daclatasvir-treatment naïve genotype 1b patients was comparable to a previous study which involved direct sequencing, when a cut-off value was introduced to our deep sequencing data, although the prevalence of NS5A mutants changed depending on the cut-off value. However, deep sequencing analysis revealed that NS5A L31M/V/F and Y93H mutations were detectable in 13/110 (11.8%) and in 34/110 (30.9%) patients, respectively, above the background error rate of 0.1% and significantly more frequently than detected by direct sequencing. These results demonstrate that deep sequencing is useful for the detection of viral mutants present as minor variants.

Do HCV populations with Y93H present as minor variants have any association with clinical characteristics? Interestingly, univariate analysis based on the relationship between the presence of theY93H variant and clinical factors or factors determining treatment efficacy to PEG-IFN/RBV combination therapy extracted three significant factors: the IL28B SNP, core aa 70 and the IRRDR (Table 4). All these factors were associated with a favorable response to PEG-IFN/RBV combination therapy in the group with the Y93H resistance mutation [26]. Despite that the difference did not reach statistical significance, the number of substitutions in the ISDR also tended to be higher in the group with the Y93H mutation, similar to the IRRDR. It was quite intriguing that multivariate analysis of the presence of Y93H extracted the IL28B major-type, the SNP was significantly associated with favorable IFN responses, as an independent factor (Table 4). On the other hand, because it is known that the IL28B SNP is closely linked with core aa 70, it is assumed that core 70R should be observed more frequently in the group with Y93H [16].

Then, do NS5A resistant variants with Y93H that are present prior to treatment affect the response to daclatasvir treatment? At present, in genotype-1b infection,

daclatasvir is scheduled to be used in combination with other DAAs but not with IFN. Considering the correlation between IL28B SNPs and Y93H, and the fact that Y93H variants might be sensitive to IFN but resistant to daclatasvir [27], patients with the IL28B major-type might be recommended to receive IFN-based therapy rather than DAA regimens including daclatasvir, because those patients have a greater chance of being infected with daclatasvir-resistant Y93H variants leading to treatment failure. In contrast, the IL28B minor-type patients who have poor responses to IFN might be more promising candidates.

The true clinical influence of Y93H on treatment responses remain unknown and further elucidation is mandatory after the approval of daclatasvir for clinical use. In particular, it is important to clarify the cut off values as to the mixture ratio of Y93H to Y93 wild type in establishing clinical resistance, if the presence of viruses with Y93H before treatment really does affect the response. If so, it is also important to clarify whether the proportion of Y93H variants changes during the clinical course (the natural course or during therapy including IFN) in order to determine the most appropriate timing for introducing daclatasvir. However, it is possible for Y93H variants to disappear after IFN treatment considering that Y93H variants might be sensitive to IFN. The mechanism of the relationship between the IL28B SNP and Y93H also is not clear at present. Considering that wild-type NS5A is known to be associated in its ISDR-region with IFN-resistance and with the IL28B minor SNPs (TG/GG) [28], it is possible that wild-type NS5A Y93 also is associated with IFN-resistance and with IL28B minor SNPs, although further elucidation is necessary.

We acknowledge that the PCR technique has a risk of producing biased amplicons according to the PCR primer sequences, and therefore, we designed novel

primers in this study by searching for the most conserved sequence regions around NS5A. We speculate that the sequence bias might have been avoided at least to some extent considering the fact that the NS5A mutation rate in this study was quite compatible with that of a previous study and that obtained from the public database.

In conclusion, we detected by deep sequencing the substantial presence of resistance mutations to daclatasvir, Y93H in particular, in daclatasvir treatment naïve patients and these were not detectable by direct sequencing. We also showed that IL28B major-type patients who have favorable responses to IFN may have a higher risk of being infected with Y93H HCV than IL28B minor-type patients, suggesting that those patients might have a higher risk of developing daclatasvir resistance, although further studies are needed.

FIGURE LEGENDS

Figure 1. The percentage of mutations in the NS5A region associated with resistance to daclatasvir is presented, classified by the response to previous pegylated-interferon/ribavirin (PEG-IFN/RBV) therapy (Naïve, Null Responder, and Relapser). (A) NS5A aa 31, (B) NS5A aa 93. Each bar indicates the mutation rate in one patient and a dot above a bar shows a patient with a mutation detected by deep sequencing.

