was significantly lower than that of cells transfected with pGFP-CMV/pRL-TK or pGFP-CMV/pRL-SV40 (Fig. 2F).

mRNA and Protein Expression Level of Luciferases in B16-BL6 Cells Transfected With Two Different Plasmids

The mRNA level was measured in B16-BL6 cells transfected with two types of plasmids, each of which expressed firefly or RL. Again, cells were transfected with 0.2 μg/mL pFL-CMV and 1.8 μg/mL RL-expressing plasmid, and the mRNA and luciferase activity were simultaneously measured at 24 h after transfection (Fig. 3). The amount of RL protein was dependent on the type of plasmids used as described above, but it was almost proportional to its mRNA level in all cases (Fig. 3A). The amount of FL protein was highly dependent on the type of RL-expressing plasmid cotransfected (Fig. 3B), as shown in Figure 2B. Moreover, the mRNA level of FL was also affected by other plasmids in a manner dependent on the promoter strength (Fig. 3B), suggesting that some processes leading to mRNA expression are saturated in cells that produce a large amount of mRNA. In addition, the degree of reduction in FL mRNA by cotransfection with pFL-CMV/pRL-CMV (about 20% of pFL-CMV/pRL-TK) was less than that of FL protein (about 8%), which implies that the amount of FL protein was disproportionate to its mRNA level.

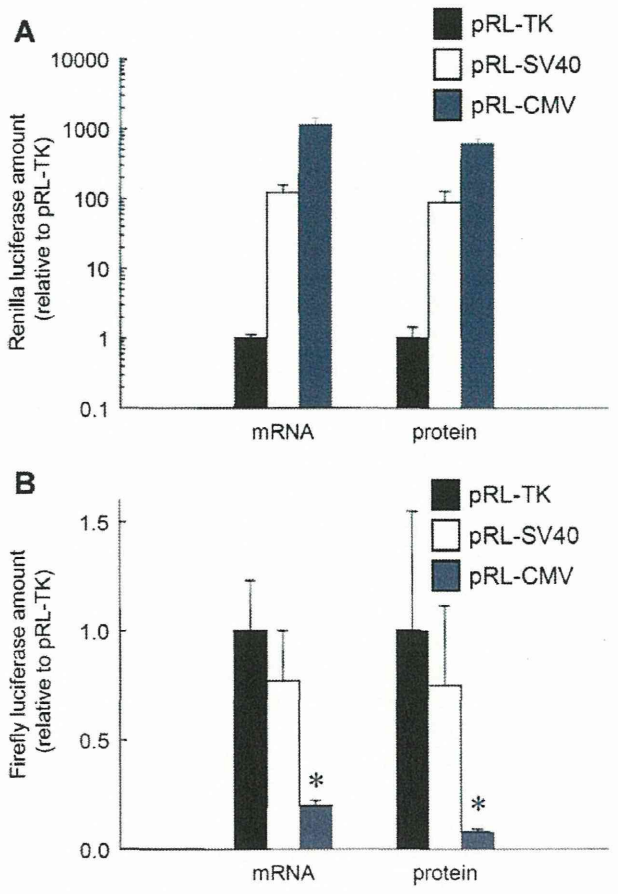

Figure 3. Effect of cotransfection on the levels of mRNA and protein in B16-BL6 cells. **A and B:** B16-BL6 cells were cotransfected with 1.8 μg/mL pRL-TK (closed bar), pRL-SV40 (open bar), or pRL-CMV (gray bar) and 0.2 μg/mL pFL-CMV. One day after transfection, mRNA and protein expression levels of renilla (A) and firefly (B) luciferase were simultaneously measured. The results are expressed as the mean ± SD ($n = 4$). $^*P < 0.05$.

Luciferase Expression in B16-BL6 Cells Transfected With siRNA and Plasmids

The results obtained thus far are consistent with the hypothesis that the synthesis of protein from mRNA is saturated when a large amount of mRNA is transcribed, and this leads to disproportionate protein synthesis to the mRNA level. To confirm this hypothesis, we degraded the mRNA of RL using siRL, an siRNA targeting RL, and measured the amount of luciferase proteins. For this purpose, B16-BL6 cells were cotransfected with 1 μg/mL siRL or siGFP, a control siRNA, in addition to 0.9 μg/mL pRL-CMV, pRL-SV40, or pRL-TK and 0.1 μg/mL pFL-CMV. As expected, the amount of RL was markedly suppressed in cells transfected with siRL compared with siGFP (Fig. 4A), which suggests that siRL successfully degraded the target mRNA under these experimental conditions. Cotransfection of siRL significantly increased the amount of FL in B16-BL6 cells transfected with pRL-CMV/pFL-CMV (Fig. 4B). These results suggest that siRNA-mediated degradation of mRNA from competing plasmids is effective in restoring transgene expression from the monitoring plasmid. However, the FL expression in the cells transfected with siRL, pFL-CMV, and pRL-CMV was lower than that of the cells transfected with siRL, pFL-CMV, and pRL-TK.

