

COMMENTS

Background

Whether or not to treat patients older than 65 years with antiviral treatment is highly debated, especially in terms of cost/benefit ratio. However, there is little data concerning the response and safety of combination treatment for a large number of older patients with chronic hepatitis C virus infection. Therefore, in an attempt to ameliorate these problems, the authors decided to treat older patients with pegylated interferon (PEG-IFN) α -2b plus ribavirin (RBV) combination therapy.

Research frontiers

The combination treatment of PEG-IFN α -2b plus RBV improved the sustained virological response rate in chronic hepatitis C patients. However, the current issue is whether or not to treat older patients because of low response and high dropout rate.

Innovations and breakthroughs

There have been four studies on the efficacy of PEG-IFN plus RBV therapy in patients 65 years or older with genotype 1. However, these studies were too small (11-93 patients) for conclusive recommendations to be made. This study is very useful for clarifying the efficacy and safety of PEG-IFN plus RBV combination therapy in older patients, because of its large scale, multicenter design.

Applications

The study demonstrated that PEG-IFN α -2b plus RBV treatment was effective in chronic hepatitis C patients 65 years or older who completed treatment with at least the minimum required treatment dosage. Furthermore, this study suggested that the combination treatment and beginning this therapy as soon as possible are important, especially in older patients.

Peer review

The study has been well conducted and includes a large number of patients. Results have been described in a lucid and informative manner and are of clinical relevance.

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Original Article

Accumulation of refractory factors for pegylated interferon plus ribavirin therapy in older female patients with chronic hepatitis C

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Aim: Several host and viral factors have been reported to influence the effectiveness of pegylated interferon plus ribavirin combination therapy for chronic hepatitis C. In Japan, where the age of treated patients is comparatively high, recent studies have reported poor response to treatment in older female patients, but little is known about the relationship between advanced age in women and previously reported factors.

Methods: Using a database of 1167 patients chronically infected with hepatitis C virus (HCV) genotype 1b, we analyzed the amino acid sequences of the HCV core protein and interferon sensitivity determining region (ISDR) and examined the relationships among predictive factors.

Results: The proportion of patients with substitutions at core 70, which is associated with poor response to pegylated interferon plus ribavirin therapy, increased with age only in female patients. A similar trend was observed for ISDR wild type (wt). We also found that core 70 wt is associated with

core 91 wt ($P = 5.4 \times 10^{-9}$) as well as ISDR wt ($P = 0.025$). HCV RNA levels were higher in patients with core and ISDR wt ($P < 0.001$). Furthermore, core amino acid mutations were associated with advanced fibrosis and higher inflammatory activity ($P = 0.028$ and 0.048 , respectively) as well as higher gamma-glutamyltranspeptidase, alanine aminotransferase and low-density lipoprotein cholesterol levels ($P < 0.001$, 0.006 and 0.001 , respectively).

Conclusion: A combination of factors account for poor response rate in older female patients in Japan. Elucidating the relationship between amino acid substitutions and metabolic alteration is an important step in understanding the mechanism of HCV interferon resistance.

Key words: combination therapy, core protein, genotype 1b, interferon sensitivity determining region, low-density lipoprotein cholesterol

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INTRODUCTION

HEPATITIS C VIRUS (HCV) is a causative agent of acute and chronic hepatitis as well as liver cirrhosis and hepatocellular carcinoma.^{1–3} The single stranded RNA genome encodes one large open reading frame that is processed into at least 10 proteins by host and viral enzymes.^{4,5} Some viral proteins are known to affect the outcome of pegylated interferon (PEG IFN) plus ribavirin combination therapy, the current standard of care for chronic hepatitis.^{6–8} The number of amino acid substitutions in the IFN sensitivity determining region (ISDR) of the NS5A protein, which was initially reported to affect IFN monotherapy,^{9,10} has recently been reported to affect PEG IFN plus ribavirin combination therapy as well.^{11–14}

NS5A PKR binding domain (PKRBD),^{15–19} variable region 3 (V3),^{20–23} IFN/ribavirin resistance determining region (IRRDR),^{24,25} and E2 PKR-eIF2 α phosphorylation homology domain (PePHD)²⁶ have also been reported to affect therapy outcome, although these results need to be confirmed. More recently, amino acid (a.a.) substitutions in the core protein have been reported to negatively affect IFN plus ribavirin therapy.^{27,28} Substitution at a.a. 70 of the core protein (core 70) has been reported to be associated with non-virological response (NVR), and this finding was confirmed by several groups.^{29–31}

Several cytokines and adipokines have also been reported to be associated with the effectiveness of therapy. For instance, tumor necrosis factor (TNF)- α expression has been reported to be elevated in patients with HCV infection, and high expression levels are associated with poor response to IFN therapy.³² IP-10 has also been reported to associate with response to therapy in patients with HCV and HIV co-infection.³³ Leptin and adiponectin levels are also reportedly associated with the effect of combination therapy.^{34,35} In addition to these factors, there are many studies reporting relationships between common polymorphisms in the human genome and outcome of IFN therapy.^{36–44} Among them, single nucleotide polymorphisms (SNP) in the interleukin (IL)-28B locus discovered through genome-wide association studies appear to have a large effect on outcome of PEG IFN plus ribavirin combination therapy^{42–44} as well as spontaneous eradication of HCV.⁴⁵

In addition to the above viral and host genetic factors, several metabolic factors such as obesity,³⁴ insulin resistance⁴⁶ and low-density lipoprotein (LDL) cholesterol levels^{28,47} have been reported to be correlated with the effect of combination therapy. Further-

more, higher gamma-glutamyltranspeptidase (γ -GTP) levels, often associated with fatty liver, have also been reported to be associated with treatment outcome.^{48,49} Although these factors may be mutually interdependent, their relationships with viral factors have not yet been analyzed.

Recent papers have reported poor response to therapy in older female patients,^{50–52} but little is known about the relationship between age, sex and other predictive factors. To analyze these associations, we constructed a database consisting of 1425 patients with chronic hepatitis C. Using this database, we analyzed the relationship between viral and metabolic data and found that a.a. substitutions in the core and ISDR are associated with metabolic change, which may be related to disease progression and response to therapy.

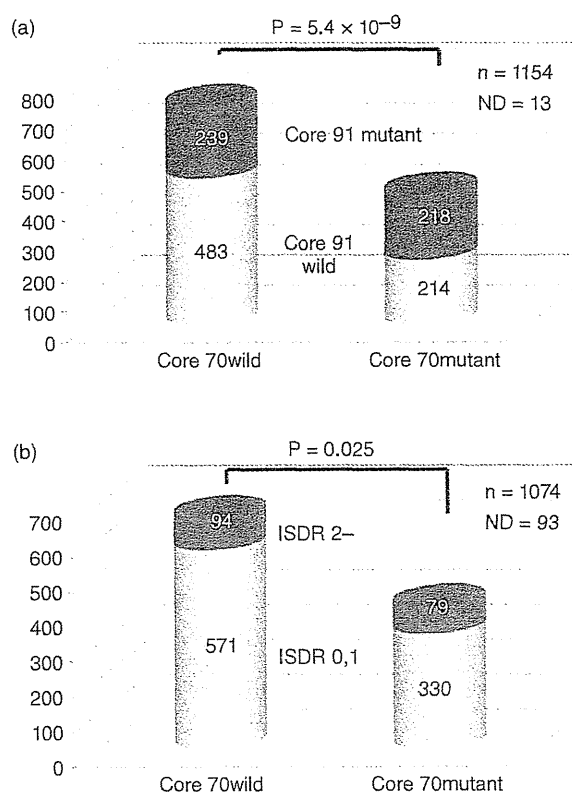


Figure 1 Association of core amino acid 70, amino acid 91 and interferon sensitivity determining region (ISDR). The relationship between hepatitis C virus core 70 and core 91 wild type and mutant amino acids (a) and the ISDR (b) were examined. Statistical significance was assessed using the χ^2 -test. ND, not determined due to polymerase chain reaction or sequence calling failure.

Table 1 Clinical profile of 1167 patients

	All patients n = 1167	Tx naive n = 570 (48.84%)	Prev. tx n = 597 (51.16%)	P-value
Sex (male/female)	606/561	259/311	347/250	1.45E-05
Age	55.1 ± 10.7	55.2 ± 11.0	55.0 ± 10.5	0.604
Body weight	60.6 ± 10.8	59.5 ± 10.5	61.7 ± 11.0	0.001
BMI	27.0 ± 7.38	24.3 ± 5.46	29.6 ± 8.02	0
Fibrosis stage (0–2/3–4/ND)	815/192/160	422/78/70	393/114/90	0.005
Activity stage (0–1/2–3/ND)	531/465/171	263/234/73	268/231/98	0.803
Steatosis (present/absent/ND)	207/428/532	103/175/292	104/253/240	0.034
White blood cells (/mm ³)	4808 ± 1428	4871 ± 1395	4748 ± 1457	0.127
Hemoglobin (g/dL)	14.1 ± 1.88	14.0 ± 1.39	14.3 ± 2.23	0.001
Platelets (×10 ⁴ /mm ³)	16.6 ± 5.06	16.5 ± 5.31	16.7 ± 4.82	0.288
ALT (IU/L)	66 ± 52	67 ± 48	65 ± 55	0.265
AST (IU/L)	65 ± 54	58 ± 37	71 ± 66	0.001
γ-GTP (IU/L)	56 ± 58	57 ± 62	55 ± 54	0.942
Albumin (g/dL)	4.00 ± 0.375	4.04 ± 0.402	3.97 ± 0.347	0.001
Total cholesterol (mg/dL)	173 ± 32.1	175 ± 32.7	172 ± 31.6	0.206
Fasting blood sugar (mg/dL)	101 ± 24.9	102 ± 27.2	99.8 ± 22.2	0.715
HCV RNA (KIU/mL: amp)	2999 ± 4523	2822 ± 4365	3169 ± 4668	0.048
ISDR (0–1/≥2/ND)	908/178/81	440/85/45	468/93/36	0.863
Core 70 (wild/mutant/ND)	722/433/12	349/218/3	373/215/9	0.509
Core 91 (wild/mutant/ND)	697/457/13	349/217/4	348/240/9	0.39

ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GTP, gamma-glutamyltranspeptidase; HCV, hepatitis C virus; ND, not determined; tx., treatment.