Figure 2. The percentage of mutations at the NS5A region for resistance to daclatasvir is presented, classified by the IL28B SNP (TT or non-TT). (A) NS5A aa 31, (B) NS5A aa 93. Each bar indicates the mutation rate in one patient and a dot above a bar shows a patient with a mutation detected by deep sequencing.

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Table 1. Patient characteristics classified by their responses to previous PEG-IFN/RBV combination therapy

	Naïve N = 59	Relapser N = 30	Null responder N = 21	*p*
Age (years)	62.3 ± 11.5	62.7 ± 9.1	61.2 ± 7.7	*0.719*
Sex F/M	35 / 24	16 / 14	9 / 12	*0.427*
AST (IU/l)	35.4 ± 12.6	43.9 ± 53.4	45.3 ± 14.6	*0.008*
ALT (IU/L)	34.6 ± 18.5	45.3 ± 73.2	51.8 ± 23.5	*<0.001*
PLT ($x10^{-4}/\mu l$)	15.1 ± 5.6	14.3 ± 3.8	13.8 ± 4.8	*0.582*
Alb (g/dl)	4.2 ± 0.4	4.3 ± 0.3	4.2 ± 0.5	*0.334*
γGTP (IU/L)	35.2 ± 37.7	37.6 ± 45.1	67.1 ± 55.2	*<0.001*
AFP (ng/ml)	5.7 ± 6.3	4.5 ± 3.6	14.7 ± 29.0	*<0.001*
Core aa 70 R	35 (59.3%)	23 (76.7%)	6 (28.6%)	*0.003*
Core aa 91 L	41 (69.5%)	18 (60.0%)	14 (66.7%)	*0.672*
ISDR 2-	14 (23.7%)	5 (16.7%)	2 (9.5%)	*0.340*
IRRDR 5-	29 (49.2%)	13 (43.3%)	8 (38.1%)	*0.181*
IL28B SNP TT	38 (64.4%)	27 (90.0%)	6 (25.6%)	*<0.001*

PEG-IFN/RBV, pegylated-interferon/ribavirin; ISDR, interferon sensitivity-determining region; IRRDR, interferon-ribavirin resistance determining region.

Table 2. Amplicon read numbers obtained by deep sequencing

	N	Average reads ± SD*(range) / sample
Naïve	59	3603.9 ± 1758.4 (655-10293)
Relapser	30	3980.4 ± 3295.9 (445-14330)
Null responder	21	4601.6 ± 2385.5 (1187-9579)
Plasmid	7	5448.3 ± 1299.1 (2277-7000)

*SD; standard deviation.

Table 3. Presence of daclatasvir-resistance amino acid substitutions in daclatasvir-treatment naïve patients, determined by deep sequencing

	Naïve	Relapser	Null responder	Naïve vs. Relapser	Naïve vs. Null	Relapser vs. Null
	N = 59	N = 30	N = 21	*p*	*p*	*p*
L31M/V/F %, median (range) *	2.0 (0.0-99.8)	4.1 (0.0-100.0))	0.2 (0.0-3.4)	*0.895*	*0.295*	*0.317*
Pts with L31M/V/F (%)**	8 (13.6%)	4 (13.3%)	1 (4.8%)	*1.000*	*0.510*	*0.612*
P32L %, median (range) *	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	*1.000*	*1.000*	*1.000*
Pts with P32L (%)**	0 (0%)	0 (0%)	0 (0%)	*1.000*	*1.000*	*1.000*
Y93H %, median (range) *	11.7 (0.0-99.1)	7.9 (0.0-100.0)	4.1 (0.0-45.3)	*0.824*	*0.190*	*0.301*
Pts with Y93H (%)**	21 (35.6%)	10 (33.3%)	3 (14.3%)	*1.000*	*0.112*	*0.224*

* Median proportion per patient

** Number of patients with the mutant

Table 4. Univariate and multivariate analysis of factors associated with NS5A-Y93H