Dose-Dependent mRNA and Protein Expression of Luciferase in Mice After Hydrodynamics-Based Administration and its Effect on GAPDH Expression

Figure 5A shows the amounts of mRNA and protein of FL in mouse liver 6 h after hydrodynamic injection of pFL-CMV at different doses. Results were normalized to those of mice that received 1 μg/mouse pFL-CMV administration. A linear correlation was observed between the FL mRNA and the plasmid dose over the dose range investigated, that is, from 1–300 μg/mouse. At the dose of 10 μg/mouse, the relative amount of luciferase protein was comparable with that of mRNA expression, indicating that the efficiency of protein synthesis from mRNA was similar at doses of 1 and 10 μg/mouse. However, at a dose of 30 μg/mouse or higher, the ratio of FL protein to its mRNA was smaller than at the low doses. Thus, these results indicate that the amount of luciferase protein becomes disproportionate to the amount of its mRNA in mouse liver, when the mRNA level is

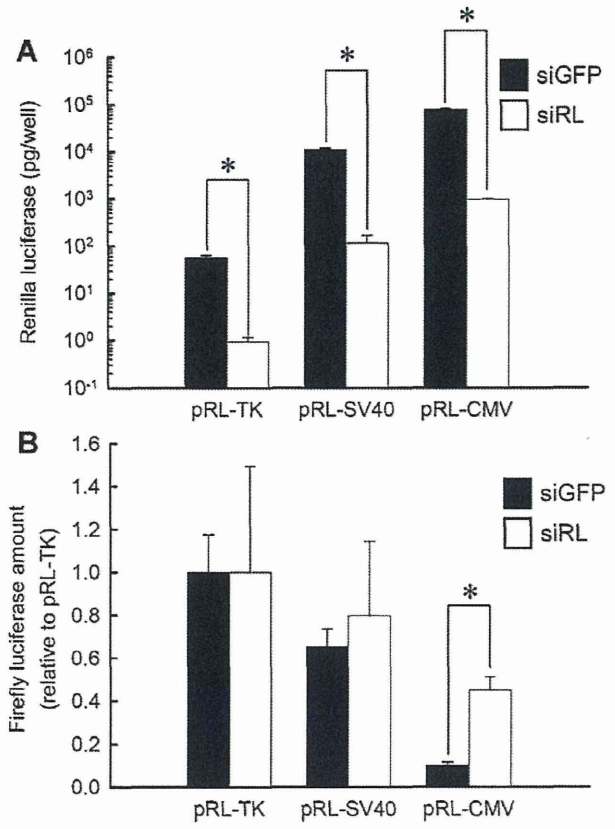

Figure 4. Effect of siRNA-mediated degradation of mRNA expressed from the competing plasmid on the expression from the monitoring plasmid. **A and B**: B16-BL6 cells were cotransfected with siGFP (closed bar) or siRL (open bar) (1 μg/mL), 0.9 μg/mL pRL-CMV, pRL-SV40, or pRL-TK and 0.1 μg/mL pFL-CMV. One day after transfection, renilla (A) and firefly (B) luciferase activities were simultaneously measured. The results are expressed as the mean ± SD ($n = 4$). *$P < 0.05$.

increased. Then, the mRNA and protein amounts of FL were divided by the dose, and the values obtained were used to evaluate the efficiency or saturation of the expression. The efficiency of mRNA expression was not significantly affected by the dose, whereas that of protein expression significantly decreased with an increase in the dose of plasmid DNA.

To evaluate whether endogenous gene expression process is affected by large amount of mRNA expressed from plasmid vectors, the mRNA and protein expression of FL and GAPDH in mouse liver were simultaneously measured 6 h after hydrodynamic administration of 0, 1, or 100 μg pFL-CMV (Fig. 5B). As is the case of the experiment above, the difference in mRNA amount of FL between 1 and 100 μg was bigger than that in protein amount, which suggests the existence of saturation of protein synthesis process of transgene. No significant changes in GAPDH mRNA and protein expression were observed among all treatment group despite the fact that transgene expression process was saturated.

mRNA and Protein Expression of Luciferases in Mouse Liver After Hydrodynamic Injection of Naked Plamid Vectors

Finally, mice received a hydrodynamic injection of 1 μg pRL-CMV and 10 μg pFL-CMV, pFL-SV40, or pFL-TATA, and the levels of mRNA and protein were simultaneously measured (Fig. 5C and D). The amount of FL was dependent on the strength of promoter and proportional to its mRNA level (Fig. 5C). The mRNA level of RL was almost the same in all cases (Fig. 5D), reflecting the fact that the same amount of pRL-CMV was used for hydrodynamic injection. In contrast, the amount of RL protein in the liver of mice receiving pRL-CMV/pFL-CMV was significantly lower than that of mice receiving pRL-CMV/pFL-TATA (Fig. 5C).

Discussion

Transgene expression from monitoring plasmid was reduced by cotransfection with competing plasmid driven by a strong promoter and this phenomenon was independent of the type of cDNA encoded in the monitoring and competing plasmids (Fig. 2). These results suggest that saturation of transgene expression is not dependent on the type of transgene product. In the experiment using pGFP-CMV, cells with a very high fluorescence intensity, which was found after cotransfection of cells with pRL-SV40 or pRL-TK, were not detected when cells were cotransfected with pRL-CMV. The cells with high fluorescence intensity should have been delivered with a large amount of pGFP-CMV and, therefore, they could have also efficiently taken up coexisting plasmids. Therefore, it is reasonable to suggest that transgene expression is saturated especially in cells that encounter a large amount of plasmid DNA. Although the both experiments exhibited a similar trend, the degree of the reduction by co-transfection with pRL-CMV was much less in the GFP experiment than that in the luciferase experiment. The discrepancy may be explained by the difference in the detection sensitivity of GFP and luciferase expression.

