^{-4}), which was further confirmed in an independent sample set (overall P=1.67×10^{-4}). We also found by an *in silico* analysis that the risk allele of rs17650301 increased the transcription of *POLG2*. In conclusion, rs17650301 is a good candidate marker for UBC invasiveness in Japanese males.
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Keywords: genotyping; invasiveness; *POLG2*; single nucleotide polymorphism; urinary bladder cancer

INTRODUCTION

Urinary bladder cancer (UBC) is one of the most common cancers of the urinary system and is much more common in males worldwide, including in Japan. UBC is classified by the stage and grade of the tumor, which are highly correlated with recurrence, progression and patient survival rates. Tumor stage is determined by the degree of invasiveness and metastasis, whereas tumor grade classification is based on the degree of differentiation. Non-invasive UBCs are designated as stage Ta, whereas stages T1, T2, T3 and T4 refer to invasion into the subepithelial connective tissue, muscle, perivesical tissue and adjacent organs, respectively. With regard to tumor grades, the degrees of differentiation are based on tumor histology: well differentiated (G1), moderately differentiated (G2) and poorly differentiated (G3) cancer.[1,2] Most of the UBC cases clinically fall into one of the two distinct tumor entities at diagnosis related to the risk of patients.[3,4] The first comprises non-invasive low-grade carcinoma of relatively well-differentiated histology (G1 or G2) with stage Ta, which rarely progresses to a higher stage and pose a minimal risk for the patients. On the other hand, the second refers to aggressive tumors corresponding to stages T1 to T4 with higher grades (G2 to G3), showing high recurrence and progression rates, and therefore pose a serious risk for the patients.

Carcinogen exposure is one of the major risk factors contributing to UBC incidence.[5] Genes involved in DNA repair mechanisms, DNA replication, transcription, DNA damage signaling, cell cycle and metabolic processes influence the development and progression of UBC. A number of genetic polymorphisms in these types of genes were reported to be associated with genetic susceptibility to UBC.[6–8] However, genetic association analyses focusing on the invasiveness of UBC are yet to be performed.

In this study, we focused on 121 genes acting on the four pathways of DNA repair (base excision repair, nucleotide excision repair, double strand break repair and mismatch repair), as well as those related to DNA synthesis, cell cycle and metabolism. We designed a panel of 1536 single nucleotide polymorphism (SNP) markers from these genes for extensive genotyping study, of which 802 SNPs corresponding to 114 genes that passed quality control process were statistically analyzed for their association with UBC invasiveness in Japanese patients.

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MATERIALS AND METHODS

Study population

DNA samples were collected at the Department of Urology, Kyoto University, Kyoto, Japan. The stage and the grade of UBC were judged by the histological examinations at diagnosis. Non-invasive group (group-N) corresponds to stage Ta patients with grades G1 or G2, whereas invasive group (group-I) refers to those of stages T1 to T4 with grades G2 or G3. The characteristics and the detailed clinical diagnosis of the study populations were summarized in Table 1 and Supplementary Table 1, respectively. All the patients provided their written informed consents according to the protocols approved by the ethical review board of Kyoto University Graduate School of Medicine.

Selection of candidate genes and construction of SNP genotyping panel

Identification of SNPs was performed by resequencing exons and flanking regions of 121 genes (summarized in Supplementary Table 2), using 32 each of DNA samples of Japanese, French, Congolese and Thai populations as reported.[9] Alignment and genotyping were performed using the Genalys software (Centre National de Génotypage, Institut Génomique, Commissariat à l'Énergie Atomique (CEA), Evry, France, http://software.cng.fr/).[10] As for the selection of SNP markers, SNPs located in linkage disequilibrium (LD) blocks encompassing the 121 genes and essentially covering all the major haplotypes with frequencies greater than 5% were initially chosen from the International HapMap Project data of Japanese, Caucasians and Africans.[11] Additional SNPs identified in at least one of the four populations by the SNP discovery were included in the selection. Finally, we generated a panel of 1536 SNPs, which were chosen as tag SNP markers for genetic analyses of multiple populations using GoldenGate technology (Illumina, San Diego, CA, USA).

SNP genotyping

Genomic DNAs were prepared from peripheral blood leukocytes using Qiagen DNA Extraction kits (Qiagen, Hilden, Germany). The first screening was performed using GoldenGate assay (Illumina). The validation analysis of marker rs17650301 was performed with additional samples by sequencing. The forward primer (5′-AGGCTGGTAGGGTCCAAAGT-3′) and the reverse primer (5′-AGGGTTAGGTTGAGCATCCC-3′) were used for the PCR and an internal primer (5′-GAAGTTTTCACCGTGTTGCC-3′) was used for the sequencing with BigDye Terminator v3.1 Cycle Sequencing kit (Life Technologies, Foster City, CA, USA).

Quality control and association analysis

Among the 1536 SNPs genotyped, 20 SNPs on X-chromosome and one triallelic SNP were initially removed before quality control process. During the quality control, we first examined the presence of DNA samples showing high degrees of kinship by plink (Center for Human Genetic Research, Massachusetts General Hospital, Boston, MA, USA, http://pngu.mgh.harvard.edu/~purcell/plink/)[12] and found there were no such samples. Three DNA samples with call rates smaller than 0.90 were removed. Regarding the SNP markers, 104 SNPs with calling rates smaller than 0.95 and 609 SNPs with minor allele frequencies smaller than 0.01 were also excluded. After these steps, the remaining 802 SNP markers corresponding to 114 genes were used for statistical analyses. The association was examined by trend exact test for genotype distribution. Odds ratios and 95% confidence intervals were estimated with additive model. The heterogeneity of the effect size between the first screening and the validation analysis was evaluated by Breslow–Day test.[13] The LD structure of the *POLG2* locus was established by using Japanese data from the International HapMap Project.[11]

Table 1 Characteristics of UBC patients used for statistical analysis

Study	*Group-N*	*Group-I*
First screening		
Number	106	93
Number of males (%)	75 (70.8)	70 (75.3)
Age at diagnosis (mean ± s.d.)	68.5 ± 11.9	68.8 ± 10.5
Validation analysis		
Number	65	114
Number of males (%)	50 (76.9)	98 (86.0)
Age at diagnosis (mean ± s.d.)	67.9 ± 13.3	71.1 ± 10.0
Combined analysis		
Number	171	207
Number of males (%)	125 (73.1)	168 (81.2)
Age at diagnosis (mean ± s.d.)	68.3 ± 12.4	70.1 ± 10.3

Abbreviations: Group-I, invasive group; Group-N, non-invasive group; UBC, urinary bladder cancer.

Expression quantitative trait loci analysis

Allele-specific effect on the expression of rs17650301 was performed using Japanese data set GSE6536 of expression profile Gene Expression Omnibus (National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD, USA, http://www.ncbi.nlm.nih.gov/geo/).[14] The association *P*-values were obtained by Jonckheere-Terpstra method using R software (Department of Statistics, The University of Auckland, Auckland, New Zealand, http://www.r-project.org/) or SPSS (version 18, IBM, Armonk, NY, USA, http://www-01.ibm.com/software/data/spss/products/statistics/statistics-desktop/).

RESULTS

First screening

Among 1536 SNPs corresponding to 121 candidate genes in the genotyping panel, a total of 802 SNPs derived from 114 candidate genes were examined for their association with UBC invasiveness, using 106 group-N samples and 93 group-I samples (for details, see Materials and Methods). Although no SNPs showed significant association (P=6.23×10^{-5}) after Bonferroni's correction for multiple testing, we identified 55 SNPs with nominal *P*-values smaller than 0.05, of which nine SNPs showed *P*-values smaller than 0.01 (Table 2). The strongest *P*-value was obtained for rs17650301 (P=3.40×10^{-3}) located in intron 5 of DNA polymerase subunit γ2 gene (*POLG2*) at 17q24.1. Five SNPs in the *POLL* gene, rs3730476, rs1055364, rs3730465, rs3730472 and rs3095795, were in complete LD with each other.

We then performed a subgroup analysis by dividing the UBC patients according to their gender. In the male study, six SNPs showed *P*-values smaller than 0.01, of which the strongest association was again obtained for rs17650301 (P=6.93×10^{-4}) (Table 2). However, rs17650301 did not show association in the female study. In addition, rs2518968, which showed the second lowest *P*-value in the joint study (P=4.50×10^{-3}), showed association in the male study (P=4.81×10^{-3}), although it was much weaker than that of rs17650301.