METHODS

Study subjects

WE COLLECTED DATA from 1425 participating patients with chronic hepatitis C from 16 centers in Japan. Inclusion criteria included testing positive for

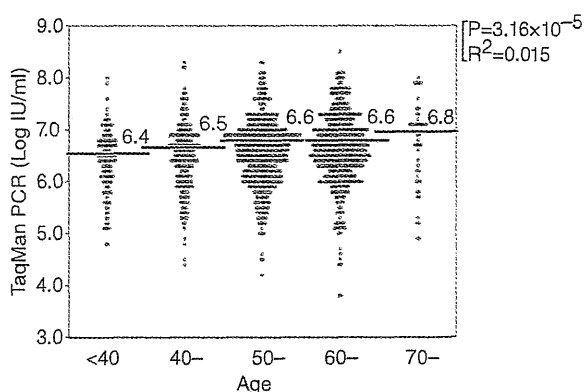


Figure 2 Relationship between age and virus titer. Virus titers were plotted according to age. The median titer within each 10-year age group is shown as horizontal bars.

HCV RNA over a period of more than 6 months and testing negative for both hepatitis B virus surface antigen and anti-HIV antibody. Patients with confounding liver conditions were excluded, as well as patients who were lost to follow up or who did not have high viral load (≥ 5 log IU/mL) for HCV genotype 1b (Fig. 1). Patient data was not used when we failed to determine core 70, core 90 and ISDR sequences. In total, data from 1167 patients were included in the analysis. All subjects gave written informed consent to participate in the study according to the process approved by the ethical committee of each hospital and conforming to the ethical guidelines of the 1975 Declaration of Helsinki.

Patients received weekly injections of PEG IFN- α -2b for either 48 or 72 weeks using the following doses: 60 μ g for 35–45 kg bodyweight; 80 μ g for 46–60 kg; 100 μ g for 61–75 kg; 120 μ g for 76–90 kg; and 150 μ g for 91–120 kg. Ribavirin was administered p.o., and the dose was determined based on the patient's bodyweight (600 mg for <60 kg, 800 mg for 60–80 kg, 1000 mg for >80 kg). Ribavirin dosage was reduced when hemoglobin levels reduced to 10.0 g/dL and stopped if hemoglobin levels reached 8.5 g/dL. Bio-

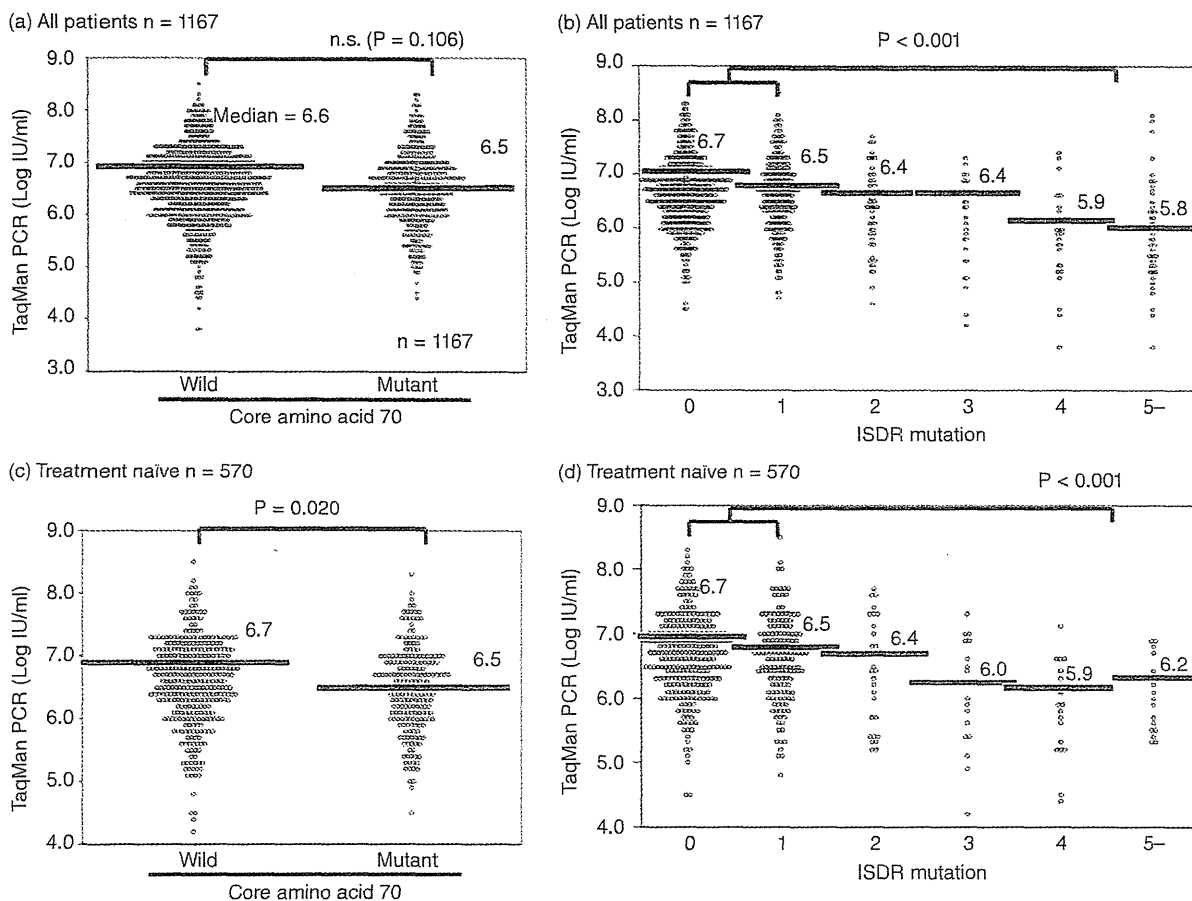


Figure 3 Analysis of virus load by core amino acid 70 substitution and number of amino acid substitutions in the interferon sensitivity determining region (ISDR). Virus titers of all 1167 patients were classified according to core 70 wild type and mutant amino acids (a) or by the number of substitutions in the ISDR (b). The 570 interferon therapy naïve patients were also examined separately (c,d).

chemical tests were performed by center, and pathological diagnosis was made according to the criteria of Desmet *et al.*⁵³ Successful treatment was ascertained based on sustained virological response (SVR), defined as HCV RNA negative 6 months after cessation of therapy.

Analysis of viral titer and a.a. sequences in the core and ISDR region

The HCV RNA level was analyzed using reverse transcription polymerase chain reaction (RT-PCR)-based methods (Amplicor Hepatitis C Virus test: Roche Diagnostics, Basel, Switzerland; high range test: Cobas Amplicor, Roche Diagnostics, Basel, Switzerland; or TaqMan RT-PCR test: Applied Biosystems, Foster city,

CA, USA). The measurement ranges of these assays were 5–5000 KIU/mL and 1.2–7.8 log IU, respectively. For values exceeding the measurable range, the titer was determined after dilution of the serum samples.

Sequences were determined by direct sequencing of PCR fragments following extraction and RT of serum HCV RNA. For core 70 and 91, arginine and leucine were considered wild type (wt) according to Akuta *et al.*^{27,28} The number of a.a. substitutions in the ISDR was determined as described previously.^{9,10,53}

Statistical analysis

The χ^2 -test and Mann–Whitney *U*-test were applied to detect significant associations using PASW ver. 18