Variables	No. of patients	NS5A-Y93H substitution		Univariate Analysis (N = 110)		Multivariate Analysis (N = 110)	
		Positive (N = 34)	Negative (N = 76)	Odds Ratio (95% CI)	p Value	Odds Ratio (95% CI)	p Value
Age (years) ≥65	48	16 (47.1%)	32 (42.1%)	1.22 (0.54-2.76)	0.629		
Sex Male	50	16 (47.1%)	34 (44.7%)	1.10 (0.49-2.47)	0.821		
AST (IU/L) ≥41	38	11 (32.4%)	27 (35.5%)	0.87 (0.37-2.05)	0.746		
ALT (IU/L) ≥41	33	9 (26.5%)	24 (31.6%)	0.78 (0.32-1.92)	0.590		
Platelets ($x10^4/mm^3$) ≤12	35	12 (35.3%)	23 (30.3%)	1.43 (0.61-3.33)	0.601		
Albumin (g/dL) ≤4	25	9 (26.5%)	16 (21.1%)	0.69 (0.28-1.70)	0.422		
γGTP (IU/L) ≥41	30	10 (29.4%)	20 (26.3%)	1.25 (0.51-3.08)	0.628		
AFP ≥10	16	5 (14.7%)	11 (14.5%)	1.02 (0.32-3.20)	0.974		
IL28B TT	71	29 (85.3%)	42 (55.3%)	4.70 (1.64-13.43)	0.004	3.67 (1.05-12.88)	0.042
Core aa 70 R	64	25 (73.5%)	39 (51.3%)	2.64 (1.09-6.38)	0.032	1.19 (0.40-3.55)	0.759
Core aa 91 L	73	24 (70.6%)	49 (64.5%)	1.32 (0.55-3.17)	0.531		
ISDR* ≥2	21	8 (23.5%)	13 (17.1%)	1.49 (0.55-4.02)	0.430		
IRRDR** ≥5	54	23 (67.5%)	32 (42.1%)	2.88 (1.23-6.73)	0.015	2.37 (0.98-5.74)	0.056
NS5A L31 M/V/F positive	11	2 (5.9%)	9 (11.8%)	0.46 (0.10-2.28)	0.345		
History of IFN therapy	59	21 (61.8%)	38 (50.0%)	1.62 (0.71-3.69)	0.255		