Validation analysis

We focused on rs17650301 and performed a validation analysis by direct sequencing, using DNA samples of an independent set of UBC patients consisting of 65 group-N samples and 114 group-I samples. Although the association was not observed in the male–female joint study (P=0.432), we obtained a marginal *P*-value (P=0.0396) in the male study (Table 3). The combined analysis by pooling the genotypes of the two studies returned a *P*-value of 9.45×10^{-3} and 1.67×10^{-4} in the joint study and in the male study, respectively (Table 3). The *P*-value of heterogeneity did not show evidence of differentiation in effect size (P=0.17 for the joint study and P=0.47 for the male study). A logistic regression analysis adjusted for age and sex returned a *P*-value of 5.28×10^{-3} for the joint study.

Table 2 Results of association analysis in the first screening

Study	SNP ID	A1/A2[a]	Gene	Non-invasive (N)				Invasive (I)				P-value[b]
				A1/A1	A1/A2	A2/A2	Freq. A1	A1/A1	A1/A2	A2/A2	Freq. A1	
Male and female												
	rs17650301	A/C	*POLG2*	55	41	10	0.712	31	43	19	0.565	3.40×10^{-3}
	rs2518968	C/G	*BLM*	30	55	21	0.542	46	36	11	0.688	4.50×10^{-3}
	rs3730476	A/G	*POLL*	81	23	0	0.889	85	5	1	0.962	7.29×10^{-3}
	rs1055364	C/A	*POLL*	83	23	0	0.892	87	5	1	0.962	7.31×10^{-3}
	rs3730465	A/G	*POLL*	83	23	0	0.892	87	5	1	0.962	7.31×10^{-3}
	rs3730472	T/G	*POLL*	83	23	0	0.892	87	5	1	0.962	7.31×10^{-3}
	rs3095795	T/C	*POLL*	83	23	0	0.892	87	5	1	0.962	7.31×10^{-3}
	rs1801127	G/A	*OGG1*	98	7	1	0.958	93	0	0	1.000	7.63×10^{-3}
	rs2276332	A/C	*ADH1A*	84	22	0	0.896	86	7	0	0.962	8.95×10^{-3}
Male												
	rs17650301	A/C	*POLG2*	43	26	6	0.747	22	32	16	0.543	6.93×10^{-4}
	rs8192772	T/C	*CYP2E1*	38	30	5	0.726	53	15	1	0.877	1.79×10^{-3}
	rs2518968	C/G	*BLM*	19	41	15	0.527	36	25	9	0.693	4.81×10^{-3}
	rs2070676	G/C	*CYP2E1*	4	23	45	0.215	1	10	57	0.088	6.34×10^{-3}
	rs9634161	T/C	*RAD52*	65	10	0	0.933	46	21	1	0.831	6.84×10^{-3}
	rs4937	T/C	*POLR2C*	25	38	11	0.595	13	34	22	0.435	8.68×10^{-3}
Female												
	rs12611088	G/A	*XRCC1*	12	18	1	0.677	18	5	0	0.891	4.40×10^{-3}
	rs2023614	C/G	*XRCC1*	26	5	0	0.919	12	9	2	0.717	9.92×10^{-3}
	rs3213266	G/A	*XRCC1*	26	5	0	0.919	12	9	2	0.717	9.92×10^{-3}

Abbreviation: SNP, single nucleotide polymorphism.
SNP markers that showed statistical *P*-values smaller than 0.01 are shown.
[a]The alleles A1 and A2 represent the reference and non-reference allele, respectively, in NCBI build 36.
[b]Statistical *P*-values were calculated by trend exact test.

Table 3 Summary of the association analysis of rs17650301 with UBC invasiveness

Study	Non-invasive (N)				Invasive (I)				OR (95% CI)	P-value[a]
	AA	AC	CC	Freq. A	AA	AC	CC	Freq. A		
First screening										
Male and female	55	41	10	0.712	31	43	19	0.565	1.91 (1.26–2.89)	**3.40×10^{-3}**
Male	43	26	6	0.747	22	32	16	0.543	2.48 (1.51–4.08)	**6.93×10^{-4}**
Validation analysis										
Male and female	32	26	7	0.692	49	49	16	0.645	1.24 (0.78–1.97)	0.432
Male	28	19	3	0.750	39	45	14	0.628	1.78 (1.04–3.05)	**0.0396**
Combined analysis										
Male and female	87	67	17	0.705	80	92	35	0.609	1.53 (1.13–2.08)	**9.45×10^{-3}**
Male	71	45	9	0.748	61	77	30	0.592	2.04 (1.43–2.93)	**1.67×10^{-4}**

Abbreviations: CI, confidence intervals; OR, odds ratio; UBC, urinary bladder cancer.
Reference (A) and variant (C) alleles are based on NCBI build36.
P-values that are less than 0.05 are shown in bold characters.
[a]Statistical *P*-values were calculated by trend exact test.

Also, the *P*-value did not change ($P=1.67\times10^{-4}$) after adjusting for age in the male study.

Linkage disequilibrium block

The LD block of the DNA region encompassing rs17650301 in the Japanese population was established using genotype data of the International Hapmap Project.[11] In the LD block encompassing a 34-kb DNA between rs8068311 and rs6504238, there were no SNP markers showing strong LD ($r^2\geqslant0.8$) with rs17650301, except rs1991401 ($r^2=1$). Rs1991401 is located in the 5′ untranslated region of the *DDX5* (DEAD box polypeptide 5) gene adjacent to *POLG2* (Figure 1).

Rs17650301 and expression of *POLG2*

Although rs17650301 is located in an intronic region, it may have functional impact in the transcription of *POLG2*. We performed an expression quantitative trait loci (eQTL) analysis by examining the expression profiles of 44 lymphoblastoid cells of Japanese origin in Gene Expression Omnibus database[14] and found an increase of

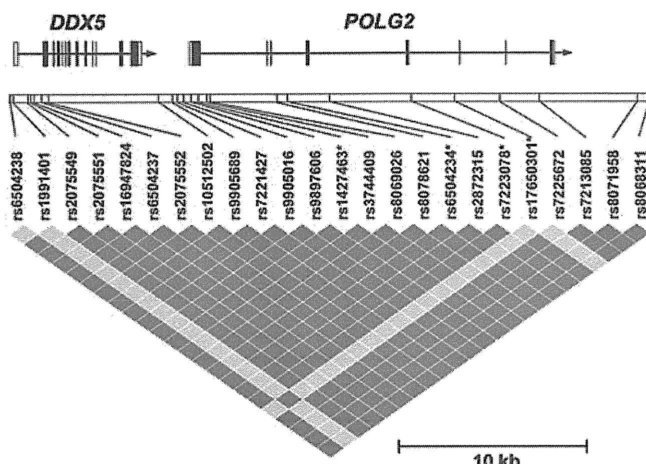

Figure 1 LD structure of the 34-kb region spanning the *POLG2* and *DDX5* genes. The structure and transcriptional polarity of *POLG2* and *DDX5* are shown according to NCBI Reference Sequence Build 36. Exons are shown by filled and open rectangles representing the coding and untranslated regions respectively. Pairwise LD estimation scores between SNPs within the region were converted into colors according to the color scheme of Haploview. The SNPs included in the genotyping panel for the first screening are indicated by an asterisk.

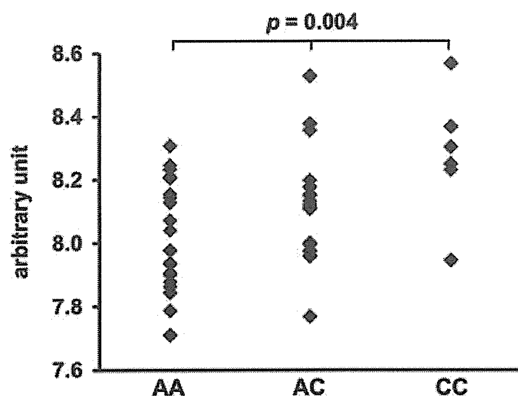

Figure 2 Allelic expression of *POLG2* with the genotypes of rs17650301. The levels of POLG2 transcription in cell lines carrying the three genotypes are indicated by a square. 'C' and 'A' represent the risk and the alternative alleles, respectively.

POLG2 transcription by the risk allele (C) of rs17650301 (P=0.004, Figure 2). However, a search for *cis*-regulatory elements using Jaspar database (The Bioinformatics Centre, Department of Biology, University of Copenhagen, Copenhagen, Denmark, http://jaspar.genereg.net/)[15] failed to identify any known motifs covering rs17650301.