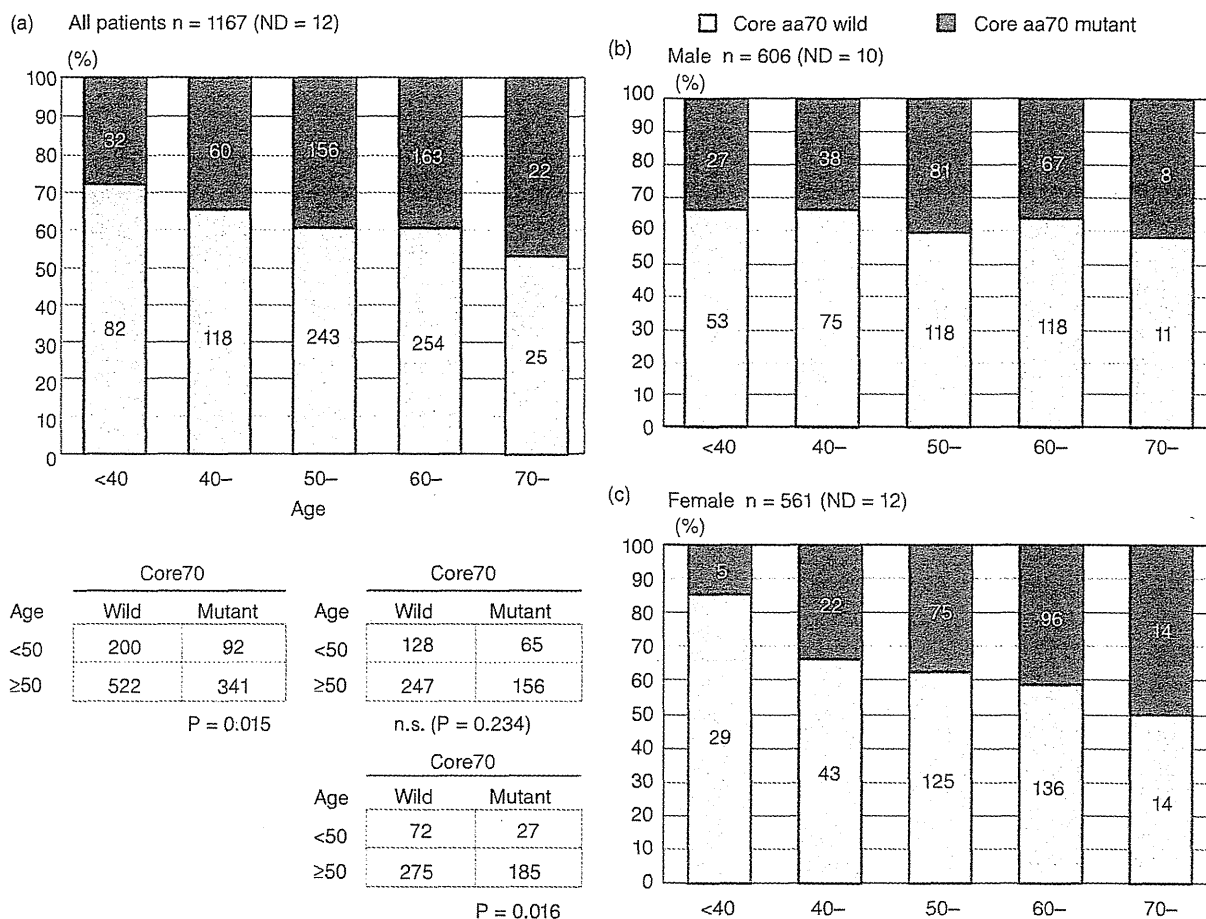


Figure 4 Age-dependent increase in core amino acid 70 mutants in female patients. Percentages of core wild type (arginine) and mutant amino acids for all patients (a), as well as for male (b) and female (c) patients are shown. Note that the age-dependent increase in mutant frequency was observed only in female patients. Statistical analysis was performed by χ^2 -test. ND, not determined.

(SPSS, Chicago, IL, USA). All statistical analyses were two sided, and $P < 0.05$ was considered significant. Simple and multiple regression analyses were used to examine the association between viral substitutions and clinical factors using $P < 0.05$ as the criterion for inclusion in the multivariate model. Continuous variables were split into indicator variables based on the median, except for age which was divided into 10-year intervals. Multivariate logistic regression analysis was performed using the Design package in R (www.r-project.org) with fast backward elimination and validation based on AIC score.

RESULTS

Patient characteristics

PATIENT PROFILES ARE shown in Table 1. Results are presented separately for patients who were naive to IFN therapy and those who had had previous IFN therapy but failed to eradicate the virus.

Virus titer and a.a. substitutions in the core and the ISDR

We found a significant positive correlation between patient age and virus titer ($P = 3.16 \times 10^{-5}$, $R^2 = 0.015$,

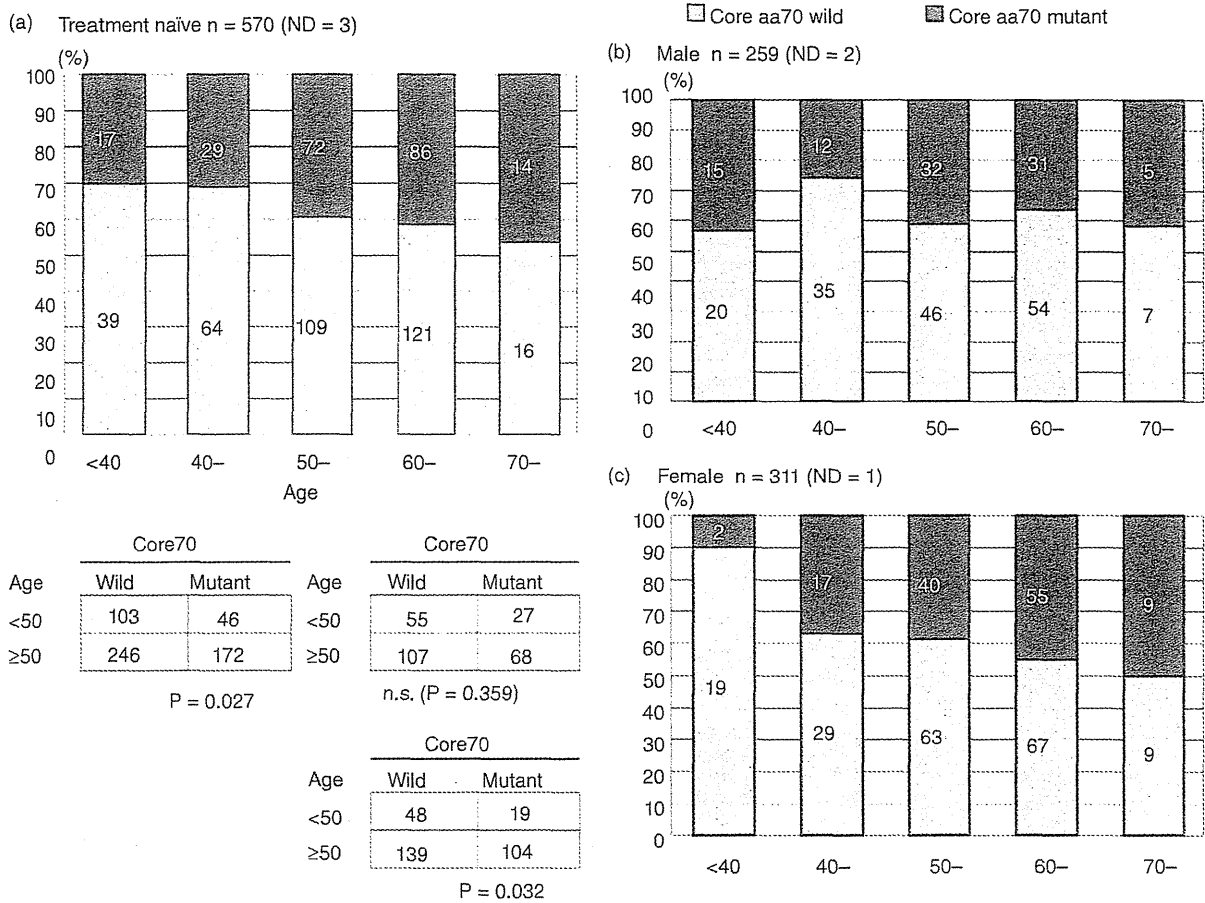


Figure 5 Age-dependent increase in core amino acid 70 mutants in treatment-naïve female patients. Percentage of core wild type (arginine) and mutant amino acid were analyzed as in Figure 5 using only interferon treatment-naïve patients. Results for all 561 patients (a), as well as for male (b) and female (c) patients are shown. ND, not determined.

Fig. 2). Wt core 70 was associated with wt core 91, with 40% of patients wt for both core 70 and core 91 and 20% of patients non-wt for both (Fig. 1, $P = 5.4 \times 10^{-9}$). Virus titer did not differ in patients with wt core 70 compared to non-wt when all patients were included (Fig. 3a), but when treatment-naïve patients were analyzed separately, virus titer was significantly higher in patients with core 70 wt ($P = 0.02$, Fig. 3c). We found a significant negative linear relationship between virus titer and the number of substitutions in the ISDR ($P < 0.001$, Fig. 3b), regardless of treatment history ($P < 0.001$, Fig. 3d).

Amino acid substitution and age

The proportion of patients with core 70 substitutions increased with age among female patients (Figs 4,5), and the proportion of patients without substitutions in the ISDR tended to increase with age among treatment-naïve females ($P = 0.0581$, Fig. 6).