* ISDR mutation number

** IRRDR mutation number

Figure 1A

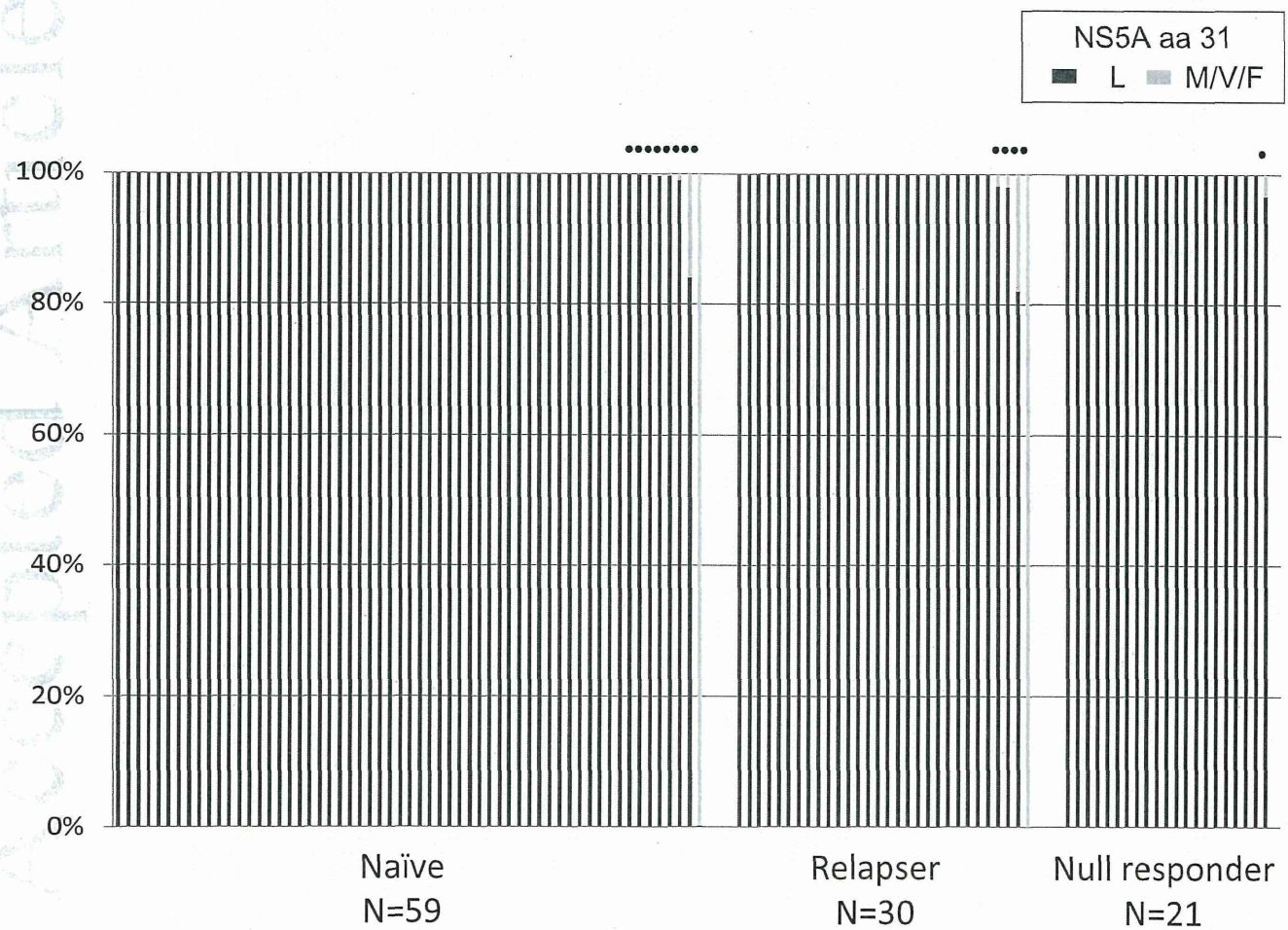

NS5A aa 31
L
M/V/F
100%
80%
60%
40%
20%
0%
Naïve
N=59
Relapser
N=30
Null responder
N=21