DISCUSSION

In this study, we conducted a case-control association analysis of UBC invasiveness by taking a candidate gene approach of 1536 SNPs in 121 genes related to DNA repair mechanisms, DNA synthesis and metabolic processes. To our knowledge, this is the first genetic study focusing on the invasiveness of UBC. Statistical analysis was performed for 802 SNPs in 114 genes that passed quality control. The majority of the SNP markers (627 out of 734) excluded from the statistical test showed minor allele frequencies smaller than 0.01 in the Japanese population. This is due to the design of the genotyping panel for trans-ethnic study by covering SNPs identified in at least one of the four populations (Japanese, French, Thai and Congolese). Indeed, there were as many as 934 SNPs whose minor allele frequencies in the SNP discovery were smaller than 0.01 in Japanese and greater than 0.01 in at least one of the three other populations.

There was no SNP showing a significant association after Bonferroni's correction for multiple testing ($P=6.23\times10^{-5}$). The Bonferroni method is a very stringent test; although the possibilities of false positives are decreased, it comes together with the risk of losing potential candidates with true associations. In order to overcome this drawback, we took a strategy to screen SNP markers for potential associations in two stages. The most important aspect of our results is that the association of rs17650301 with UBC invasiveness was reproducibly obtained in two independent sample collections, without evidence of differentiation in effect size. However, the detection of significant association will require replication analyses using other sample collections.

The genotyping of 802 SNP markers followed by the validation analysis identified rs17650301 located in intron 5 of the *POLG2* gene as the strongest candidate for the invasiveness of UBC in Japanese male patients. *POLG2* encodes the 55-kDa accessory subunit of mitochondrial DNA polymerase. This subunit, together with the 140-kDa catalytic subunit (POLG), stimulates the polymerase and exonuclease activities in the replication process of mitochondrial DNA.[16,17] Although the association between *POLG2* polymorphisms and bladder cancer has not yet been established, its association with the risk of head and neck cancer was demonstrated in the French population.[18] There is no strong biological evidence to support the functional importance of *POLG2* to the invasiveness of UBC. However, the increase of transcription level with the risk allele of rs17650301 may be a reason for the association. Because there are no other known SNPs in strong LD ($r^2 \geqslant 0.8$) with rs17650301 in the *POLG2* locus, rs17650301 is a good candidate marker for the invasiveness of UBC in Japanese males.

The strong association of rs17650301 with the UBC invasiveness was observed only in males. As such, the reasons for the observed higher UBC incidence in males than females remain uncertain. Different non-genetic risk factors including sex hormones, life style and environment may contribute to the disease onset, resulting in the sex-specific association. For instance, involvement of androgen and its receptor in bladder cancer is demonstrated in a mouse study.[19] Epidemiologically, postmenopausal women have a higher risk of development and progression of UBC than premenopausal women.[20] Also, smoking is one of the major risks of urinary tract cancer and considered to increase the cancer risk by approximately threefold,[21,22] and the percentage of smokers is much higher in males than in females in Japan (36.8 versus 9.1%, as of 2008 in the National Survey by the Ministry of Health, Labour and Welfare of Japan). If such environmental and life style-related factors contribute to the predisposition of bladder cancer together with *POLG2*, this may explain the observed effect of *POLG2* with UBC invasiveness in males only. However, further functional characterization of *POLG2* for its involvement in carcinogenesis of indolent and aggressive tumors are required for the elucidation of the molecular mechanism underlying the prognosis of UBC.

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1 Greene, F. L., Compton, C. C., Fritz, A. G., Shah, J. & Winchester, D. P. *AJCC Cancer Staging Atlas* (Springer, New York, 2006).
2 Meiden, A.v.d. in *Invasive Bladder Cancer* (eds Bassi, P. & Pagano, F.) 67–76 (Springer, London, 2007).
3 Gofrit, O. N., Pode, D., Lazar, A., Katz, R. & Shapiro, A. Watchful waiting policy in recurrent Ta G1 bladder tumors. *Eur. Urol.* **49,** 303–306 discussion 306-307 (2006).
4 McNally, M. L., Rashid, H. H. & Messing, E. M. in *Invasive Bladder Cancer* (eds Bassi, P. & Pagano, F.) 15–38 (Springer, London, 2007).
5 Taylor, J. A., Umbach, D. M., Stephens, E., Castranio, T., Paulson, D., Robertson, C. *et al.* The role of N-acetylation polymorphisms in smoking-associated bladder cancer: evidence of a gene-gene-exposure three-way interaction. *Cancer Res.* **58,** 3603–3610 (1998).
6 Sanyal, S., Festa, F., Sakano, S., Zhang, Z., Steineck, G., Norming, U. *et al.* Polymorphisms in DNA repair and metabolic genes in bladder cancer. *Carcinogenesis* **25,** 729–734 (2004).
7 Garcia-Closas, M., Malats, N., Silverman, D., Dosemeci, M., Kogevinas, M., Hein, D. W. *et al.* NAT2 slow acetylation, GSTM1 null genotype, and risk of bladder cancer: results from the Spanish Bladder Cancer Study and meta-analyses. *Lancet* **366,** 649–659 (2005).
8 Figueroa, J. D., Malats, N., Rothman, N., Real, F. X., Silverman, D., Kogevinas, M. *et al.* Evaluation of genetic variation in the double-strand break repair pathway and bladder cancer risk. *Carcinogenesis* **28,** 1788–1793 (2007).
9 Danoy, P., Sonoda, E., Lathrop, M., Takeda, S. & Matsuda, F. A naturally occurring genetic variant of human XRCC2 (R188H) confers increased resistance to cisplatin-induced DNA damage. *Biochem. Biophys. Res. Commun.* **352,** 763–768 (2007).
10 Takahashi, M., Matsuda, F., Margetic, N. & Lathrop, M. Automated identification of single nucleotide polymorphisms from sequencing data. *J. Bioinform. Comput. Biol.* **1,** 253–265 (2003).
11 The International HapMap Consortium. A haplotype map of the human genome. *Nature* **437,** 1299–1320 (2005).
12 Purcell, S., Neale, B., Todd-Brown, K., Thomas, L., Ferreira, M. A., Bender, D. *et al.* PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am. J. Hum. Genet.* **81,** 559–575 (2007).
13 Breslowk, N. E. & Day, N. E. Statistical methods in cancer research. Volume I—The analysis of case-control studies. *IARC Sci. Publ.* 5–338 (1980).
14 Stranger, B. E., Forrest, M. S., Dunning, M., Ingle, C. E., Beazley, C., Thorne, N. *et al.* Relative impact of nucleotide and copy number variation on gene expression phenotypes. *Science* **315,** 848–853 (2007).
15 Portales-Casamar, E., Thongjuea, S., Kwon, A. T., Arenillas, D., Zhao, X., Valen, E. *et al.* JASPAR 2010: the greatly expanded open-access database of transcription factor binding profiles. *Nucleic Acids Res.* **38,** D105–D110 (2010).
16 Lim, S. E., Longley, M. J. & Copeland, W. C. The mitochondrial p55 accessory subunit of human DNA polymerase gamma enhances DNA binding, promotes processive DNA synthesis, and confers N-ethylmaleimide resistance. *J. Biol. Chem.* **274,** 38197–38203 (1999).
17 Yakubovskaya, E., Chen, Z., Carrodeguas, J. A., Kisker, C. & Bogenhagen, D. F. Functional human mitochondrial DNA polymerase gamma forms a heterotrimer. *J. Biol. Chem.* **281,** 374–382 (2006).
18 Michiels, S., Danoy, P., Dessen, P., Bera, A., Boulet, T., Bouchardy, C. *et al.* Polymorphism discovery in 62 DNA repair genes and haplotype associations with risks for lung and head and neck cancers. *Carcinogenesis* **28,** 1731–1739 (2007).
19 Miyamoto, H., Yang, Z., Chen, Y. T., Ishiguro, H., Uemura, H., Kubota, Y. *et al.* Promotion of bladder cancer development and progression by androgen receptor signals. *J. Natl. Cancer Inst.* **99,** 558–568 (2007).
20 McGrath, M., Michaud, D. S. & De Vivo, I. Hormonal and reproductive factors and the risk of bladder cancer in women. *Am. J. Epidemiol.* **163,** 236–244 (2006).
21 Zeegers, M. P., Tan, F. E., Dorant, E. & van Den Brandt, P. A. The impact of characteristics of cigarette smoking on urinary tract cancer risk: a meta-analysis of epidemiologic studies. *Cancer* **89,** 630–639 (2000).
22 Puente, D., Hartge, P., Greiser, E., Cantor, K. P., King, W. D., Gonzalez, C. A. *et al.* A pooled analysis of bladder cancer case-control studies evaluating smoking in men and women. *Cancer Causes Control* **17,** 71–79 (2006).