Transgene expression from plasmid DNA requires a number of processes, such as delivery of plasmid DNA to the nucleus, mRNA transcription from the DNA, export of mRNA from the nucleus, processing of mRNA, translation, and post-translational modification of protein. In the present study, we found that the level of mRNA and protein expression from monitoring plasmids was affected by the type of competing plasmids cotransfected in B16-BL6 cells (Fig. 3). Moreover, co-transfection of siRL with pFL-CMV (monitoring plasmid) and pRL-CMV (competing plasmid) significantly rescued FL protein expression compared with the co-transfection of siGFP with pFL-CMV and pRL-CMV. These experimental results suggest for the first time that the translational process is highly likely to be saturated when a large amount of protein is produced. A careful comparison of the results showed that FL expression in the cells

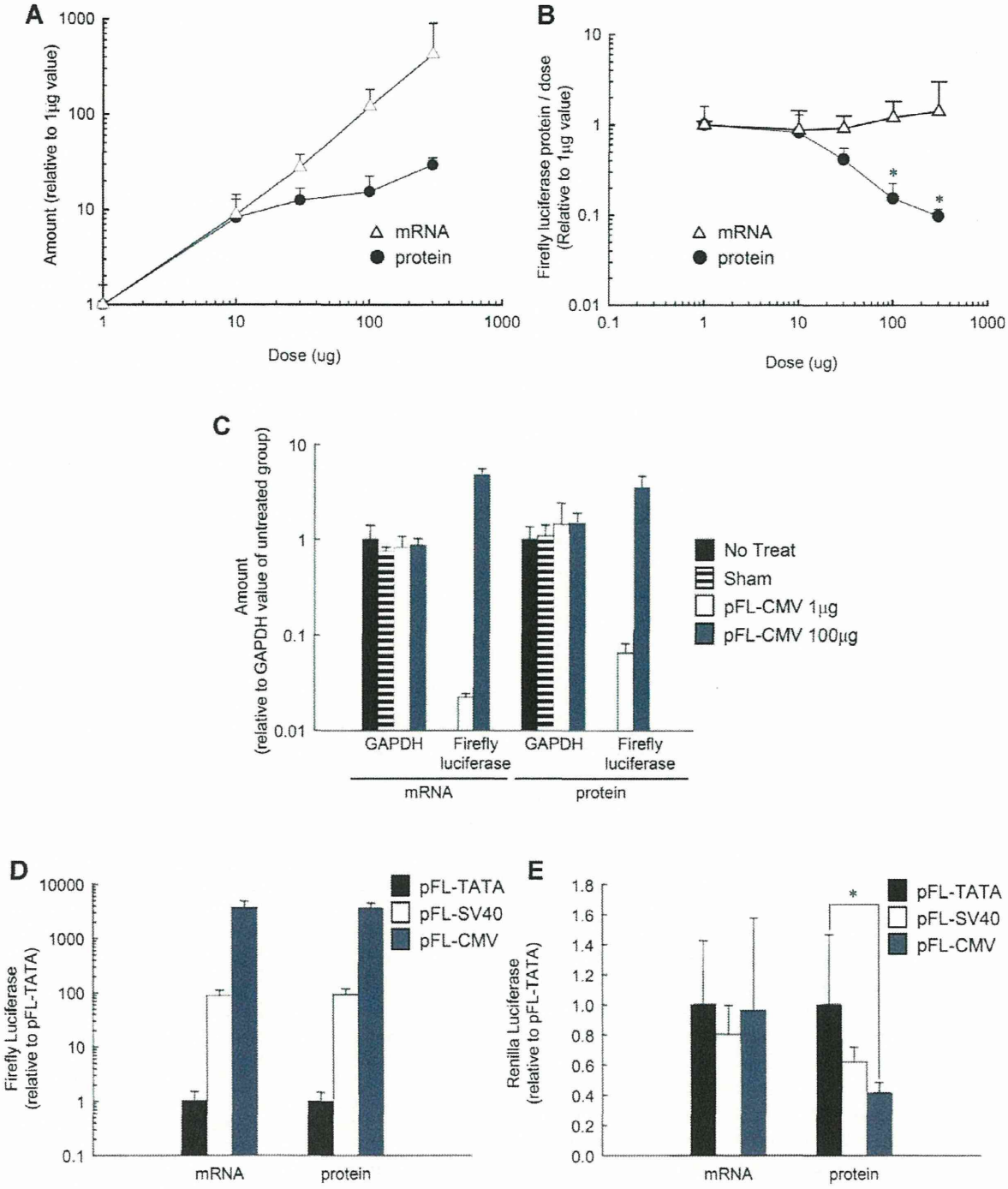

Figure 5. Effect of level of transgene expression from competing plasmid DNA on the expression from monitoring plasmid DNA and endogenous gene in mouse liver. **A**: Mice received a hydrodynamic injection of pFL-CMV at indicated doses. At 6 h after injection, the level of mRNA (open triangle) and protein (closed circle) expression of firefly luciferase were simultaneously measured. The results are expressed as the mean + SD ($n = 4$). **B**: The amount of mRNA or protein expression in (A) was divided by the dose. The results are expressed as the mean + SD of the relative value to that of 1 μg group ($n = 4$). $^*P < 0.05$ versus 1 μg group. **C**: Mice received a hydrodynamic injection of 0 (hatched bar), 1 (open bar), or 100 μg pFL-CMV (gray bar) or were untreated (closed bar). At 6 h after injection, the level of mRNA and protein expression of firefly luciferase and GAPDH were simultaneously measured. The results are expressed as mean ± SD of the relative value to that of GAPDH of the no treatment groups ($n = 4$). **D and E**: Mice received a hydrodynamic injection of 1 μg pRL-CMV and 10 μg pFL-TATA (closed bar), pFL-SV40 (open bar), or pFL-CMV (gray bar). At 6 h after injection, mRNA and protein expression of firefly (C) and renilla (D) luciferase were simultaneously measured. The results are expressed as the mean ± SD ($n = 4$). $^*P < 0.05$.

transfected with pFL-CMV was lower when cells were cotransfected with siRL and pRL-CMV than that with siRL and pRL-TK. As RL amount of the siRL/pFL-CMV/pRL-CMV group was much higher than that of the siRL/pFL-CMV/pRL-TK group, a simple explanation is that siRL-mediated reduction in renilla mRNA was not enough to completely rescue the FL expression. We found a reduced mRNA expression of FL in the pFL-CMV/pRL-CMV group compared to the pFL-CMV/pRL-TK group (Fig. 3B). In addition, siRNA degrades its target mRNA in the cytoplasm so that siRNA does not affect the earlier processes such as transcription. Therefore, the siRL-mediated incomplete rescue of FL protein expression may suggest that not only translation but also the earlier processes such as transcription are also saturated under the experimental conditions used.