Figure 1B

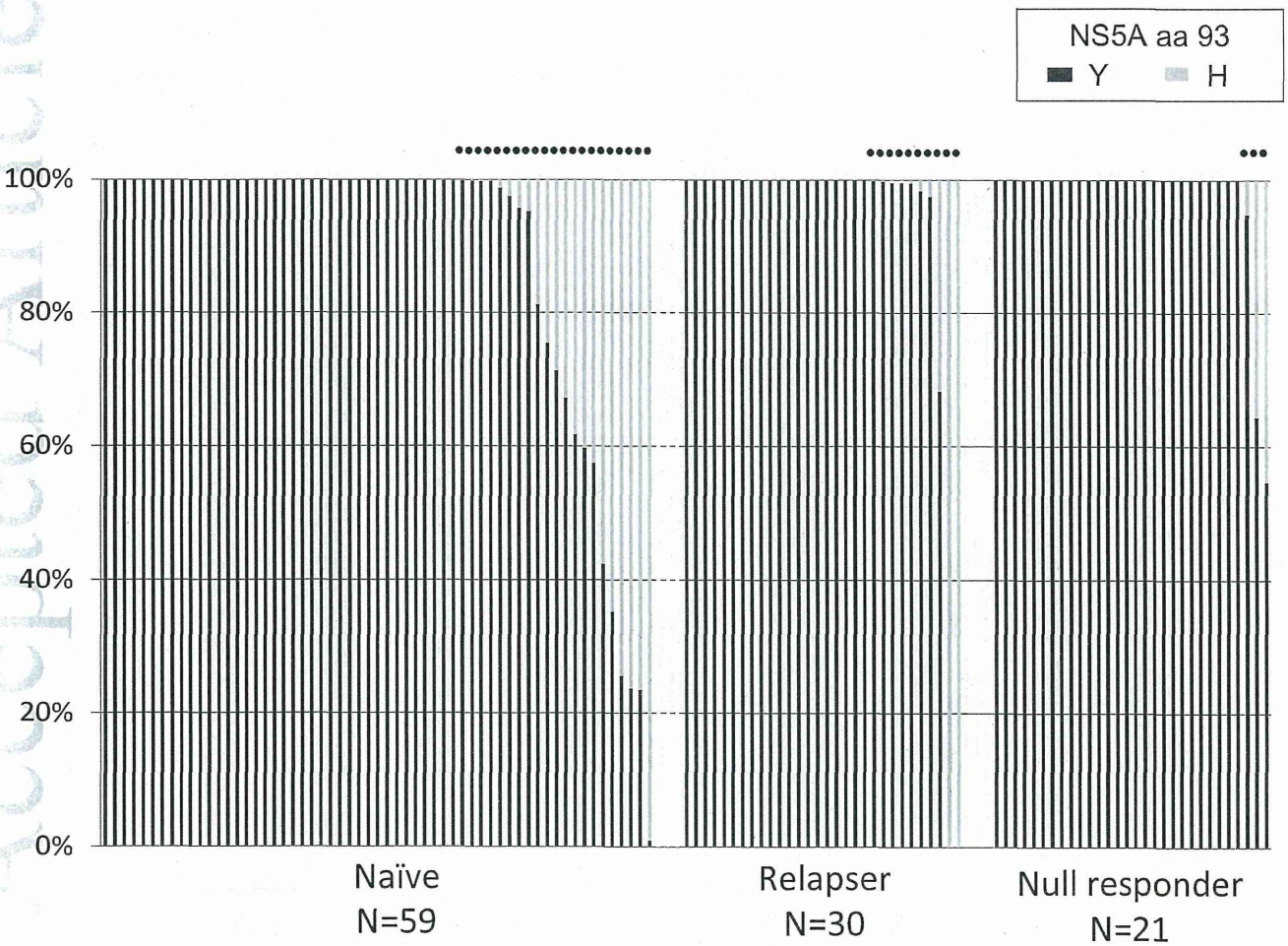


Figure 2A