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Dysregulation of IFN System Can Lead to Poor Response to Pegylated Interferon and Ribavirin Therapy in Chronic Hepatitis C

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Abstract

Background: Despite being expensive, the standard combination of pegylated interferon (Peg-IFN)- α and ribavirin used to treat chronic hepatitis C (CH) results in a moderate clearance rate and a plethora of side effects. This makes it necessary to predict patient outcome so as to improve the accuracy of treatment. Although the antiviral mechanism of genetically altered IL28B is unknown, IL28B polymorphism is considered a good predictor of IFN combination treatment outcome.

Methodology: Using microarray, we quantified the expression profile of 237 IFN related genes in 87 CH liver biopsy specimens to clarify the relationship between IFN pathway and viral elimination, and to predict patients' clinical outcome. In 72 out of 87 patients we also analyzed IL28B polymorphism (rs8099917).

Principal Findings: Five IFN related-genes (IFI27, IFI 44, ISG15, MX1, and OAS1) had expression levels significantly higher in nonresponders (NR) than in normal liver (NL) and sustained virological responders (SVR); this high expression was also frequently seen in cases with the minor (TG or GG) IL28B genotype. The expression pattern of 31 IFN related-genes also differed significantly between NR and NL. We predicted drug response in NR with 86.1% accuracy by diagonal linear discriminant analysis (DLDA).

Conclusion: IFN system dysregulation before treatment was associated with poor IFN therapy response. Determining IFN related-gene expression pattern based on patients' response to combination therapy, allowed us to predict drug response with high accuracy. This method can be applied to establishing novel antiviral therapies and strategies for patients using a more individual approach.

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Introduction

Hepatitis C virus (HCV) infection affects more than 3% of the world population. Without suitable treatment, chronic hepatitis C (CH) frequently leads to the development of chronic liver diseases such as liver cirrhosis (LC) and hepatocellular carcinoma (HCC) [1]. The current standard treatment for CH is a combination of pegylated-IFN (Peg-IFN)- α and ribavirin (hereafter CH combination therapy). Over a 15-year observation period, the rate of hepatocarcinogenesis was found to be significantly lower in sustained viral responders (SVR) and relapse (R) patients than in non responders (NR) and interferon (IFN) untreated patients [2]. However, CH combination therapy achieves a sustained virological response in 50–55% of patients with HCV genotype 1b infection [3]. Consequently, this creates a pressing need to develop alternative strategies for treating CH.

IFN Type-I and III play various important immunomodulatory roles in both innate immune and acquired immune responses. Four main effector pathways of the IFN-mediated antiviral response have been recognized by gene targeting studies: the Mx GTPase pathway, the 2′, 5′-oligoadenylate-synthetase-directed ribonuclease L (OASL) pathway, the protein kinase R (PKR) pathway and the interferon stimulated gene (ISG) 15 ubiquitin-like pathway. These effector-pathways individually block viral

transcription, degrade viral RNA, inhibit translation and modify protein function to control all steps of viral replication [4–5].

IFN treatment for CH usually results in a high incidence of side effects; therefore, it is important to adjust IFN treatment accurately using a prediction method. Viral factors (HCV genotype, pretreatment viral load, and sequence of HCV gene core and NS5A), [6–7] host factors (obesity, cirrhosis, ethnic background, serum cytokine levels, liver fibrosis grades) [8], and treatment factors (adequate course of treatment, adherence to the treatment, management of side effects) [9] has been utilized in prior research to predict the outcome of combination therapy. Hepatic microRNA expression pattern before anti-viral treatment has also been utilized as a prediction biomarker of drug response in CH [10], while other studies have shown that there is a possible association between two SNPs near the gene interleukin 28B (IL28B) on chromosome 19 and lack of response to combination therapy [11–13].

In this study, we evaluated the IFN related gene expression profiles in CH patients before administering CH combination treatment. After the anti-viral therapy, patients were classified according to their clinical outcome: sustained viral response (SVR), relapse (R), and non responder (NR). It was observed that in the NR group, the expression level of some IFN related genes was significantly higher than that in normal liver (NL) groups, and that the expression level of the other IFN related genes was significantly lower than in NL. Moreover, the significantly high expression of IFN related genes was associated with low response to combination therapy. This suggests that dysregulation of the IFN system can be related to cases of CH combination therapy failure.

Results

In order to provide specific information with less data analysis, we developed a custom-made focused DNA microarray called Genopal (Mitsubishi Rayon, Tokyo, Japan) using genes that target human innate-immunity. Based on the results from the expression profiles, we carefully selected 237 gene probes (materials and methods) by activating RIG-I with Agilent DNA microarray. A microarray platform was used to establish IFN-related gene expression profiles in the specimens collected from the 87 CH and 5 NL samples (Table 1). The results of the analysis of these genes using the DNA chip strongly correlated with those obtained by real-time PCR (Pearson's correlation coefficient $R2 = 0.996$, $P<0.0001$; data not shown).

Table 1. Clinical characteristics of patients.

Characteristics	SVR (n = 38)	R (n = 26)	NR (n = 23)	NL (n = 5)
Age	56.7±10.3	61.3±8.6	60.8±7.8	57.2±9.5
Male (%)	28 (61%)	11 (39%)	9 (36%)	3(60%)
Weight (kg)	59.5±8.9	57.2±10.3	55.7±7.2	ND
HCV RNA (x10^6 copies/ml)	2.00±2.07	1.79±1.02	1.55±0.95	ND
Fibrosis stage				
F 0	1	1	1	
F 1	29	13	10	
F 2	9	7	5	
F 3	6	4	6	
F 4	0	0	1	
WBC(×10^3/mm^3)	5.42±1.63	5.23±1.25	4.69±1.13	ND
Hemoglobin (g/dl)	14.3±1.14	13.5±1.35	13.6±1.09	ND
Platelet (×10^4/mm^3)	16.7±5.3	16.6±4.0	15.0±5.7	ND
AST (IU/L)	59.2±51.0	48.7±30.1	57.4±29.7	ND
ALT (IU/L)	80.8±93.7	49.3±29.6	69.1±44.4	ND
γGTP (IU/L)	60.3±74.2	41.2±29.7	76.2±60.2	ND
ALP (IU/L)	255±74.0	246±71.3	314±144	ND
Total bilirubin (mg/dl)	0.66±0.22	0.73±0.31	0.69±0.19	ND
Albumin (g/dl)	4.20±0.34	4.14±0.25	4.02±0.48	ND

Abbreviations; NR, non-virological responder; R, relapse; SVR, sustained virological responder; AST, aspartate aminotransferase;ALT, alanine aminotransferase; WBC, white blood cell; ALP, alkaline phosphatase; γGTP, gamma-glutamyl transpeptidase; ND, not detected.
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IFN related genes associated with the final response to combination therapy

We determined unique IFN gene expression patterns for liver specimens with or without HCV based on the final virological response to the combination therapy. The expression level of 66 genes significantly differed among NR, R, SVR, and normal liver (NL) groups (Figure 1). To clearly identify the IFN-related genes associated with the clinical outcome, we extracted genes that showed significant differences ($p<0.05$). It was observed that the expression level of 5 genes (myxovirus (influenza virus) resistance 1 (MX1), 2″,5″-oligoadenylate synthetase 1 (OAS1), ISG15 ubiquitin-like modifier (ISG15), interferon, alpha-inducible protein 27 (IFI27), and interferon, alpha-inducible protein 44 (IFI44)) were significantly higher in NR than in SVR samples (Table 2). The expression levels of 3 genes (MX1, IFI27, and ISG15) were significantly higher in NR than in R samples (Table 2). We also analyzed the IFN-related genes expression pattern according to the grade of inflammation or stage of fibrosis, however, no significant differences was observed between the two (data not shown).

Comparison of IFN related genes between CH and NL

We also compared the gene expression pattern in NR and NL. After extracting genes with a fold change $<1/3$, $3<$ and p-value<0.05, we found that the expression level of 6 genes (growth arrest and DNA-damage-inducible, beta (GADD45B), hairy and enhancer of split 1 (HES1), B-cell CLL/lymphoma 3 (BCL3), signal transducer and activator of transcription 3 (STAT3), suppressor of cytokine signaling 3 (SOCS3), and DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 11 (DDX11)) was significantly lower in NR than in NL. The expression level of SOCS3 and DDX11 in NR was significantly lower than in SVR. The expression level of 25 genes were significantly higher in NR than in NL. The expression levels of most of these genes were significantly higher in NR than in SVR, but the expression level of tumor necrosis factor (ligand) superfamily, member 10 (TRAIL), major histocompatibility complex, class I, C (HLA–C), major histocompatibility complex, class I, B (HLA–B), and chemokine (C-X-C motif) ligand 10 (CXCL10 (IP10)) were similar in NR and SVR samples (Table 3).