The studies using mice led to a slightly different conclusion. Transgene expression was also saturated when a large amount of plasmid DNA was delivered, but the transcription was hardly affected by the dose (Fig. 5A). Therefore, the translational process is likely to be saturated in mouse liver (Fig. 5C and D), which is different from the in vitro results. This apparent discrepancy might be due to the difference in the type of transgene-expressing cells or in the delivery method of plasmid DNA. Further studies are needed to identify factors explaining the discrepancy. The firefly gene sequence in pFL-CMV was not fully codon-optimized for expression in mice and 5% of the codons in the sequence are ones with low frequency (<30%) in mice, so this could be a bottleneck during translation. To our knowledge, no previous study has reported the saturation of transgene expression processes in vivo. Our finding agrees with the results of Carpentier et al. (2007). They found that transcriptional and translational processes are saturated under optimal transfection conditions in which cells were very efficiently transfected with plasmids. In addition, they reported that the translational process is saturated at a dose of plasmid DNA that does not affect the transcriptional process. In the present study, we found that transgene expression process can be saturated when the large amount of plasmid DNA is delivered to mouse liver by hydrodynamic injection. We and others also found a similar phenomenon in lipoplex-mediated and polyplex-mediated transfection of cultured cells, where the mode of gene delivery is different from the hydrodynamic injection (Carpentier et al., 2007). In addition, it has been reported that there is a saturation in secretory pathway after the transfection by adenoviral vectors encoding secretory proteins (Marmorstein et al., 2000). Therefore, we believe that transgene expression could be saturated when cells are transfected using gene vectors with strong promoters.

When the transcription of endogenous genes is inhibited by adenovirus vectors because of the sharing of transcription factors, the expression of endogenous genes was inhibited at the mRNA level, and cells were damaged (Lin et al., 2007). As we observed saturation in the translational process, but not in the transcriptional process, this "promoter squelching" will not be the case. Regarding toxicity, we did not observe any B16-BL6 cell death at 24 h after transfection. As an earlier study by Lin et.al reported that cellular toxicity by promoter squelching was observed at 48 and 72 h after transfection but not at 24 h after transfection, any toxicity in B16-BL6 cells might be observed at later time points. At least 6 h after the hydrodynamic delivery of plasmid DNA, no changes in the mRNA and protein amount of model endogenous gene, GAPDH, was observed. In addition, no damage was observed in the liver of mice at 6 h after hydrodynamic injection. In the case of hydrodynamic gene transfer, peak time of the transgene expression is usually about 6–24 h and transgene expression level declines after the peak time. Therefore, transgene expression after hydrodynamic gene delivery is considered to be not long enough to affect endogenous gene expression.

In conclusion, we demonstrated that the transgene expression process can be saturated both in cultured cells and in mouse liver and that translation is a major process that can be saturated in transgene expression. Therefore, such an approach that can potentiate the gene expression processes of cells such as transcription and translation would be desirable to achieve higher gene expression if the delivery efficiency is high enough to saturate gene expression machinery.