Core 70 a.a. substitution and histological findings

Fibrosis stage and activity were higher in patients with core 70 mutants ($P = 0.028$ and $P = 0.048$, respectively; Fig. 7). There was no apparent correlation between his-

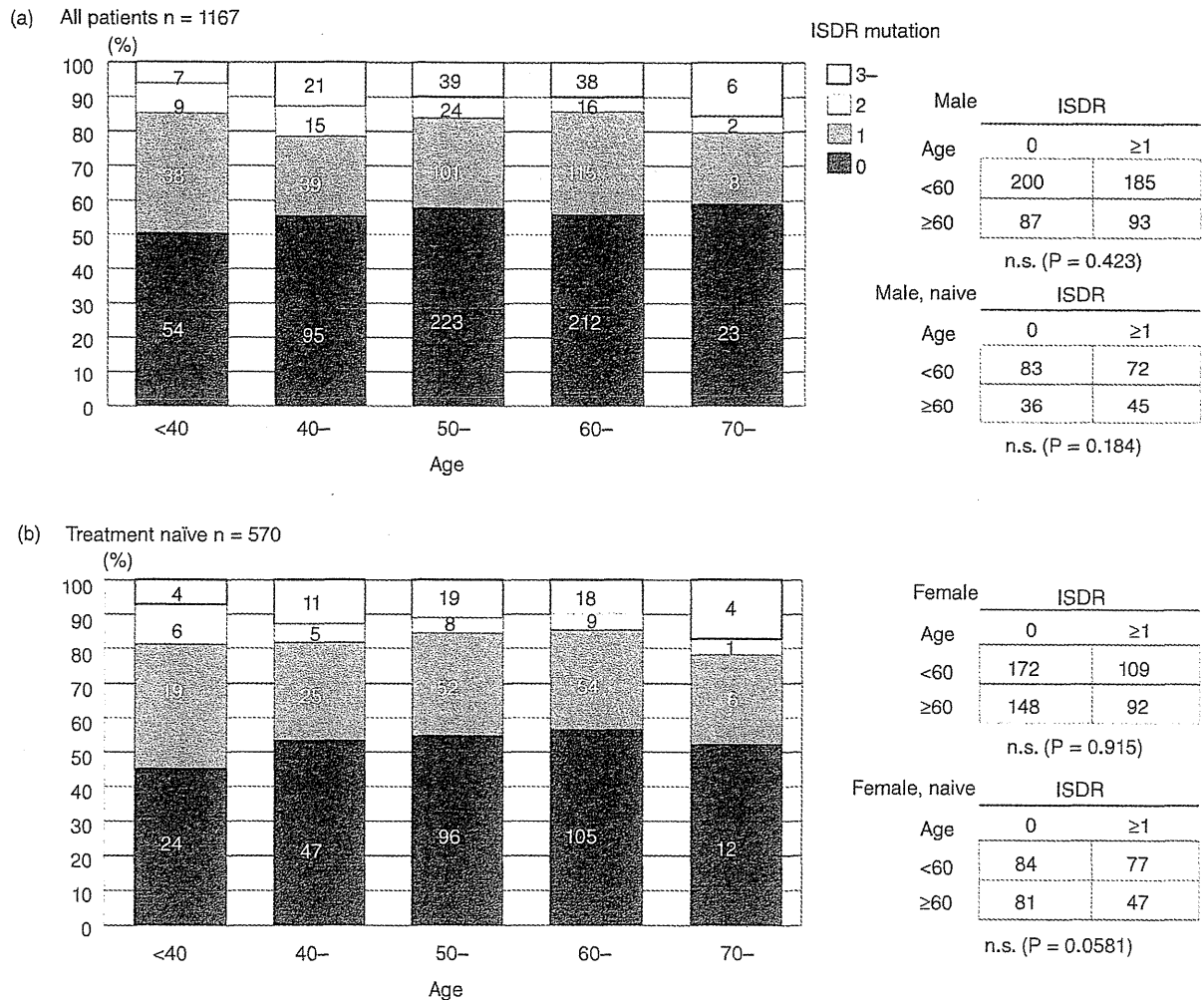


Figure 6 Age-dependent increase in number of amino acid substitutions in the interferon sensitivity determining region (ISDR). The relationship between age and the number of amino acid substitutions in the ISDR was examined. All patients (a) and only naive patients (b) were analyzed. Statistical analysis was performed using the χ^2 -test.

tological findings and the number of a.a. substitutions in the ISDR (data not shown).

Correlation between viral a.a. substitutions and clinical conditions

We compared γ -GTP, ALT, LDL cholesterol levels and other clinical conditions between patients with core 70 wild and mutant types (Fig. 8). ALT and γ -GTP levels were significantly higher in patients with core 70 substitutions (Fig. 8a,b). In contrast, LDL cholesterol levels and platelet counts were significantly higher in patients with core 70 wt (Fig. 8c,d). However, only sex, fibrosis, γ -GTP and core 91 substitution were independently

associated with core 70 substitution (Table 2). Only viral load and core 70 substitutions are independent predictive factors for the presence of two or more ISDR substitutions (Table 3).

DISCUSSION

WE FOUND THAT factors previously reported to be associated with poor response to IFN-based treatment for chronic hepatitis C tended to be most strongly associated with older female patients. Studies on difficult-to-treat older female patients have so far only been reported in Japan, probably due to the rela-

Table 2 Factors associated with HCV core protein amino acid 70 substitutions

Variable	Simple			Multiple			
	<i>n</i>	OR	<i>P</i>	<i>n</i>	OR	(95% CI)	<i>P</i>
Age (in 10-year increments)	331	1.1	0.3536				
Sex (male vs female)	365	1.58	0.04178	214	2.09	(1.11–3.95)	0.0234
BMI (kg/m ²)	363	0.763	0.2229				
Diabetes	312	1.77	0.08053				
Fibrosis (F0–1 vs F2–4)	252	2.12	0.007444	214	2.18	(1.15–4.13)	0.017
Activity (A0–1 vs A2–4)	246	1.73	0.04849				
ALT (IU/L)	329	0.866	0.5461				
Platelets (×10 ⁴ /mm ³)	329	0.937	0.7836				
γ-GTP (IU/L)	305	1.69	0.03427	214	1.59	(0.841–3.02)	0.153
Albumin (g/dL)	190	0.765	0.3981				
Fasting blood sugar (mg/dL)	250	0.898	0.6878				
TaqMan PCR (log IU/mL)	327	0.748	0.2232				
HDL cholesterol (mg/dL)	202	1.64	0.1025				
LDL cholesterol (mg/dL)	165	1.25	0.5085				
Total cholesterol (mg/dL)	321	0.907	0.6847				
Core 91 (wild vs others)	365	2.22	0.000393	214	2.68	(1.43–5.02)	0.002
ISDR (0, 1 vs >1)	343	1.82	0.03102	214	1.85	(0.853–4)	0.1197

Simple and multiple logistic regression were used to examine the association between substitution at core amino acid 70 and patient and viral factors.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CI, confidence interval; γ-GTP, gamma-glutamyltranspeptidase; HCV, hepatitis C virus; HDL, high-density lipoprotein; ISDR, interferon sensitivity determining region; LDL, low-density lipoprotein; ND, not determined; OR, odds ratio.

Table 3 Factors associated with viral ISDR substitutions (0–1 vs >1 mutations)

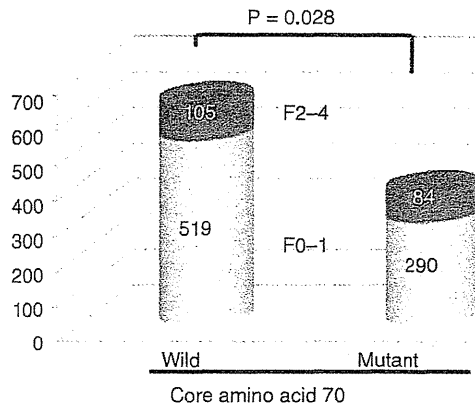
Variable	Simple			Multiple			
	<i>n</i>	OR	<i>P</i>	<i>n</i>	OR	(95% CI)	<i>P</i>
Age (in 10-year increments)	311	1	0.9735				
Sex (male vs female)	345	0.644	0.1247				
BMI (kg/m ²)	343	1.14	0.6254				
Diabetes	293	0.818	0.6509				
Fibrosis (F0–1 vs F2–4)	235	1.28	0.4545				
Activity (A0–1 vs A2–4)	229	1.3	0.4281				
ALT (IU/L)	309	1.15	0.646				
Platelets (×10 ⁴ /mm ³)	309	0.668	0.1707				
γ-GTP (IU/L)	287	1.47	0.2115				
Albumin (g/dL)	172	0.979	0.9622				
Fasting blood sugar (mg/dL)	233	1.36	0.3641				
TaqMan PCR (log IU/mL)	307	0.517	0.02527	305	0.529	(0.30–0.95)	0.03223
HDL cholesterol (mg/dL)	189	1.23	0.617				
LDL cholesterol (mg/dL)	152	0.463	0.1199				
Total cholesterol (mg/dL)	303	0.656	0.1537				
Core 70 (wild vs others)	343	1.82	0.03102	305	1.82	(1.01–3.3)	0.04763
Core 91 (wild vs others)	344	0.699	0.2038				

Simple and multiple logistic regression was used to examine the association between the number of substitutions in the ISDR region and patient and viral factors.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CI, confidence interval; γ-GTP, gamma-glutamyltranspeptidase; HCV, hepatitis C virus; HDL, high-density lipoprotein; ISDR, interferon sensitivity determining region; LDL, low-density lipoprotein; ND, not determined; OR, odds ratio.