Validation of the microarray result by real-time qPCR

The five genes (ISG15, MX1, OAS1, IFI27 and IFI44) with the largest difference in fold change between NR and SVR groups were chosen to confirm the microarray results using real-time

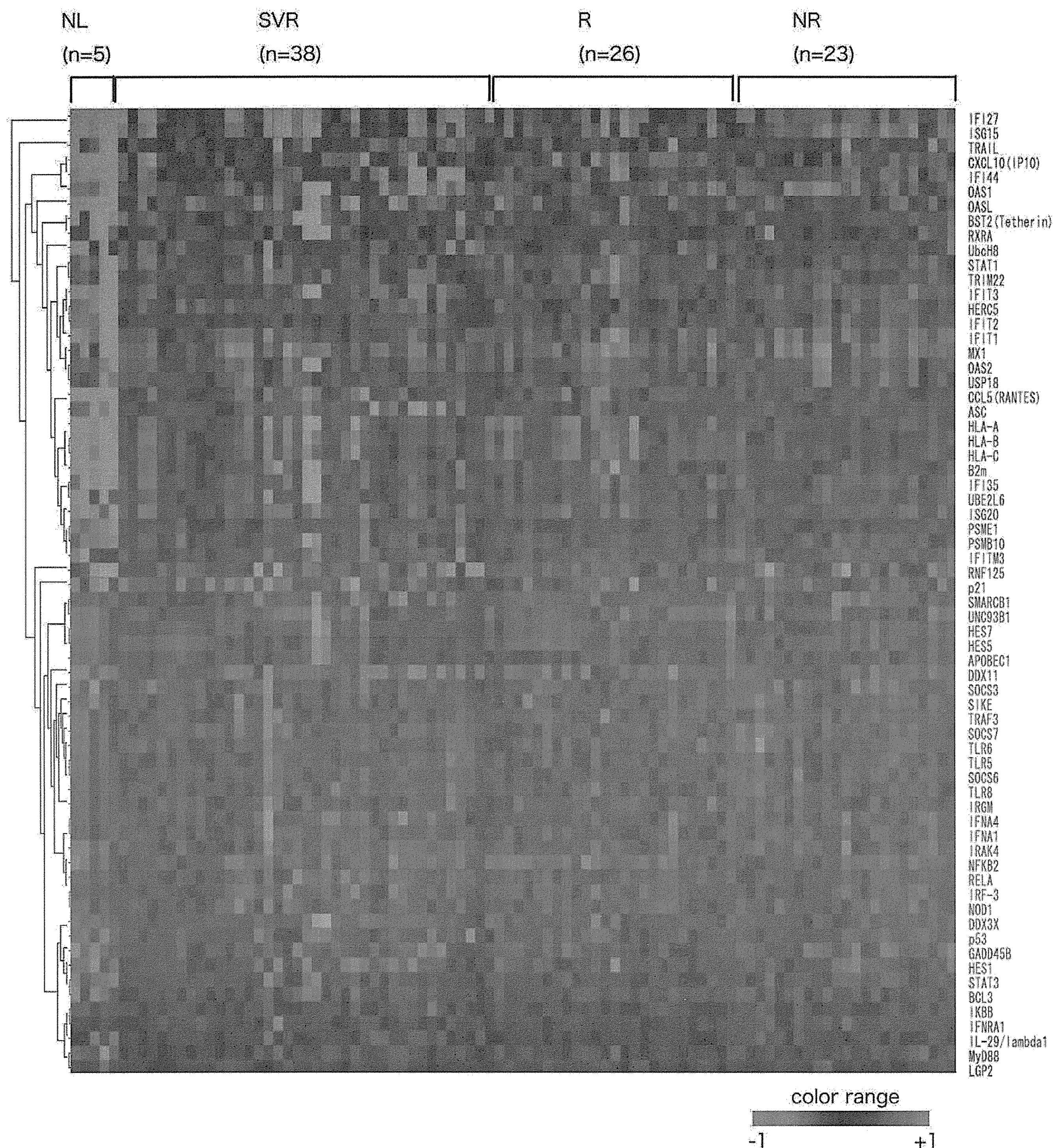

Figure 1. Clustering of IFN related gene expression. Clustering of CH patients according to the expression profiles of the 66 genes that showed significant differences among SVR, R, NR, and NL. Vertical bars represent the IFN related genes and the horizontal bars represent the samples. Green bars reflect down-regulated genes and red bars up-regulated genes.
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qPCR. The result from real-time qPCR supported the results from the microarray analysis (Figure S1).

Prediction of the clinical outcome by DLDA

We attempted to simulate the clinical outcome of the CH combination therapy using diagonal linear discriminant analysis (DLDA). Patients were randomly divided into TS (training set) and VS (validation set) (Table 4) in the order in which their samples were obtained. Samples within each group were then classified as NR or non-NR (SVR+R). DLDA showed that the accuracy, sensitivity, specificity, positive and negative predictive value of these two classifications were 86.1%, 87.5%, 81.8%, 93.3%, and

Table 2. Extracted genes related to the clinical outcome with a fold change greater than or equal to 1.5 between two groups (NR/SVR, NR/R) ($p<0.05$).

Accession No.	gene	symbol	fold change (NR/SVR)	p-value
NM_006417.4	interferon, alpha-inducible protein 44	IFI44	2.13	2.01E-03
NM_005532.3	interferon, alpha-inducible protein 27	IFI27*	2.37	2.01E-03
NM_016816.2	2',5'-oligoadenylate synthetase 1, 40/46kDa, transcript variant 1	OAS1	2.51	1.36E-02
NM_005101.2	ISG15 ubiquitin-like modifier	ISG15*	2.68	1.18E-03
NM_002462.2	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	MX1*	2.71	1.57E-03
Accession No.	gene	symbol	fold change (NR/R)	p-value
NM_002462.2	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	MX1*	2.27	1.11E-03
NM_005532.3	interferon, alpha-inducible protein 27	IFI27*	2.33	1.69E-03
NM_005101.2	ISG15 ubiquitin-like modifier	ISG15*	2.5	1.11E-03

Asterisk deposits extracted genes that are common to both SVR and NR and to NR and R.
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69.2% respectively (Table 5). Additionally, we attempted to predict (1) SVR and nonSVR (R+NR), and (2) SVR, R, and NR by DLDA. The accuracy with which patients were classified as SVR and nonSVR, was 56.8% and as SVR, R, and NR was 56.9%.

Genetic variation of IL28B is correlated with the expression of IFN related genes

To examine the relationship between the genetic variation of IL28B and IFN related gene expression, we determined the IL28B polymorphism in 72 patients (Table 6). Patients with the minor genotype of IL28B displayed higher levels of hepatic ISGs expression, whereas patients with the major genotype showed significantly lower expression levels (Figure 2A). In order to further widen our understanding of the above relationship, we significantly identified individual genetic variations in IL28B at the clinical outcome (Figure 2B). We then individually compared the expression level of several IFN-lambda related genes at the clinical outcome with the genetic variation of IL28B. The expression level of interleukin 28A (IL28A), IL28B, interleukin 29 (IL29), interleukin 10 receptor, beta (IL10RB), signal transducer and activator of transcription 1 (STAT1), STAT5A, and tyrosine kinase 2 (TYK2) in IL28B genotype minor allele and major allele did not differ; however, the expression level of STAT5A and IRF9 was significantly higher in IL28B minor allele cases than in major allele (Figure 3A). The expression levels of these nine genes did not significantly differ among the clinical outcomes (NR, R, and SVR) (Figure 3B).

Finally, in regards to genes which contribute to IFN production (interferon regulatory factor 7 (IRF7), interleukin-1 receptor-associated kinase 1 (IRAK1), myeloid differentiation primary response gene (MyD88), and toll-like receptor 7 (TLR7)) there was not much difference in their expression level prior to CH combination treatment and their expression level at the clinical outcome (Figure 4A) [14]. Unlike IRF7 and MyD88, there was no significant difference in the expression level of IRAK1 and TLR7 according to the IL28B genetic variation (Figure 4B). When we attempted to predict NR and nonNR by using ISG genes with and without IL28B polymorphism using DLDA by using 72 patients (36 patients for training set, 36 patients for validation set). DLDA with IFN related gene and IL28B polymorphism showed that the accuracy, sensitivity, specificity, positive and negative predictive value of these two classifications were 83.3%, 85.1%, 77.8%, 92.0%, 63.6%, respectively (Table 7). DLDA with IFN related gene only showed that the accuracy, sensitivity, specificity, positive and negative predictive value were 83.3%, 81.5%, 88.9%, 95.7%, 61.5%, respectively (Table 8).