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Development of safe and effective nonviral gene therapy by eliminating CpG motifs from plasmid DNA vector

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1. ABSTRACT

Nonviral gene therapy is expected to become a regular treatment for a variety of difficult-to-treat diseases, such as cancer and virus infection. Plasmid DNA, which is used in most nonviral gene delivery systems, usually contains, unmethylated cytosine-guanine dinucleotides, so called CpG motifs. CpG motifs are recognized by immune cells as a danger signal, leading to an inflammatory response. Such inflammatory responses could affect the safety and effectiveness of nonviral gene therapy. Therefore, reducing the number of CpG motifs in plasmid DNA has been used to increase the potency of plasmid DNA-based gene therapy. Previous studies have demonstrated that CpG reduction can extend the time period of transgene expression from plasmid DNA after *in vivo* gene transfer. In this review, the biological functions of the CpG motif are briefly summarized. Then, safety issues of nonviral gene therapy are discussed from the viewpoint of the inflammatory response to the CpG motif in plasmid DNA, and the effects of the CpG motif in plasmid DNA on the transgene expression profile of nonviral gene transfer are reviewed.

2. INTRODUCTION

The success of gene therapy is much lower than initially expected. Successful application of gene therapy has been hampered by many factors including the acute inflammatory response to gene vectors, carcinogenesis and a limited therapeutic effect (1, 2). In performing gene therapy, the vector that carries the transgene is one of the most important components that determine the therapeutic outcome. Some serious side effects in clinical studies of gene therapy using viral vectors as well as improvements in the efficacy of nonviral gene transfer methods have greatly increased the importance of nonviral gene transfer methods that use no potentially harmful viral vectors in their clinical applications (1-4).

Plasmid DNA is the most frequently used DNA in nonviral gene therapy. As the properties of plasmid DNA, such as its promoter, enhancer and poly adenylation site greatly affect the level and time-course of transgene expression, optimization of the properties of plasmid DNA has been performed by modifying these functional regions (5). In particular, the promoter region has been the most

attractive region for optimization. By selecting an appropriate promoter, the period of transgene expression from the plasmid DNA is extended (6-8). Moreover, drug-inducible promoters and tissue-specific promoters have been developed to control the temporal and special profile of the transgene expression (9-11). Not only these functional sequences in plasmid DNA, but a small sequence of unmethylated CpG dinucleotides has been identified as an important factor that requires great attention. In this review, the biological role of the CpG sequence in the mammalian body is discussed with regard to inflammation and the regulation of endogenous gene expression. Then, the effect of CpG sequences in plasmid DNA on the inflammatory response to administered plasmid DNA is discussed. Finally, the relationship between the CpG sequences in plasmid DNA and transgene expression from plasmid DNA is summarized based on the recent results obtained in animal experiments.

3. BIOLOGICAL ROLE OF CpG MOTIFS IN THE BODY

In vertebrate animals, the frequency of CpG dinucleotides in genome DNA is 1 out of about 50 bases, which is much lower than the mathematical frequency of 1 out of 16 bases. Moreover, most of CpGs are methylated at the carbon 5-position of the cytosine residue, which further reduces the frequency of the unmethylated CpG dinucleotides (12). In contrast, bacterial DNA contains CpG dinucleotides almost as frequently as expected from the mathematical frequency, and most of bacterial CpGs are unmethylated (13). Mammals use this difference in CpGs between bacterial DNA and mammalian DNA, i.e., unmethylated CpG dinucleotides are recognized as a danger signal in mammals. Despite the low frequency of CpG motifs in mammalian DNA, there are some regions in mammalian genome DNA with many CpGs, called CpG islands, which have been recently discovered to play important roles in regulating the expression of a variety of genes. In this section, the latest view of the DNA recognition in mammals and the role of CpGs in gene regulation are briefly summarized.

3.1. Response to CpG dinucleotides in mammals

Unmethylated CpG dinucleotides, or CpG motifs, are recognized as a danger signal by Toll-like receptor 9 (TLR9), one of the pattern recognition receptors that recognize a pathogen-associated molecular pattern (14). It is known that a limited number of cells express TLR9. In humans, a constitutively high level of TLR9 is expressed in plasmacytoid dendritic cells and B cells and, in mice, macrophages and dendritic cells also express high level of TLR9 (15-17). Therefore, the TLR9-mediated response is mainly induced by a limited number of cells. We showed that depletion of phagocytic cells using clodronate liposomes markedly reduced the inflammatory response induced by the intravenous injection of CpG DNA complexed with cationic liposomes (18). This study also demonstrated that splenectomy hardly reduced the response, which suggests that cells outside the spleen are at least involved in the CpG DNA-induced response.

TLR9 is composed of an extracellular domain, a transmembrane domain and a cytoplasmic domain. TLR9 is localized in the endoplasmic reticulum and is delivered to the endolysosomal compartments, where TLR9 meets CpGs (19). As TLR9 recognizes CpG DNA in the endosome, the intracellular localization of CpG-containing DNA is also an important factor that determines the inflammatory response (20). Binding of CpGs to TLR9 recruits signaling adaptor molecules, such as MyD88, which leads to the activation of nuclear factor-κB and activation of gene expression of cytokines and co-stimulatory molecules (21, 22). In addition, recognition of CpG motifs by TLR9 has been reported to be related to the development of autoimmunity (23, 24). An example is the activation of B cells by the immune complexes of DNA and autoantibody in an autoimmune mouse model (25), which led to the secretion of rheumatoid factors through the TLR9-dependent signaling pathway.