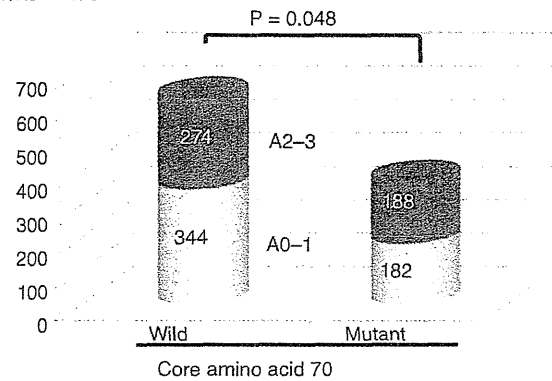
(a) Fibrosis (F0-1 vs F2-4) n = 1167

※ND = 169



(b) Activity (A0-1 vs A2-3) n = 1167

※ND = 179



(c) Activity (A0-2 vs A3) n = 1167

※ND = 179

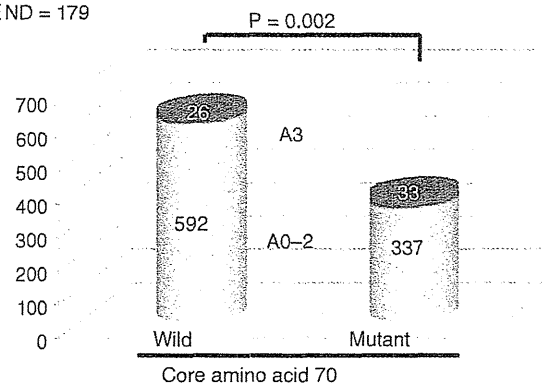


Figure 7 Histological findings and core amino acid 70 substitutions. Relationships between core amino acid 70 (wild type or mutant) and degree of fibrosis (F0-1 and F2-4) (a) and activity (b,c) were examined. Activity was divided into A0-1 and A2-3 (b) or A0-2 and A3 (c) and compared with amino acid 70. ND, not determined.

tively higher age at treatment. The mechanism underlying this association is unknown. Recently, SNP in the IL-28B locus were found to be associated with response to combination therapy as well as to spontaneous eradication of the virus,⁴²⁻⁴⁴ although differences in the eradication rate between men and women have not been reported so far. We have previously reported that incidence of wt core 70 is significantly higher in patients with the IL-28 protective allele.⁵⁴ Therefore, it seems reasonable that the wt core 70 confers a selective advantage for the virus in patients with the IL-28 protective allele. During the time when IFN monotherapy was still the standard treatment, female sex, or perhaps the lower iron concentration associated with female sex, had been reported as one of the predictive factors for a favorable response to monotherapy.⁵⁵⁻⁵⁷ It is pos-

sible that spontaneous eradication of the virus occurs during the natural course of chronic hepatitis through IFN produced naturally as a result of liver inflammation in young female patients with wt core 70, resulting in accumulation of core mutant viruses as the patient ages. Further prospective observations are necessary to address this issue.

In this study, we found that each of the previously reported predictive factors that we examined also correlated with HCV a.a. substitutions. Interestingly, a.a. substitutions in the virus are associated with metabolic factors such as LDL and high-density lipoprotein cholesterol and fatty liver-related γ -GTP, and in particular, we found that substitution in the core protein (and possibly ISDR) is correlated with LDL cholesterol. The virus appears to influence expression of genes involved

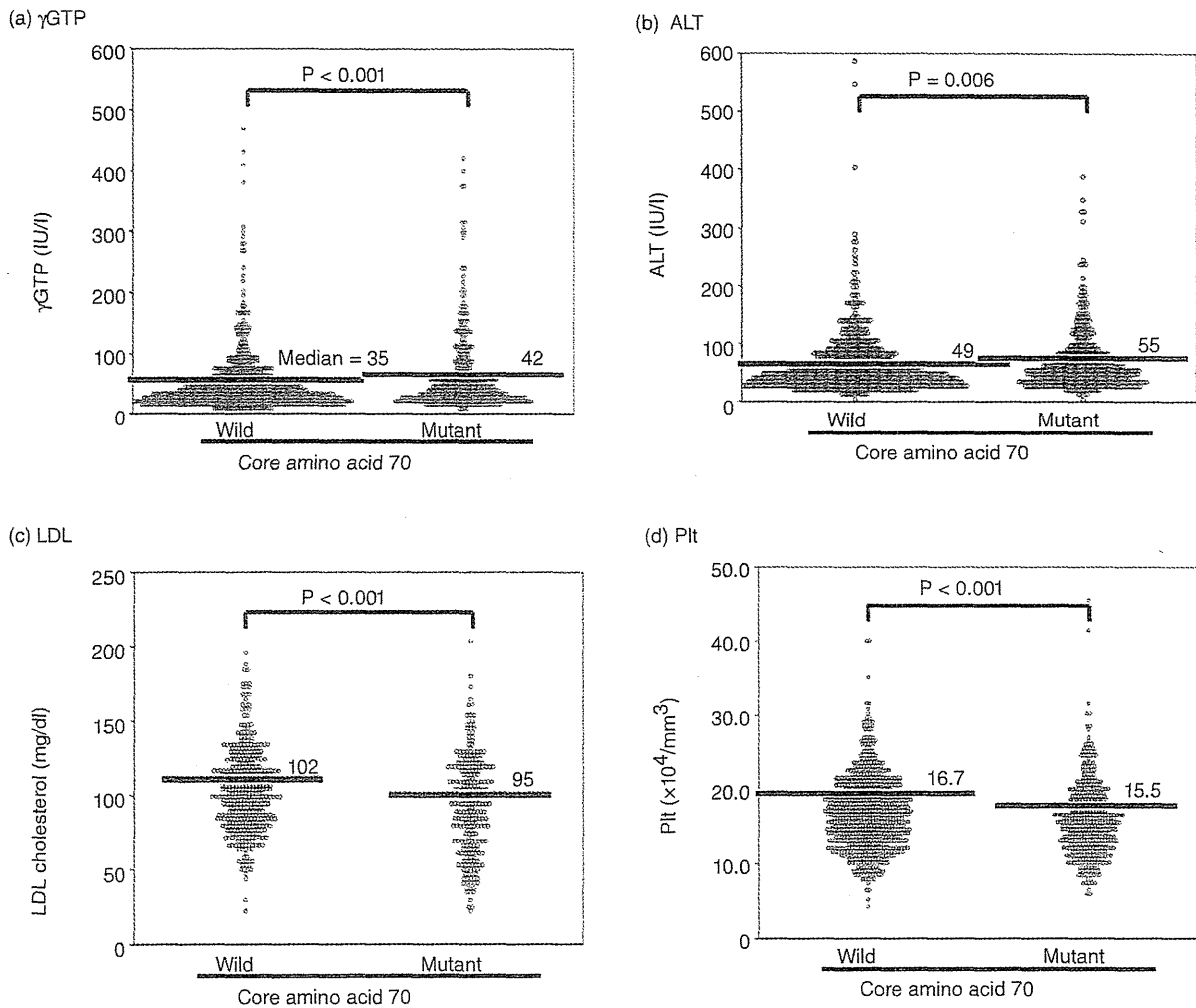


Figure 8 Relationship between blood test findings and core amino acid 70 substitutions. Relationships between core amino acid 70 (wild type or mutant) and gamma-glutamyltranspeptidase (γ -GTP) (a), alanine aminotransferase (ALT) (b), low-density lipoprotein (LDL) cholesterol (c) and platelet count (Plt) (d) were examined. Bars represent the median.

in host cell lipid metabolism to enhance its own replication and secretion.⁵⁶ Consequently, metabolic changes induced by infection by different strains of HCV should be investigated further to understand viral mechanisms of IFN resistance and to develop effective personalized therapies.

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Kaposi's sarcoma-associated herpesvirus genome replication, partitioning, and maintenance in latency

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Kaposi's sarcoma-associated herpesvirus (KSHV) is thought to be an oncogenic member of the γ -herpesvirus subfamily. The virus usually establishes latency upon infection as a default infection pattern. The viral genome replicates according to the host cell cycle by recruiting the host cellular replication machinery. Among the latently expressing viral factors, LANA plays pivotal roles in viral genome replication, partitioning, and maintenance. LANA binds with two LANA-binding sites (LBS1/2) within a terminal repeat (TR) sequence and is indispensable for viral genome replication in latency. The nuclear matrix region seems to be important as a replication site, since LANA as well as cellular replication factors accumulate there and recruit the viral replication origin in latency (ori-P) by its binding activity to LBS. KSHV ori-P consists of LBS followed by a 32-bp GC-rich segment (32GC). Although it has been reported that LANA recruits cellular pre-replication complexes (pre-RC) such as origin recognition complexes (ORCs) to the ori-P through its interaction with ORCs, this mechanism does not account completely for the requirement of the 32GC. On the other hand, there are few reports about the partitioning and maintenance of the viral genome. LANA interacts with many kinds of chromosomal proteins, including Brd2/RING3, core histones, such as H2A/H2B and histone H1, and so on. The detailed molecular mechanisms by which LANA enables KSHV genome partitioning and maintenance still remain obscure. By integrating the findings reported thus far on KSHV genome replication, partitioning, and maintenance in latency, we will summarize what we know now, discuss what questions remain to be answered, and determine what needs to be done next to understand the mechanisms underlying viral replication, partitioning, and maintenance strategy.

Keywords: Kaposi's sarcoma-associated herpesvirus, human herpesvirus 8, latency-associated nuclear antigen, ori-P, DNA replication, genome maintenance, pre-replication complex, nuclear matrix

INTRODUCTION

Kaposi's sarcoma (KS)-associated herpesvirus (KSHV) is a gamma-2 herpesvirus discovered from KS specimens in 1994 (Chang et al., 1994). KSHV is closely associated with KS and several non-Hodgkin lymphomas, including primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD; Cesarman et al., 1995, 1996; Soulier et al., 1995). While KS is the most common cancer in acquired immune deficiency syndrome patients (Potthoff et al., 2010), KSHV is detected in about 95% of all types of KS lesions by PCR analysis (Dupin et al., 1995; Huang et al., 1995; Moore and Chang, 1995). PEL is a rare B cell lymphoma originated from preterminal B cells, and PEL in AIDS patients is often associated with KSHV as well as EBV. Several KSHV-infected PEL cell lines have been established, and EBV is frequently lost in the course of establishment (Arvanitakis et al., 1996; Gaidano et al., 1996; Renne et al., 1996; Said et al., 1996; Carbone et al., 1997, 1998; Katano et al., 1999). MCD is a plasmacytic lymphadenopathy with polyclonal hyper-immunoglobulinemia and high levels of serum IL-6 (Frizzera et al., 1983; Yoshizaki et al., 1989).