Discussion

Our comprehensive analysis identified 66 genes with expression levels that consistently differed depending on the drug response of 87 CH patients and 5 normal liver specimens (Figure 1). Comparing the gene expression pattern in NR and NL showed the expression levels of 31 genes were significantly different (Table 3). In addition, most genes with expression levels in NR that were higher or lower than in NL, also differed between NR and SVR. Therefore, it is possible that innate immunity in the early period of HCV infection strongly influences IFN reaction.

HCV infection induces the impairment of cell subset number and the function of plasmacytoid dendritic cells (PDC) and natural killer cells [15]. The amount of PDC, which are the most potent producers of antiviral Type-I and III IFN [16], decreased in patients' peripheral blood [17], however, PDC was trapped in the HCV infected liver tissue. Therapeutic non-responders had increased PDC migration to inflammatory chemokines before therapy, compared with therapeutic responders [18]. This situation resulted in elevated expressions of IFN-related genes in the CH samples and was associated with their inability to eliminate the virus [19].

Inadequate expression of IFN related genes has been associated with several diseases. High expression of ISG can induce a refractory state in IFN therapy [20] and impaired IFN production leads to high risk of HCV-related hepatocarcinogenesis [21]. Lymphocyte IFN signaling was less responsive in patients with breast cancer, melanoma, and gastrointestinal cancer and these defects may represent a common cancer-associated mechanism of immune dysfunction. Alternately, since immunotherapeutic strategies require functional immune activation, such impaired IFN signaling may hinder therapeutic approaches designed to stimulate anti-tumor immunity [22]. In this way, the dysregulation of the IFN system can influence the progression of diseases and decrease curative effects.

Table 3. List of genes that had significantly different expression levels in NR and NL (fold change <1/3, 3<, and p<0.05).

symbol	NR/NL (fold change)	NR/NL (t-test)	NR/SVR (fold change)	NR/SVR (t-test)
GADD45B	0.20	1.14E-02	1.01	NS
HES1	0.26	1.26E-03	0.97	NS
BCL3	0.26	1.84E-02	1.02	NS
STAT3	0.26	5.81E-04	0.97	NS
SOCS3	0.27	7.96E-03	0.68	2.15E-02
DDX11	0.28	4.33E-05	0.59	9.52E-03
TRIM22	3.06	2.91E-03	1.37	7.97E-03
ASC	3.19	1.35E-03	1.33	4.07E-03
UBE2L6	3.32	1.06E-02	1.41	1.01E-03
STAT1	3.38	6.04E-04	1.33	1.86E-02
ISG20	3.64	2.42E-04	1.42	2.37E-03
TRAIL	3.81	2.08E-02	0.78	NS
OAS2	4.02	2.91E-03	1.89	1.07E-04
IFIT2	4.60	1.48E-03	1.56	8.34E-05
BST2(Tetherin)	5.14	8.17E-03	1.49	5.67E-04
IFI35	5.29	1.35E-03	1.63	2.37E-05
HERC5	5.32	1.16E-03	1.68	4.07E-05
MX1	6.21	1.33E-03	2.94	8.46E-07
HLA-C	6.49	6.34E-04	1.21	NS
CCL5(RANTES)	6.73	5.48E-04	1.25	3.77E-02
HLA-B	6.84	4.91E-04	1.22	NS
OAS1	7.80	5.52E-04	2.75	1.92E-04
HLA-A	8.49	5.92E-05	1.41	9.08E-04
B2m	9.09	7.78E-04	1.25	1.89E-02
IFIT1	9.42	1.86E-03	2.11	1.41E-05
OASL	10.38	3.97E-06	1.48	1.24E-02
IFIT3	10.45	4.33E-05	2.11	5.63E-06
CXCL10(IP10)	15.67	8.89E-07	1.28	NS
IFI44	17.00	9.40E-05	2.22	4.83E-06
ISG15	21.12	1.05E-04	2.85	3.99E-05
IFI27	43.74	1.80E-05	2.56	5.62E-05

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Genes which participate in IFN production (TLR7, MyD88, IRAK1, and IRF7) did not show any significant difference in their expression level prior to CH combination therapy, and their level at the clinical outcome (Figure 4A and 4B). However, the gene expression pattern of down-stream IFN pathway genes (IFI27, IFI44, ISG15, MX1, and OAS1) was significantly different among SVR, R, and NR (Table 2). IFN is usually up-regulated in HCV infected cells; however in some cases, the mechanism that controls IFN becomes abnormal, and the expression levels of IFN and ISG remain high without any curative effect [23]. The ISG family was generally up-regulated in NR compared to SVR [24–27] and this high expression of ISG related genes was associated with poor response to IFN therapy in previous, as well as in this present study. ISG15 has been linked to innate immune response to viruses and to cellular response to IFN. Although over-expression of ISG15 enhances the antiviral activity of IFN in vitro in acute infection [28], in chronic infection, extended pre-activation of IFN induced genes leads to dysregulation of the IFN system.

CH therapy is still imperfect at present and therefore suitable prediction methods are necessary to avoid adverse effects. Treatment failure using CH combination therapy is associated with up-regulation of a specific set of IFN-responsive genes thereby making it possible to predict non-response to exogenous therapy [29]. Early gene expression during anti-HCV therapy may elucidate important molecular pathways that might be influencing the probability of achieving a virological response [30]. Our study supports this fact by demonstrating that CH and NL differ fundamentally in their innate response to CH combination therapy.

IFN related gene expression suggests novel aspects of HCV pathogenesis, and form the basis for a subset of genes that can predict treatment response before initiation of combination therapy. After proper external validation, these gene sets may provide the basis for a diagnostic biomarker that can determine early on whether a patient treated with combination therapy is likely to be NR or not. In this respect, what sets our analysis apart is the effect of using DLDA to predict final response with high accuracy in NR and non-NR groups. This prediction showed that the expectation in NR (proportion of actual non-NR versus the predicted number of non-NR) was 93.3% and overall accuracy was 86.1%. In prior report, Dill et al. successfully predicted SVR, but were unable to predict R and NR with high accuracy [31]. In our experiments on the other hand, we predicted NR with high accuracy but were unable to do so for SVR and R. Possible causes for differences between our results and those received by Dill et al. may be (1) the differences in the races of subjects; European patients vs. Japanese patients in our study, (2) the composition of genotype; genotype 1 and 4 vs. genotype 1b in our study, and (3) the difference of the ISG genes extracted.

Genome-wide association studies have described alleic variants near the IL28B gene that are associated with treatment response and with spontaneous clearance of HCV [11–13]. In order to clarify the relationship between IL28B polymorphism and drug response, we compared the expression level of IFN-lambda related gene at the clinical outcome with any genetic variation in IL28B. The expression of hepatic ISG and related genes was strongly associated with treatment response and genetic variation of IL28B [32]. Classification of the patients into SVR and NR revealed that ISG expression was conditionally independent of the IL28B genotype. In CH patients in Europe, the expression pattern of genes induced by IFN more accurately predicts CH combination treatment clinical outcome than polymorphism of IL28B [31]. We observed that curative effect prediction using IFN gene expression pattern resulted in high level of accuracy, however, IFN with IL28B or IFN alone resulted in approximately similar levels of accuracy, therefore, the polymorphism of IL28B did not contribute significantly to our prediction. These findings are accordance with Dill et al. results (Table 7). There was an increased expression in NR compared to SVR irrespective of the IL28B genotype. However, there was no significant difference in their expression at the clinical outcome or in the genetic variation of IL28B (Figure 3A and 3B). Genetic variation of IL28B polymorphism is effective in predicting curative effect; however, the reason for this is not fully understood.