3.2. CpG motifs in epigenetic regulation of gene expression

In the nucleus of eukaryotes including mammalian cells, genetic information is preserved in a DNA-protein structure called chromatin. Chromatin consists of the repeat of nucleosome, which contains DNA wrapped around histone proteins. Gene expression from the genome DNA is initiated by the binding of the transcription complex to DNA. Therefore, the interaction of DNA with histone, which sterically affects the transcription complex-DNA interaction, is an important regulatory factor for gene expression. Here, DNA methylation plays a central role in the regulation of the histone-DNA interaction. DNA methylation occurs on cytocine at the CpG dinucleotides and most of the CpGs in the mammalian genome are methylated. CpG islands are the short CpG-rich regions and generally located around promoter. It is reported that methylation status of CpG motifs in CpG islands are controlled in order to regulate the expression of endogenous genes (26). DNA methylation is recognized and bound by methyl-CpG-binding domain proteins, which forms repressor complexes with histone deacetylase (HDAC). HDACs remove the acetyl group from histones. Hyperacetylated histones are generally associated with chromatine decondensation, which increases accessibility of DNA to binding proteins and the transcription activity (27, 28). However, hypoacetylated histones are generally associated with chromatin condensation, which reduces transcription activity (27, 28).

4. CpG MOTIFS IN PLASMID DNA VECTOR

As plasmid DNA is a bacteria-derived DNA, it consists of bacteria-derived regions which contains many CpG motifs other than the cassette for transgene expression such as promoter, cDNA, enhancer and polyA (Table 1). As it has been considered that bacteria derived sequences such as replication ori are not essential for transgene expression, these regions initially attracted less attention than the transgene expression cassette. Some recent studies have shown that bacteria derived sequences in plasmid DNA can also affect the profile of transgene expression after gene transfer. Li *et al.* demonstrated that the PCR-

Table 1. Number of CpG motifs in the typical functional regions of plasmid DNA

Replication ori		Drug resistance gene		Promoter		poly A	
pUC ori	45	Ampicillin	49	CMV	31	BGH poly A	3
pMB1 ori	48	Kanamycin/Neomycin	73	SV40	10	SV40 poly A	0
R6K ori	0	zeocin	51	EF1	94		
		hygromycin	103	ROSA26	213		

The number of CpG motifs in one strand of each region was summarized, so those in double stranded plasmid DNA should be doubled.

amplified fragment of the transgene expression cassette reduced the level of transgene expression but resulted in more sustained gene expression (29). Chen *et al.* reported that simply eliminating bacteria-derived regions by using restriction endonuclease was effective in obtaining more persistent transgene expression than parental plasmid DNA (30). In their following study, they further optimized their expression cassette by obtaining a minicircle DNA vector containing only the expression cassette, which showed more sustained transgene expression than the conventional plasmid and plasmid DNA fragment obtained by restriction endonuclease (31). These pieces of experimental evidence suggest that bacteria-derived sequences have negative effects on the transgene expression from plasmid DNA.

As already described, the CpG motif is a typical sequence that is frequently observed in bacteria DNA as well as plasmid DNA, a bacteria-derived DNA. Therefore, plasmid DNA usually contains many CpG motifs. Unmethylated CpG motifs could be methylated and the methylation status of CpG motifs is associated with histone modification. As these changes influence the transgene expression from plasmid DNA, the transgene expression from plasmid DNA can be regulated via CpG motifs by the same mechanism as the epigenetic regulation of endogenous DNA. In addition, CpG motifs are TLR9 ligands so that CpG motifs in plasmid DNA have been shown to induce an inflammatory response. In the following section, the effect of CpGs in plasmid DNA on the response to the administered DNA and the transgene expression is discussed.

4.1. Inflammatory response to plasmid DNA

When plasmid DNA containing CpG motifs is administered *in vivo*, it can induce an inflammatory response if CpG motifs are recognized by TLR9 (Figure 1). As TLR9 is expressed in limited types of cells and TLR9 generally interacts with CpG motifs in endosomes, the intracellular distribution of the DNA as well as the distribution of the DNA in the body determines the response against DNA. It has been shown that gene transfer of naked plasmid DNA sometimes induces an inflammatory response. Moreover, carrier-based gene delivery, in which liposomes and polymers are used to efficiently deliver plasmid DNA, is frequently associated with a more severe inflammatory response than naked plasmid DNA, which is probably due to the differences in their distribution in the body and in cells (32, 33). Compared with carrier-based gene therapy, naked DNA-based gene delivery generally results in a weaker inflammatory response to the plasmid DNA. In this section, the inflammatory responses to naked plasmid DNA or plasmid DNA/carrier complex are separately discussed, because the intracellular distribution of plasmid DNA has been reported to be quite different between the naked DNA delivery and the carrier-based DNA delivery. In addition, TLR9-independet inflammatory response against plasmid DNA is also discussed.