Like all herpesviruses, KSHV has two life cycles: latent and lytic replication phases (for review, see Boshoff and Chang, 2001). Whereas KSHV is usually in latency when it infects KS and PEL cells, in MCD some cells express lytic genes (Katano et al., 2000;

Parravicini et al., 2000). On the other hand, it has been reported that KSHV infection itself and/or viral lytic proteins promote cell proliferation and angiogenesis as well as lymphatic reprogramming (Ciufo et al., 2001; Gao et al., 2003; Carroll et al., 2004; Hong et al., 2004; Naranatt et al., 2004; Pan et al., 2004; Wang et al., 2004; Sharma-Walia et al., 2006; Qian et al., 2007, 2008; Sadagopan et al., 2007; Ye et al., 2007).

In latency, the KSHV genome is present as an episome, which is capable of autonomously replicating during S phase of the host cell cycle without integration into host chromosomes, and only limited genes are expressed during latency. Therefore, there is no generation of progeny virions. It is very important to elucidate and learn the virus's survival strategy in order to control infection and to formulate treatment for KSHV-related diseases.

In this review, we would like to focus on studies on the mechanisms underlying viral DNA replication, genome segregation and maintenance, and gene expression regulation in latency, and to discuss these topics in the light of studies on cellular mechanisms.

GENE EXPRESSION CONTROL IN KSHV LATENCY

The KSHV genome is a double-stranded linear DNA in the virion. It is circularized upon infection and is maintained as an episome in the infected nucleus. The complete genome is about 160–170 kbp,

including a 40~50 times repeated sequence called a terminal repeat (TR), which is 801 bp as a unit, at each end of the genome (Figure 1). The viral genome encodes approximately 90 ORFs in the unique region (for reviews, see Moore and Chang, 2001; Dourmishev et al., 2003). In latency, the viral genome is maintained in a cell cycle-dependent manner, and extremely limited viral genes, such as latent gene clusters, are expressed. Although the establishment of latent infection could be this virus's default infection mode, it is not good for the virus to continue latent infection for a long time, since it will vanish from the earth without progeny virus production. Thus, latency is a state in which the virus waits for an opportunity for the explosive production of progeny virions. Nevertheless, it is very interesting how KSHV establishes latency and is maintained in infected host cells without losing the genomes.

As mentioned above, the limited region within the KSHV genome is transcriptionally active in latency, and this region contains only several genes, including the latency-associated nuclear antigen (Wang and Frappier, 2009), viral cyclin (*v-CYC*), viral FLICE-inhibitory protein (*v-FLIP*), *kaposin*, 17 microRNAs (miRNAs), and viral interferon regulatory factor 3 (*v-IRF-3*; Chang et al., 1996; Thome et al., 1997; Muralidhar et al., 1998; Lubyova and Pitha, 2000; Gomez-Roman et al., 2001; Rivas et al., 2001; Staudt and Dittmer, 2003; Cai et al., 2005; Pearce et al., 2005; Pfeiffer et al., 2005; Samols et al., 2005). Such genes, except for *v-IRF-3*, are in that limited region, and *LANA*, *v-CYC*, and *v-FLIP* are in one of the unit's genes. This region forms an active locus for expression including miRNAs and *kaposin*. It is unclear why this region is active for the expression of genes and is insulated from inactive lytic genes, such as ORF69 and K14 just downstream and upstream, respectively.

Recently, it was reported that CTCF, which is the only insulator protein found in vertebrates, coupled with SMC3, endows this gene insulation. More interestingly, the binding sites are in the genes, i.e., downstream from the *LANA* transcription start sites, not at the boundary regions (Stedman et al., 2008; Kang and Lieberman, 2009). Transcriptional analysis using the KSHV-BAC system demonstrated that mutations of CTCF binding sites abolished latency-regulated transcription such as K14 and ORF74 during latency (Kang and Lieberman, 2009). CTCF usually binds at the boundary regions between active and inactive loci in mammalian genomes, forming locus control regions (LCRs; Tanimoto et al., 2003). A typical example is an LCR seen in the beta-globin locus. CTCF binds to several DNase I hypersensitivity sites (HS), called HS4 and HS5, and forms boundaries to insulate this locus from

the outside locus (Tanimoto et al., 2003; Hou et al., 2008). Thus, latent gene expression in KSHV-infected cells might be regulated differently from the mechanism observed in the beta-globin locus.

Inversely, it is interesting how the viral lytic genes are tightly inactivated in latency. Epigenetic regulation seems to be essential for inactivation as well as activation of latent genes. *LANA* recruits heterochromatin components to the TR by the interaction between *LANA* and SUV39H1, which is a key factor that methylates histone H3, which in turn recruits heterochromatin protein 1 (HP1; Sakakibara et al., 2004). Because this mechanism contributes to the propagation and maintenance of heterochromatin, it appears that heterochromatin could spread over the KSHV genome during latency. The propagation of heterochromatin into the active latent gene zone might be blocked by the boundary effect and by the enhancer-blocking activity of an insulator, CTCF which has multiple functions such as gene activation or inactivation, X-chromosome inactivation, and gene imprinting (for review, see Zlatanova and Caijafa, 2009).

Thus, it is thought that not the overall lytic genes region, except for the latent gene clusters, forms heterochromatin during latency, because recent genome-wide analysis using CHIP-on-chip showed that not only latent gene clusters but also several regions of lytic genes are enriched in activating histone marks (acetylated H3 and H3K4me3). However, H3K27me3, which is a bivalent histone marker, is widely distributed through the KSHV genome (Toth et al., 2010), meaning that the genome is poised for reactivation. Furthermore, the treatment of specific histone demethylases of H3K27me3 such as JMJD3 and UTX could induce the lytic reactivation. Immunoprecipitation of methylated DNA assay showed that the KSHV genome was methylated during latency (Gunther and Grundhoff, 2010). Gunther and Grundhoff (2010) suggested that the CpG methylation process could take a long time to prevail over the genome, and thus could not control early latency. There are several reports that DNA methylation of viral genomes is related to the regulation of the gene expression of gammaherpesviruses such as EBV and herpesvirus saimiri (HVS; Minarovits, 2006). Heterochromatin formation on the viral genomes, however, seems to be inconvenient for the rapid induction of lytic replication. Further investigations are needed to clarify how viruses are ready for lytic induction if heterochromatin and/or DNA methylation was formed on the genome.

Viral factors play key roles in maintaining gene expression profiles in latency. Otherwise, modulation by viral and cellular factors maintains viral latency. In addition to the recruitment of

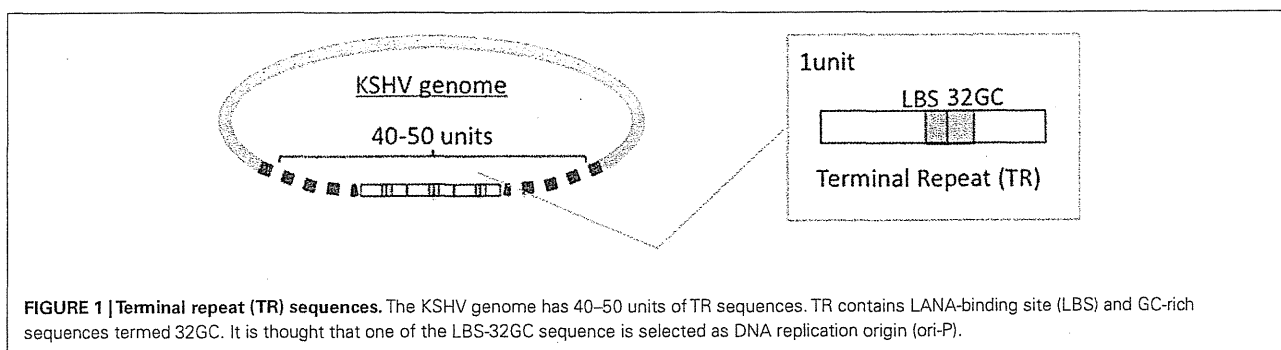


FIGURE 1 | Terminal repeat (TR) sequences. The KSHV genome has 40~50 units of TR sequences. TR contains LANA-binding site (LBS) and GC-rich sequences termed 32GC. It is thought that one of the LBS-32GC sequence is selected as DNA replication origin (*ori-P*).

heterochromatin factors on to the genome, LANA itself tends to repress viral lytic gene expression. LANA physically associates with recombination signal sequence-binding protein λ (RBP- λ) and represses the replication and transcription activator (RTA) promoter through the RBP- λ binding site existing within its promoter (Lan et al., 2005a).