In conclusion, comprehensive analysis of IFN related gene showed that dysregulation of the IFN system might be related to treatment failure and that IFN related gene expression before treatment can enable accurate prediction of CH combination therapy clinical outcome. By focusing the full course of treatment on only those patients who have the highest likelihood of achieving

Table 4. Characteristics of the training and validation set.

	non NR (SVR+R) group	non NR (SVR+R) group		NR group	NR group	
	average (training set)	average (validation set)	p-value	average (training set)	average (validation set)	p-value
No.	32	32		12	11	
Age	59.3	57.1	0.38	60.6	61.7	0.74
HCVRNA ($\times10^6$ IU/ml)	1.77	2.08	0.48	1.51	1.52	0.97
AST (IU/L)	44.6	65.3	0.06	55.3	56.9	0.89
ALT (IU/L)	50	87.3	0.05	67.7	66.8	0.96
WBC($\times10^3$/mm^3)	5220	5440	0.57	4610	4860	0.6
Platelet ($\times10^4$/mm^3)	15.8	17.6	0.15	15	15.2	0.95
Total bilirubin (mg/dl)	0.71	0.69	0.78	0.68	0.68	0.92
weight	58.1	59.2	0.67	57	53.8	0.28
ALP (IU/L)	251	249	0.92	298	326	0.64
gGTP (IU/L)	48	57.4	0.54	73.3	73.8	0.98
Hemoglobin (g/dl)	13.9	14.1	0.53	13.7	13.5	0.78
Albumin (g/dl)	4.15	4.21	0.41	4.11	3.98	0.52

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SVR, clinicians could potentially reduce the side effects and costs associated with these regimens and provide a more personalized approach to treating CH patients.

Materials and Methods

Patients and sample preparation

Eighty seven CH patients with HCV genotype 1b in the Department of Gastroenterology at the Ogaki Municipal Hospital were enrolled between 2004 and 2006 (Table 1). Patients with autoimmune hepatitis, alcohol-induced liver injury, and patients positive for hepatitis B virus associated antigen/antibody or anti-human immunodeficiency virus antibody were excluded. None of the patients had received IFN therapy or immunomodulatory therapy prior to enrollment. Five normal liver specimens were obtained by surgical resection. Three of these were obtained from Osaka City University Hospital and were taken from gall bladder cancer, cholangiocarcinoma, and hemangioma patients whose liver tissue were normal based on histological, virological and blood examination of their liver function. The remaining two normal liver samples were obtained from the Liver Transplantation Unit of Kyoto University Hospital.

Patients' serum HCV RNA was quantified before IFN treatment using Amplicor-HCV Monitor Assay (Roche Molecular Diagnostics Co., Tokyo, Japan). Histological grading and staging of liver biopsy specimens from the CH patients were performed according to the Metavir classification system. Pretreatment blood samples were analyzed to determine the level of aspartate aminotransferase, alanine aminotransferase (ALT), total bilirubin, alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (γGTP), white blood cell (WBC), platelets, and hemoglobin. Written informed consent was obtained from all patients or their guardians and provided to the Ethics Committee of the Graduate School of Kyoto University, Osaka City University and Ogaki Municipal Hospital, who approved this study in accordance with the Helsinki Declaration.

Table 5. Quality of NR-prediction by DLDA.

		Predicted		
		NR	nonNR(SVR+R)	Total
Diagnosed	NR	9	2	11
	nonNR(SVR+R)	4	28	32
	Total	13	30	43

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Treatment protocol

For all enrolled patients, treatment with PegIFN- α-2b (Schering-Plough Corporation, Kenilworth, NJ, USA) and ribavirin (Schering-Plough) was initiated at the beginning of the 1st week and lasted for 48 weeks. PegIFN was administrated at a dose of 1.5 μg kg/week and ribavirin was administrated at the dose recommended by the manufacturer.

Definition of drug response to therapy

The patients were classified into the following three groups at the completion of follow-up period (24 weeks): (1) sustained virological responder (SVR): a patient who was negative for serum HCV RNA during the 24 weeks following the completion of the

Table 6. Result of the IL28B polymorphism (rs8099917).

		rs8099917		
		TT	TG	GG
outcome	NR	7	12	1
	Relapse	18	3	0
	SVR	30	1	0
	Total	55	16	1

doi:10.1371/journal.pone.0019799.t006

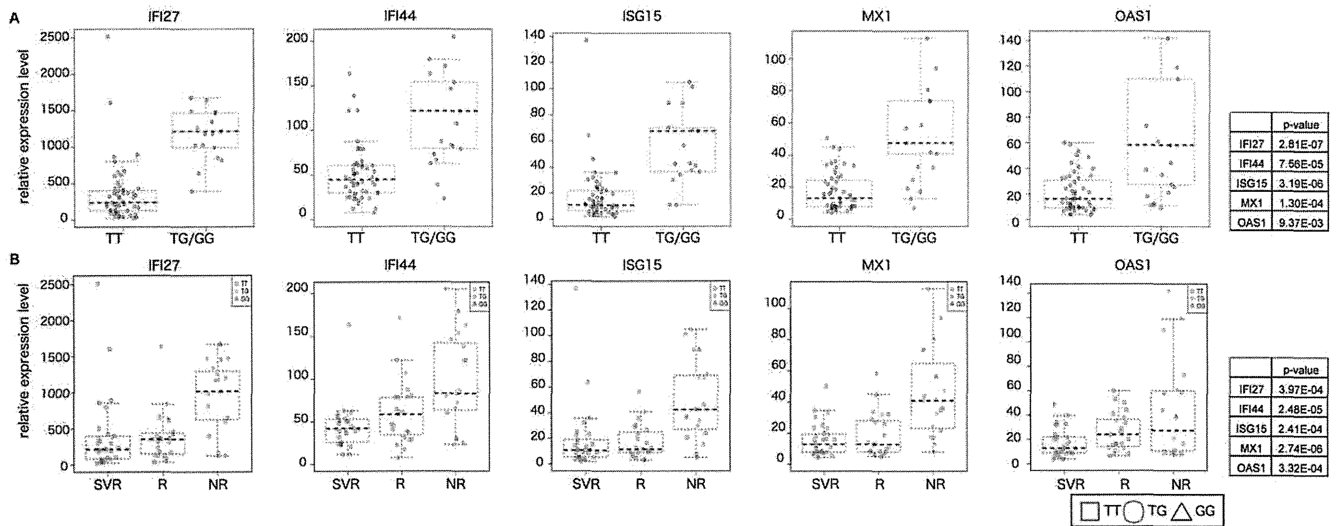

Figure 2. The relationship among the expression of IFN-related genes, IL28B polymorphism and clinical outcome. (A) The relationship between expression of ISG and five related genes (MX1, OAS1, ISG15, IFI27, and IFI44) in the liver of CH patients and IL28B with the major (TT) or minor (TG or GG) genotype (rs8099917) is shown. The p-value of the relationship between gene expression level and IL28B genotype is also depicted. (B) The relationship among the expression level of the above five genes, clinical outcome, and IL28 genotype in individual cases. Red square, green circle, and blue rectangle represent TT, TG, and GG in IL28B genotype, respectively. The p value was calculated from a linear regression employing outcome as an explanatory variable (in which SVR, R and NR are encoded to 0, 1 and 2 respectively) and expression level as the response variable. We tested the null hypothesis that the coefficient of the outcome is 0. Summary table of the p-value is also shown. NS shows no significant difference.
doi:10.1371/journal.pone.0019799.g002