4.1.1. Inflammatory response to plasmid DNA

Tissue injection of naked plasmid DNA is the simplest form of *in vivo* gene delivery. Therefore, it has often been used in nonviral gene therapy including some clinical trials (4). In particular, intramuscular injection is frequently used to deliver naked DNA (34). When a plasmid DNA containing CpG motifs is administered intramuscularly, an inflammatory response is induced in a CpG-dependent manner. It has been reported that CpG motifs in plasmid DNA vector induce the expression of chemokines and MHC class II in muscle (35). In the case of DNA vaccine, in which plasmid DNA encoding antigen is administrated to induce an antigen-specific response, the inflammatory response induced by CpG motifs is advantageous because it can boost the immune response (36). However, the inflammatory response to plasmid DNA is not favorable for other forms of gene therapy because the inflammatory response is a harmful side effect and in some cases, transgene-expressing cells are eliminated by the response. To avoid the inflammatory response following intramuscular injection of plasmid DNA, Reyes-Sandoval *et al.* administered plasmid DNA that had been methylated *in vitro* (37). As a result, they succeeded in reducing the immune response induced by plasmid DNA. In order to increase the transgene expression level, various physical stimulations, such as electroporation and sonoporation, ca be applied after local DNA administration. However, these physical stimulations may induce an inflammatory response, probably in a CpG-independent manner (38-40), although the degree of inflammation is much less compared with that induced by carrier-based delivery.

Simple intravascular injection of naked plasmid DNA generally results in little or no transgene expression. However, it can still induce an inflammatory response. For example, in mice, systemic injection of naked plasmid DNA with many CpG motifs at regular doses of about 1 mg/kg produced no detectable level of TNF-α in serum while a large naked plasmid DNA injection induced TNF-α secretion in the blood circulation, and less TNF-α was produced when large amount of plasmid DNA with few CpGs was injected (33). As a mode of naked DNA delivery for systemic administration, so called hydrodynamic

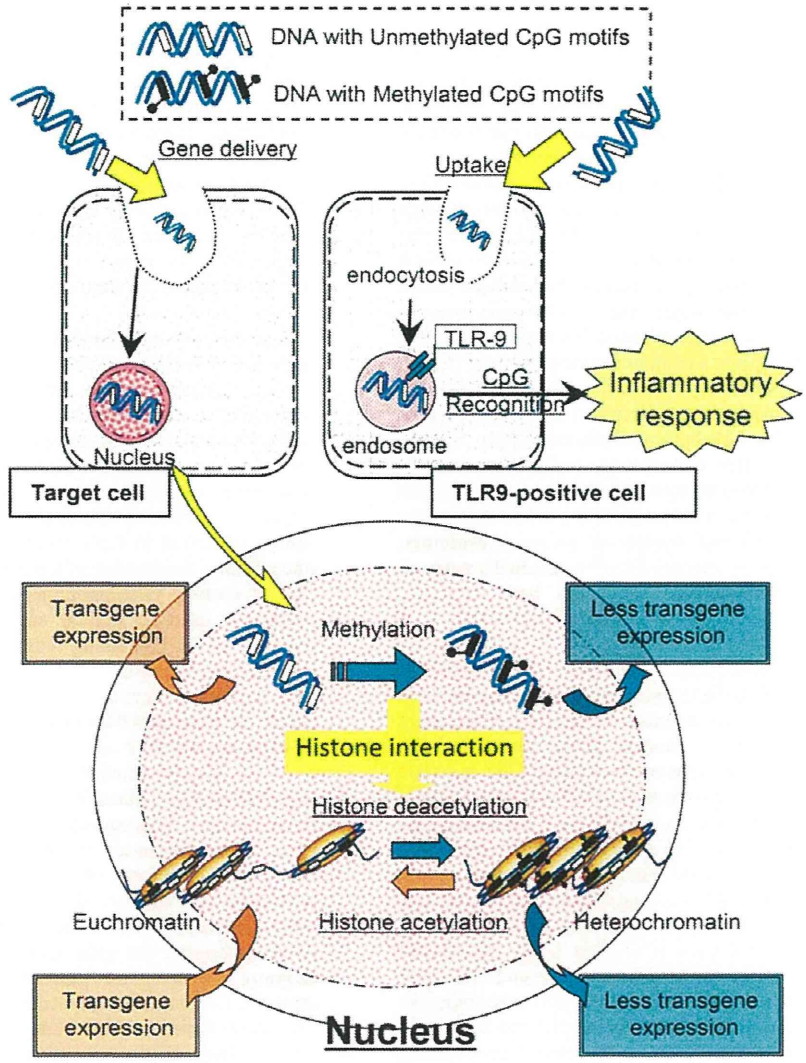

Figure 1. Schematic image of the role of CpG motifs in nonviral gene therapy. For nonviral gene therapy, plasmid DNA should be delivered to the nucleus of target cells. After intranuclear delivery of plasmid DNA, plasmid DNA may be methylated at the CpG motif, which decreases the level of transgene expression from the DNA. Plasmid DNA in the nucleus is likely to interact with histone. The level of transgene expression from plasmid DNA closely associated with histone, a heterochromatine-like structure, is lower than the DNA loosely associated with histone, a euchromatin-like structure. Plasmid DNA administered to mammals is often taken up by TLR9-positive cells. Plasmid DNA with unmethylated CpG motifs is recognized by TLR9 in endosomes, which induces an inflammatory response.

injection, in which a large volume of naked plasmid DNA solution is rapidly injected, is one of the most efficient methods to obtain a high level of transgene expression. Although the level is not very high, hydrodynamic injection of plasmid DNA at regular dose increases TNF-α in a CpG-dependent manner (41). However, hydrodynamic injection of saline without DNA increases the serum IL-6 concentration, which suggests that the gene delivery method itself may produce an inflammatory response in some situations.