Viral FLICE-inhibitory protein, also known as K13, interacts with several NF- κ B-related signaling proteins and activates the NF- κ B pathways, thus enhancing cell survival (Chaudhary et al., 1999; Field et al., 2003; Matta et al., 2003, 2007; Matta and Chaudhary, 2004). It should be noted that the effect of NF- κ B signaling on reactivation depends on cellular context (Grossmann and Ganem, 2008) and seems to be regulated by an intricate balance within the cellular environment. Previous studies, however, demonstrated that v-FLIP repressed the RTA promoter by activating NF- κ B binding to the cognate sequence, but in that case it deregulates vIL-6 and hIL-6 expression (Zhao et al., 2007). The reactivation is initiated by RTA, which is a lytic switch protein and a homolog of EBV BRLF1 (Liang et al., 2002). The RTA promoter region is highly responsive to 12-*O*-tetradecanoylphorbol 13-acetate (TPA) or phorbol 12-myristate 13-acetate (PMA), sodium butyrate (NaB), and trichostatin A (TSA), and is associated with several histone deacetylase proteins such as HDAC, which leads to chromatin remodeling of a nucleosome and then regulates KSHV reactivation from latency (Lu et al., 2003). RTA activates various viral genes through direct binding with RTA-responsive elements existing within the K8 and ORF57 promoters (Byun et al., 2002) and also through indirect mechanisms on RTA itself and vIRF-1 (Nishimura et al., 2001; Sakakibara et al., 2001; Ueda et al., 2002). Although RTA is a strong transactivator and inducer of lytic replication, it also enhances LANA expression and then is involved in the establishment of latency in the early infection phase (Lan et al., 2005b). This feedback mechanism explains the low efficiency of lytic replication and the generation of complete viral particles in KSHV-infected cell lines.

MicroRNAs (miRNAs) are single-stranded and 20- to 23-nucleotide RNA molecules that are involved in gene expression (Bartel, 2004; Bartel and Chen, 2004). Recent studies have highlighted the critical role of viral microRNAs (miRNAs) in the maintenance of KSHV latency (for review, see Ganem and Ziegelbauer, 2008; Boss et al., 2009; Lei et al., 2010a). The KSHV genome contains 17 miRNAs that are clustered and located in the intragenic region between *kaposin* and *v-FLIP* (Cai et al., 2005; Pearce et al., 2005; Samols et al., 2005; Cai and Cullen, 2006). Surprisingly, a new proteomic approach suggests that a single miRNA can directly lead to the suppression of the synthesis of hundreds of proteins at both mRNA and translation levels, although the level of suppression is mild (Baek et al., 2008; Selbach et al., 2008).

Kaposi's sarcoma-associated herpesvirus miRNAs are reported to regulate, directly or indirectly, various factors including lytic genes (Murphy et al., 2008; Bellare and Ganem, 2009) and cellular factors such as NF- κ B and I κ B α , the latter of which is directly regulated by KSHV miR-K1 (Lei et al., 2010b) and Bcl-2 associated factor (*BCLAF1*) as a target of miR-K5 (Ziegelbauer et al., 2009). miR-K12-7 (Lin et al., 2011) and miR-K9 (Bellare and Ganem, 2009) directly target RTA and contribute to the maintenance of latency. miR-K12-11 shows remarkable homology to cellular

miR-155; it inhibits a BACH-1 3'UTR-containing reporter and downregulates the expression of BACH-1, which is a broadly expressed transcriptional repressor that regulates genes involved in the hypoxia response (Gottwein et al., 2007; Skalsky et al., 2007). Thrombospondin 1 (*THBS1*), an inhibitor of angiogenesis, is targeted by multiple KSHV miRNAs, such as miR-K12-1, miR-K12-3-3p, miR-K12-6-3p, and miR-K12-11 (Samols et al., 2007). miR-K1 represses the expression of p21 via the 3'UTR and attenuates p21-mediated cell cycle arrest during KSHV latency (Gottwein and Cullen, 2010).

CELLULAR DNA LICENSING AND VIRAL DNA REPLICATION IN LATENCY

Because eukaryotic DNA replication is strictly regulated by a licensing mechanism, the genome is replicated only once per cell cycle. DNA replication starts at multiple sites on a chromosome; these sites are called the replication origin, whose number is predicted to be 30,000~50,000 (Huberman and Riggs, 1966). The genome size of eukaryotes is about 10^7 to 10^{11} bp (fungi to mammals), and the entire DNA must be replicated within a limited time (Wyrick et al., 2001). To achieve this, many proteins participate in DNA replication licensing, including origin recognition complex (ORC), Cdc6, Cdt1, and mini-chromosomal maintenance (MCM) helicase, and so on. First, ORC recognizes and binds to the origins and then recruits a Cdc6 followed by the association of another replication protein, called Cdt1. Finally, the MCM helicase is loaded onto the complex to establish a complete pre-replication complex (pre-RC) (reviews in Nishitani and Lygerou, 2002; DePamphilis, 2003, 2005).

KSHV ORIGIN OF REPLICATION IN LATENCY

The features of DNA replication origins have been reported. Although there are no consensus sequences for the replication origin, recent studies showed that CpG islands, promoter regions, DNA topology, and nucleosome positioning are involved in origin selection (Mechali, 2010). *Saccharomyces cerevisiae* (*S. cerevisiae*) has autonomous replication sequence (ARS) elements that are specific 12 bp consensus sequences and has origin activity (Stinchcomb et al., 1979; Bell and Stillman, 1992). *S. pombe* ARS also has been identified, but it does not share a consensus sequences as in *S. cerevisiae* (Segurado et al., 2003; Dai et al., 2005; Heichinger et al., 2006). Substitution experiments showed that the ARS region could be replaced with a 40-bp poly (dA/dT) fragment (Okuno et al., 1999).

In higher eukaryotes, no consensus sequences are identified, though known origin sequences have been reported (for review, see Aladjem, 2004). It is not yet known why there is no consensus sequence among ORC binding sites of higher eukaryotes, or how they are selected. Replication origins should be determined by different mechanisms, and recent genome-wide analyses show that the origin sequences are closely related with transcriptional regulatory elements and CpG islands but not sequence motifs (Cadoret et al., 2008; Sequeira-Mendes et al., 2009).

The KSHV genome appears to replicate once per cell cycle during latency, as cellular DNA replication. The number of the genome copies is supposed to be 50–100 per KSHV-infected PEL cell and the copy number is kept at the same number, at least

appears to be kept at the same copy number (Cesarman et al., 1995; Ballestas et al., 1999; Ueda et al., 2006). This observation suggests that the KSHV genome uses cellular replication machinery so that viral DNA replication synchronizes with the cell cycle. The KSHV origin of replication in latency, called ori-P, consists of two LANA-binding sites (LBS), in which LBS1 has a higher affinity with LANA (Garber et al., 2002), and the following 32 bp GC-rich segment (termed 32GC in this manuscript) and the ori-P is in the TR region of the KSHV episome (Hu and Renne, 2005; **Figure 1**). LANA directly binds to LBS and supports viral DNA replication (Garber et al., 2002; Hu et al., 2002; Fejer et al., 2003; Grundhoff and Ganem, 2003). The components of pre-RC, such as ORC, Cdc6, and MCM were recruited to the TR sequences in a LANA-dependent manner (Lim et al., 2002; Ohsaki et al., 2004; Stedman et al., 2004; Verma et al., 2006).

In the case of EBV ori-P, the dyad symmetry (DS) and family of repeats (FR) are essential for the ori-P activity (Reisman et al., 1985), though FR rather works for viral genome maintenance. The DS element contains two EBNA1 binding sites and is the functional replicator in the presence of EBNA1 (Wysokenski and Yates, 1989; Harrison et al., 1994). The FR element contains 20 copies of a 30-bp repeat sequence and has an essential role in the long-term maintenance of ori-P-containing plasmid (Krysan et al., 1989; Marechal et al., 1999). It is suggested that cellular replication factors bind to the sequences adjacent to EBNA1 binding sites through the interaction with EBNA1 (Yates et al., 2000; Koons et al., 2001). A chromatin immunoprecipitation assay suggested that the ORC complex and EBNA1 bound to chromatin and ori-P in G0-arrested cells (Ritzi et al., 2003). Nucleosome assembly proteins, such as NAP1 and TAF-I, interact with EBNA1 and are recruited to the ori-P regions. These proteins contribute to the activation of transcription, although TAF-I negatively regulates DNA replication (Wang and Frappier, 2009). The EBNA1 LR1 and LR2 domains are critical for the interaction with ORC and for disrupting this association by binding with G-rich RNA (Norseen et al., 2009). Thus, the latent replication of KSHV and EBV totally depends on cellular DNA replication machinery with the only exception of the requirement of LANA and EBNA1, respectively.

HOW LANA WORKS

LANA is a nuclear protein with 1162 amino acids. It shows a functional homology to EBNA1 of EBV and, in part, to E1/E2 of human papillomavirus and to SV40 large T antigen. Especially, the C-terminus of LANA and that of EBNA1 conserve secondary and tertiary structures (Han et al., 2010). The N-terminus of LANA contains a chromosome binding site (CBS) and a nuclear localization signal (NLS), and the C-terminus contains a DNA binding domain and a dimerization domain, called DBD. In the DBD, there appears to be another NLS, but this NLS is rather cryptic and weak, since an N-terminal deleted mutant, which contains 108–1162 aa, is localized in the cytoplasm (Ohsaki et al., 2009). The central region is composed of a proline-rich region, an aspartate (D)- and glutamate (E)-rich repetitious region, and a glutamine-rich domain (Garber et al., 2001; Piolot et al., 2001; **Figure 2**). DBD (923–1162 aa) is necessary and partially sufficient to support ori-P replication compared to the full-length LANA (Hu et al., 2002;

Ohsaki et al., 2009), and when considering their expression levels in *in vitro* study.