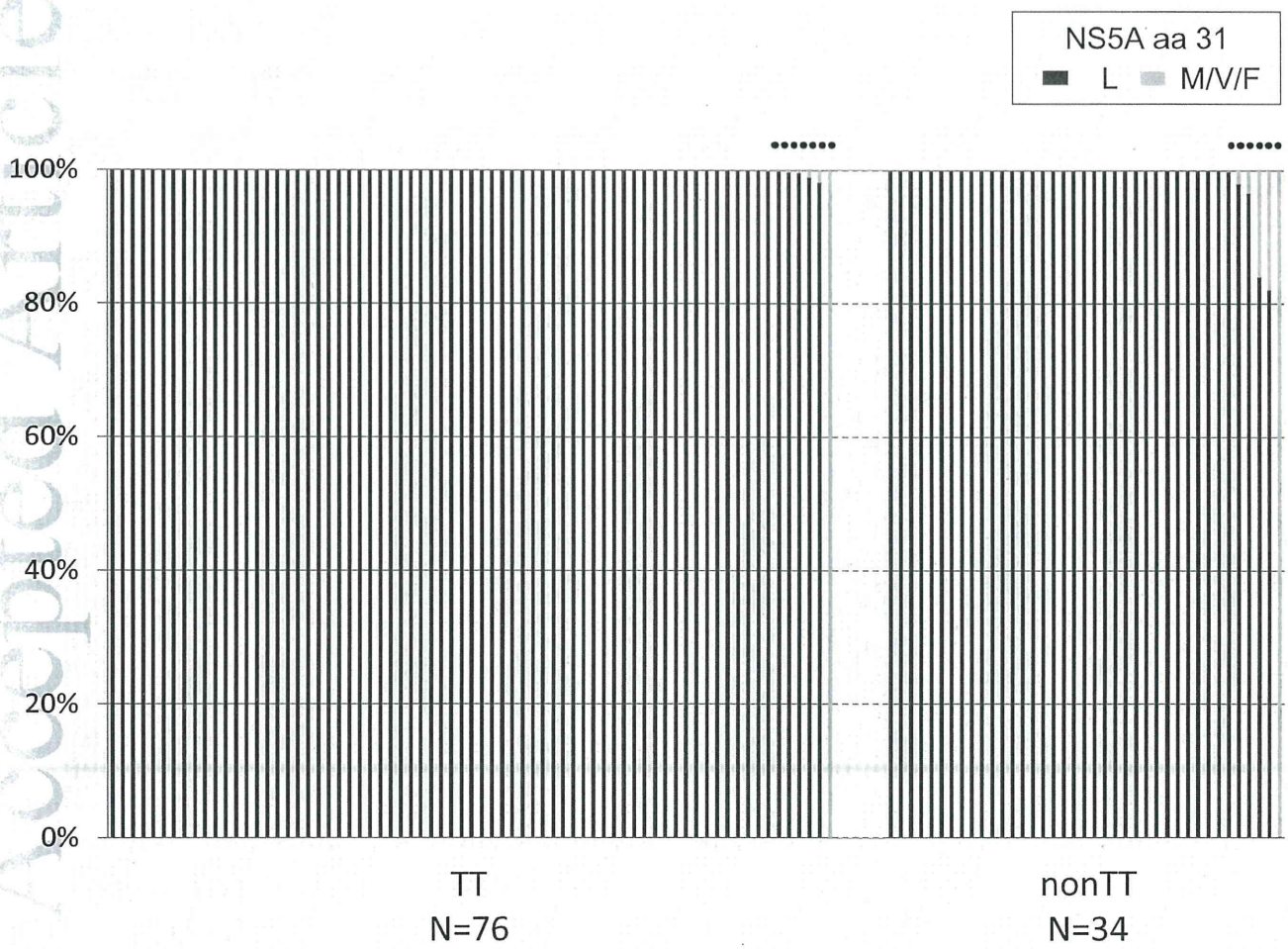
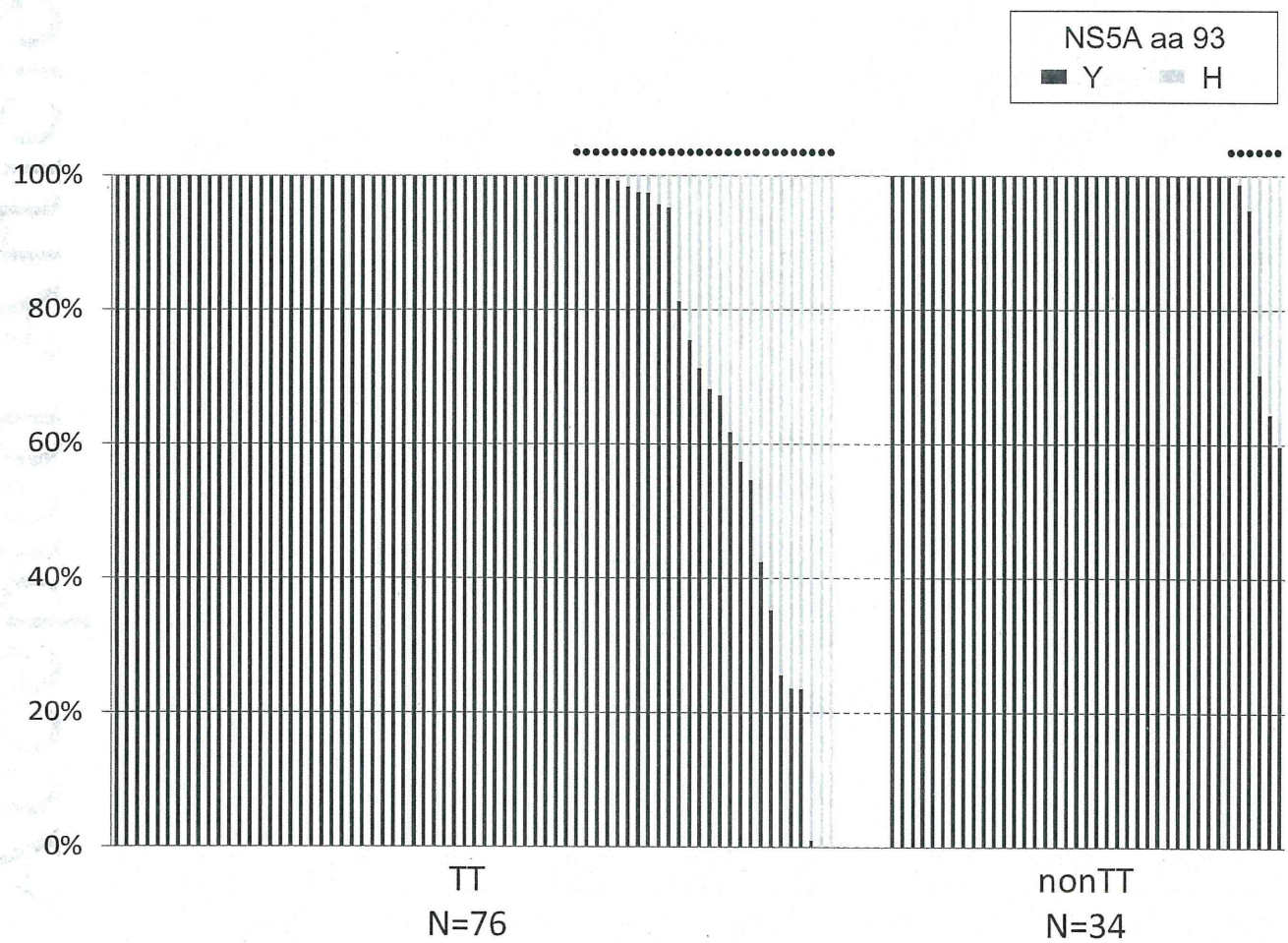