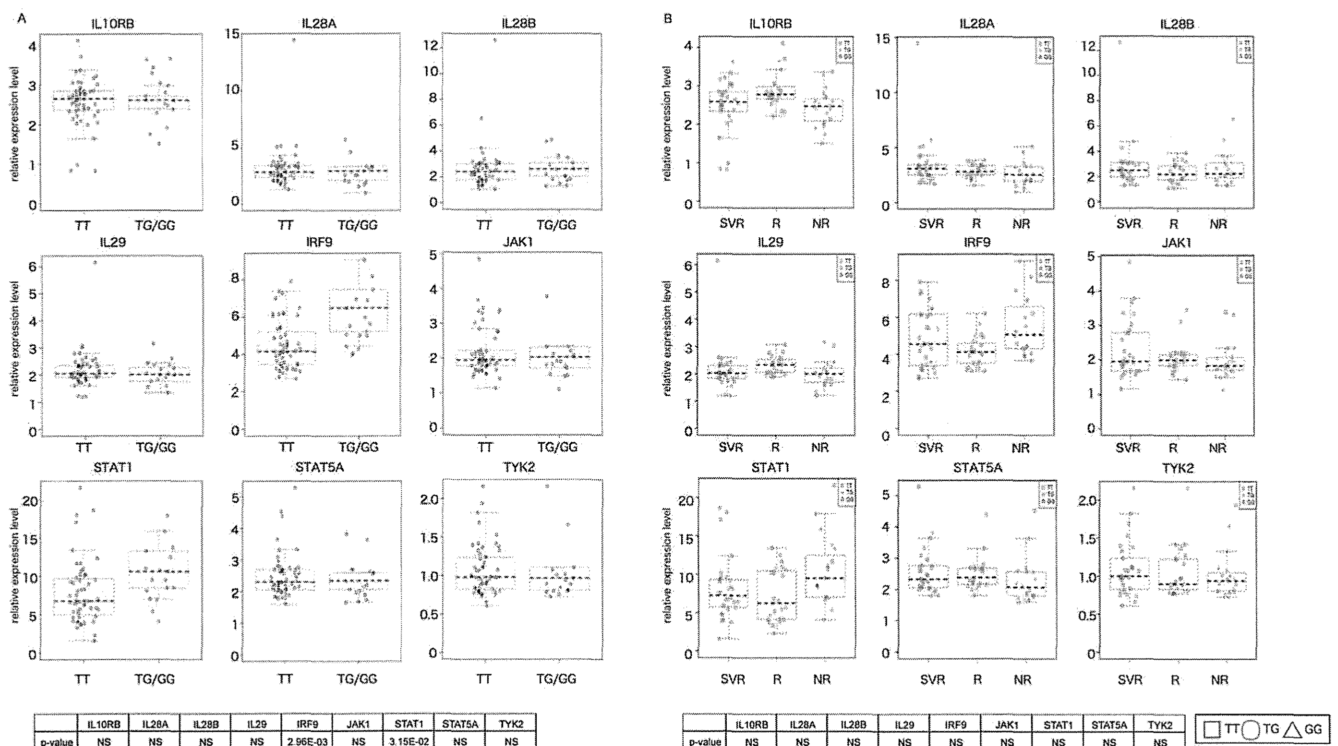

Figure 3. The relationship among the expression of IFN lambda-related genes, IL28B polymorphism and clinical outcome. (A) The relationship between the expression level of IFN lambda related genes (TYK2, STAT5A, STAT1, IL10RB, IL29, IL28A, IL28B, JAK1, and IRF9) in the liver of CH patients and IL28B with genotype. The p-value of the relationship between gene expression level and IL28B genotype is also presented. (B) The relationship among IFN lambda related genes, clinical outcome, and IL28 genotype in individual cases. Summary table of the p-value is also shown. NS was not significantly different.
doi:10.1371/journal.pone.0019799.g003

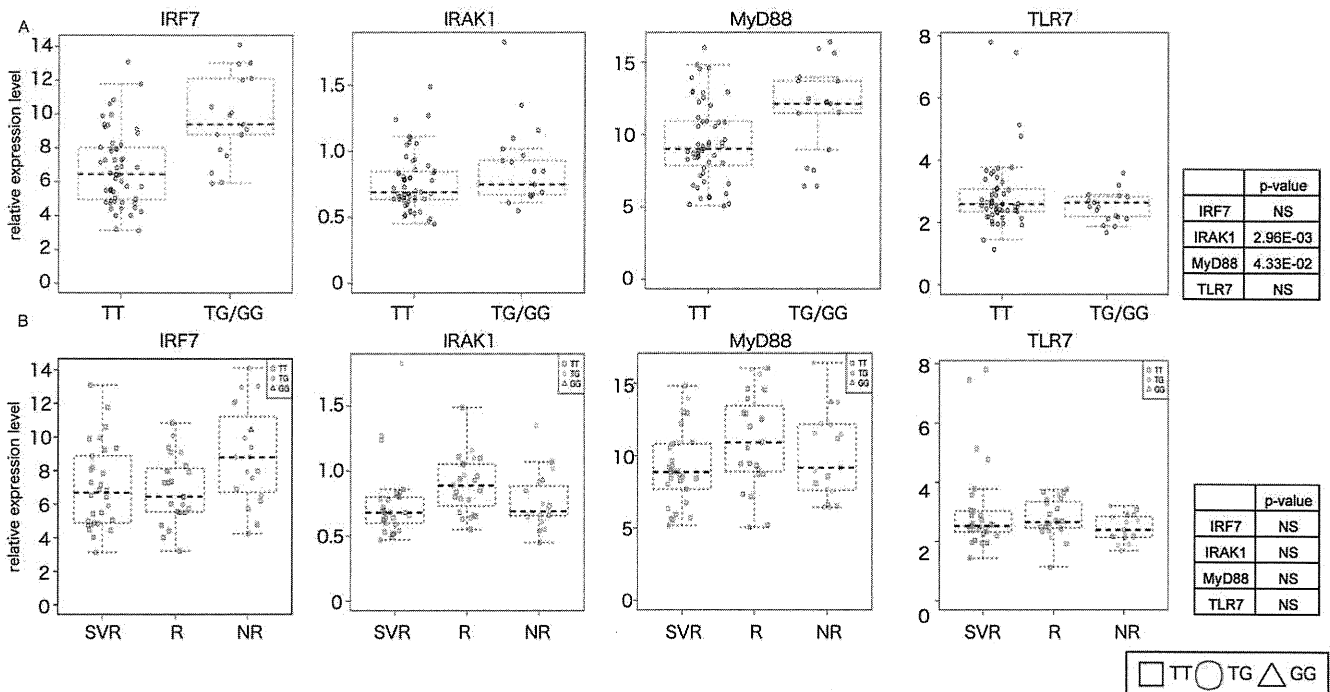

Figure 4. The relationship between the expression level of genes which participate in IFN production (TLR7, MyD88, IRAK1, and IRF7) in the liver of CH patients and IL28B genotype. (A) The relationship between IFN early response genes and clinical outcome is shown. A summary table of the p-value is also presented. NS shows no significant difference. (B) The relationship between IFN early response genes and IL28 genotype is shown. The p-value is also presented.
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combination therapy; (2) relapse (R): a patient whose serum HCV RNA was negative by the end of the combination therapy but reappeared during the 24 week observation period; and (3) non responder (NR): a patient who was positive for serum HCV RNA during the entire course of the combination therapy (Figure 5). No patients were withdrawn from the study due to side effects or any other reason.

RNA preparation and real-time qPCR

Total RNA from tissue samples was prepared using a mirVana miRNA extraction Kit (Ambion, Austin, TX, USA) according to the manufacturer's instruction. cDNA was synthesized by Transcriptor High Fidelity cDNA synthesis Kit (Roche, Basel, Switzerland). Total RNA (2 μg) in 11 μl of nuclease free water was added to 1 μl of 50 μM random hexamer and denatured for 10 min at 65°C. The denatured RNA mixture was added to 4 μl of 5x reverse transcriptase buffer, 2 μl of 10 mM dNTP, 0.5 μl of 40 U/ml RNase inhibitor, and 0.5 μl of reverse transcriptase (FastStart Universal SYBR Green Master (Roche) in a total volume of 20 μl. cDNA synthesis was performed for 30 min at 50°C, and enzyme denaturation for 5 min at 85°C. Chromo 4 detector (Bio-Rad, Hercules, CA, USA) was used to detect mRNA expression. Assays were performed in triplicate, and the expression levels of target genes were normalized to that of the ß-actin gene, as quantified using real-time qPCR as internal controls. Nucleotide sequences of primers were as follows: IFI27 (sense) 5′-ctaggccacggaattaaccc-3′, IFI27 (anti-sense) 5′-gactgcagagtagccacaag-3′, IFI44 (sense) 5′-gcatgtaacgcatcaggctt-3′, IFI44 (anti-sense) 5′-ccacaccagcgtttaccaac-3′, ISG15 (sense) 5′-ctttgccagtacaggagctt-3′, ISG15 (anti-sense) 5′-gcccttgttattcctcacca-3′, MX1 (sense) 5′-aatcagcctgctgacattgg-3′, MX1 (anti-sense) 5′-gtgatgagctcgctggtaag-3′, OAS1 (sense) 5′-gtgcgctcagcttcgtactg-3′, OAS1 (anti-sense) 5′-actaggcggatgaggctctt-3′, and β-actin (sense) 5′-ccactggcatcgtgatggac-3′, β-actin (anti-sense) 5′-tcattgccaatggtgatgacct-3′.

Table 7. Quality of NR-prediction by DLDA with IFN related gene and IL28B polymorphism A.IFN+IL28B.

		Predicted		
		NR	**nonNR**	**Total**
Diagnosed	NR	7	2	9
	nonNR	4	23	27
	Total	11	25	36

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Table 8. Quality of NR-prediction by DLDA with IFN related gene only.

		Predicted		
		NR	**nonNR**	**Total**
Diagnosed	NR	8	1	9
	nonNR	5	22	27
	Total	13	23	36

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