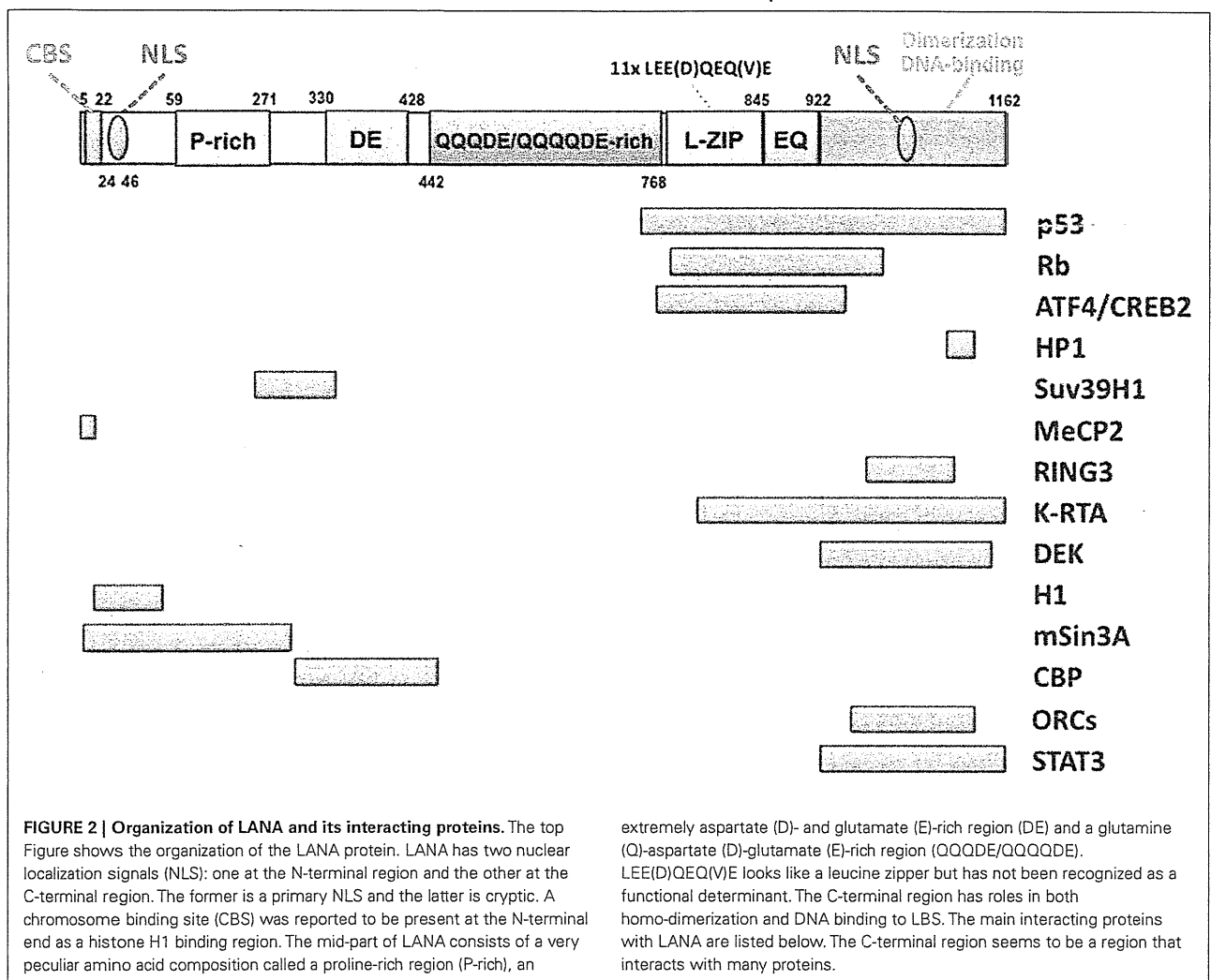
Many studies show that LANA binds to LBS and recruits ORC to the origin as described above. What is the mechanism by which ORC is recruited to ori-P? One possible mechanism is that LANA directly interacts with ORC so that ORC is loaded to the origin (Lim et al., 2002; Stedman et al., 2004; Verma et al., 2006). If LANA could directly interact with ORC and recruit to the origin, we are confronted with this question: why is 32GC required for ori-P activity despite LANA's ability to bind to the ori-P? It could be that the 32GC is required to load ORC and the other pre-RC components to the region next to LBS, although the underlying molecular mechanism is not yet known. Further study is needed to resolve this question.

LANA is highly expressed in KSHV-related malignancies, and plays an essential in episomal maintenance. It interacts with multiple cellular proteins, including tumor suppressors such as p53 (Friborg et al., 1999) and Rb (Radkov et al., 2000), as well as transcription factors such as ATF4/CREB2 (Lim et al., 2000) and STAT3 (Muromoto et al., 2006), chromatin-associated proteins such as HP1 (Lim et al., 2003), histone H2A/B (Barbera et al., 2006b), MeCP2 (Krithivas et al., 2002; Matsumura et al., 2010), and Brd4 (Ottinger et al., 2006), in addition to signal transducers such as GSK-3 β (Fujimuro and Hayward, 2003) and so on (**Figure 2**). Almost all of these proteins interact with the C-terminal domain of LANA, implying the functional importance of this domain. However, careful analysis of protein–protein interaction is required, because only DBD sometimes shows non-specific binding with other proteins and may exhibit different properties from the full-length LANA (our personal observation).

REGULATION OF KSHV ori-P ACTIVATION

The origin number varies from species to species. In mammals, it, 30,000–50,000 origins are thought to exist at each cell cycle (Huberman and Riggs, 1966). However, not all of these origins start DNA synthesis at the same time. Some origins are activated early in the S phase, whereas others are activated in the late S phase; that is, the DNA replication timing is controlled (Dimitrova and Gilbert, 1999; Cimbara et al., 2000). How is the timing of DNA replication determined? Some groups have provided possible answers to this question. It was reported that heterochromatin could change the timing of DNA replication by transgene insertion into a mammalian genome (Lin et al., 2003). Sir proteins, which are silencer proteins, can delay replication and correlate with transcriptional silencing (Zappulla et al., 2002). Thus, various studies suggest that heterochromatin modulates replication timing (Goren et al., 2008; Klochkov et al., 2009; Schwaiger et al., 2010) and EBV replication in latency occurs in mid-late S phase (Zhou et al., 2009). In the case of KSHV, the replication timing of the viral genome is not yet known. Considering that ori-P is present in the proximity of heterochromatin because of LANA-dependent accumulation of heterochromatin, DNA replication of the KSHV genome may start at the middle or late S phase.

The frequency of origin usage also differs from origin to origin; some origins are used in every cell cycle, whereas others are used rarely. Various studies including in *Drosophila*, *Xenopus*, and mammals demonstrate how specific origins are selected, but several



questions about the decision mechanism of origins remain unanswered. Considering the genome size of this virus, one origin is enough to complete replication within the S phase; and because of the existence of “origin interference” by the ATR and ATM pathways (Shechter et al., 2004), a single origin must be chosen.

The micrococcal nuclease digestion pattern at TR in G1-arrested cells leads to change, suggesting that the chromatin structure became more accessible to enzymatic digestion (Stedman et al., 2004). Therefore, the chromatin structure may be changed by the recruitment of the replication machinery during the late G1 phase.

A recent study shows that the cellular deubiquitylating enzyme USP7 stimulates EBNA1 binding to its recognition sites so that histone modification at the EBV ori-P is changed by EBNA1 mediating the recruitment of USP7 (Sarkari et al., 2009).

THE ROLES OF NUCLEAR ARCHITECTURES NUCLEAR MATRIX AS A SCAFFOLD FOR DNA REPLICATION AND TRANSCRIPTION

The nucleus consists of a well-organized structure and is highly complex. The structures of nuclear matrix proteins such as

lamins, nuclear mitotic apparatus (NuMA), hnRNP, and so on, are important for the organization of chromatin, DNA replication, and transcription (Dechat et al., 2008). The nuclear matrix, isolated by Berezney and Coffey (1974), is believed to support the spatial distribution of several nuclear factors, such as DNA replication machinery and transcription factors. The nuclear matrix fraction contains DNase I-resistant and high salt-resistant proteins. Because the nuclear matrix can be visualized only after chromatin extraction, there has been a debate that such a nuclear matrix is an essential component of *in vivo* nuclear architectures. In previous studies, replication origins have come to the nuclear matrix (van der Velden et al., 1984; Amati and Gasser, 1990; Adom et al., 1992; Brylawski et al., 1993; Fallaux et al., 1996). Eukaryotic DNA is organized into DNA loops generated by the attachment of chromatin to the nuclear matrix via specific regions, referred to as scaffold/matrix attachment regions (Pardoll et al., 1980; Vogelstein et al., 1980; Laemmli et al., 1992; Roberge and Gasser, 1992). DNA loop formation is essential for DNA replication, transcription, and chromosomal packaging (Gasser and Laemmli, 1987; Berezney et al., 1995; Bode et al., 1995; Nickerson et al., 1995; Razin et al., 1995; Jackson, 1997; Volpi et al.,