Figure 2B



Hepatocellular carcinoma risk assessment using gadoxetic acid-enhanced hepatocyte phase magnetic resonance imaging

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Short running title: HCC risk assessment using EOB-enhanced MRI

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FOOTNOTES

Competing interests

All authors have no conflict of interest related to this manuscript.

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List of Abbreviations

HCC: Hepatocellular Carcinoma

MRI: Magnetic Resonance Imaging

US: Ultrasonography

CT: Computed Tomography

HBV: Hepatitis B Virus

HCV: Hepatitis C Virus

AASLD: American Association for the Study of Liver Diseases

T1WIs: T1-Weighted Images

T2WIs: T2-Weighted Images

ALT: Alanine Aminotransferase

γ-GTP: γ - Glutamyl Transpeptidase

AFP: Alpha-fetoprotein

HR: Hazard Ratio

CI: Confidence Interval

DWIs: Diffusion-Weighted Images

ABSTRACT

Aim: To investigate whether the patients with hypovascular liver nodules determined on the arterial phase and hypointensity on the hepatocyte phase gadoxetic acid-enhanced magnetic resonance imaging (hypovascular hypointense nodules) are at increased risk of hepatocarcinogenesis, we assessed subsequent typical hepatocellular carcinoma (HCC) development at any sites of the liver with and without such nodules.

Methods: One hundred and twenty-seven patients with chronic hepatitis B or C and without a history of HCC, including 68 with liver cirrhosis, were divided into those with (non-clean liver group, n = 18) and without (clean liver group, n =109) hypovascular hypointense nodules. All the patients were followed-up for 3 years, and HCC development rates and risk factors were analyzed with the Kaplan-Meier method and the Cox proportional hazard model, respectively.

Results: A total of 17 patients (10 in the non-clean liver group and 7 in the clean liver group) developed typical HCCs. Cumulative 3-year rates of HCC development were 55.5% in the non-clean liver group and 6.4% in the clean liver group ($p < 0.001$), and those at the different sites from the initial nodules was also higher in the non-clean liver group (22.2%) than the clean liver group (6.4%) ($p=0.003$). Multivariate analysis identified older age ($p = 0.024$), low platelet counts ($p = 0.017$) and a non-clean liver ($p < 0.001$) as independent risk factors for subsequent HCC development.

Conclusions*:* Patients with hypovascular hypointense liver nodules are at a higher risk for HCC development at any sites of the liver than those without such nodules.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide and is a major cause of death in patients with chronic viral liver disease. Despite many advances in multidisciplinary treatment, complete curative treatment of early-stage HCC remains the only possible therapeutic choice for long-term survival. Therefore, surveillance programs for patients at a high-risk for HCC that include imaging-based evaluations are crucial for the detection and treatment of early-stage HCC.

The newly introduced magnetic resonance imaging (MRI) contrast agent, gadolinium ethoxybenzyl diethylenetriaminepentaacetic acid (gadoxetic acid), has enabled concurrent assessment of tumor vascularity and unique hepatocyte-specific contrast (hepatocyte phase) (1-3). This has led to the frequent identification of hypovascular nodules determined on the arterial phase with hypointensity on the hepatocyte phase (hypovascular hypointense nodules) (4-8), while many of these nodules are difficult to be detected by ultrasonography (US) or computed tomography (CT). Recently, the natural history of hypovascular hypointense nodules themselves were reported in several studies (9-12), revealing the high risk of subsequent progress to typical HCCs from these nodules. However, it is not well known whether patients with such nodules has a higher risk of developing typical HCCs at any sites of the liver, including at the different sites from initial nodules, compared to those without such nodules.

If patients with these nodules may have a high risk of developing typical HCCs not only at the same sites but also at the different sites from initial nodules, significant proportion of these nodules are precancerous lesions or early-stage HCC as reported