4.1.2. Inflammatory response to plasmid DNA delivered by carrier

Carrier-based nonviral gene delivery is also an accepted delivery method because it is associated with multiple functions such as the controlled delivery of plasmid DNA. Cationic liposomes and polymers are frequently complexed with plasmid DNA as carriers to increase delivery efficiency. However, complexing plasmid DNA with cationic lipids not only increases the delivery efficiency, but also increases inflammatory responses.

Incubation of cationic lipids/plasmid DNA complex, or lipoplex, with macrophages or dendritic cells stimulates the cells to release more inflammatory cytokines than that with naked plasmid DNA (42, 43). Like the situation *in vitro*, intravenous administration of lipoplex to mice results in more inflammatory cytokine production than that produced by naked plasmid DNA (18, 33). Moreover, systemic delivery of plasmid DNA complexed with cationic lipid induces an acute inflammation with adverse hematologic changes and liver damage (44, 45). Therefore, the inflammatory response to plasmid DNA is a more serious problem for carrier-based gene therapy than that for naked gene therapy. On the other hand, little inflammatory response was observed when plasmid DNA was complexed with polyethyleneimine, a cationic polymer frequently used as a nonviral vector (46). Such differences in the inflammatory response were discussed in relation to the altered intracellular distribution of plasmid DNA. Saito *et al.* reported that after endocytocis, polyplexes tend to escape from the endosome more effectively than lipoplexes (47). As the endosome is the place where TLR9 recognizes CpG DNA, an efficient escape of polyplex from the endosome may be a reason for the less inflammatory response against polyplexes.

Results from *in vitro* experiments using cultured macrophages and dendritic cells show that the secretion of inflammatory cytokine from these cells induced by lipoplex is CpG-dependent. Therefore, to avoid the inflammatory response against lipoplex, Yew *et al.* developed a CpG-depleted plasmid DNA vector. As a result, the systemic delivery of a CpG depleted plasmid DNA/cationic lipid complex induced a weaker inflammatory response, less liver damage and fewer hematologic changes (48). The effect of CpGs in plasmid DNA on the inflammatory response was more dramatically demonstrated by Hyde *et al* (49). They constructed plasmid DNAs with different numbers of CpG dinucleotides (from 0 to 317 CpGs), and complexed the plasmid DNAs with a cationic lipid formulation. The complex was administered to the airways of mice as an aerosol. Administration of plasmid DNA with 317 and 193 CpGs induced almost the same degree of lung inflammation. Plasmid DNA with 1 CpG still induced lung inflammation, although the level of inflammation was lower than that produced by plasmid DNA with 193 and 317 CpGs. Administration of plasmid DNA without CpG hardly induced lung inflammation compared with the mock group. These results suggest that even one CpG in plasmid DNA can induce an inflammatory response. Therefore, elimination of CpG sequences is an effective approach to reducing the degree of inflammatory response to plasmid DNA delivered as a complex with carriers.

4.1.3. TLR9-independent recognition and inflammatory response to plasmid DNA

Although TLR9 is the receptor for CpG DNA, other proteins are also involved in the recognition of both endogenous and exogenous DNA in a CpG motif-independent manner. Recently, several candidates for the receptors recognizing DNA in the cytosol have been reported. Ishii *et al.* reported the importance of TANK-binding kinase 1 in the immunostimulation by DNA vaccines (50). Separately, Takaoka *et al.* found that DAI (DNA-dependent activator of IFN-regulatory factors) recognizes cytosolic DNA irrespective of the presence of CpGs (51). They demonstrated that recognition of non-CpG DNA by DAI in fibroblasts resulted in type I interferon production. More recently, Unterholzner *et al.* identified IFI16, a member of the PYHIN protein family, as a cytosolic censor for DNA (52). They found that microbial DNA is recognized by IFI16 in monocytes, which results in type I interferon production. Therefore, inflammatory responses induced by TLR9-independent pathway should be considered in the future development of gene therapy.

4.2. Effect of CpG motifs in plasmid DNA on transgene expression from the DNA

Recently, CpG depletion from plasmid DNA is regarded to be effective not only in reducing the inflammatory response but also in increasing the duration of transgene expression from the DNA. Prolongation of transgene expression by eliminating bacteria-derived regions to produce a minicircle vector may also be because of the reduction in CpG motifs. In the following section, the effect of the number of CpG motifs in plasmid DNA on the transgene expression from the plasmid DNA is discussed first followed by an examination of how CpG-elimination is related to the transgene expression from plasmid DNA.

4.2.1. Relationship between transgene expression profile from plasmid DNA and the number of CpG motifs

The profile of transgene expression after the administration of plasmid DNA with different numbers of CpG by the hydrodynamics-based procedure has been investigated frequently regarding the transgene expression from plasmid DNA in naked form (41, 48, 53-55). In those studies, administration of CpG-depleted plasmid DNA by the hydrodynamics-based procedure resulted in prolongation of the gene expression in most cases. Our group has also reported that the number of CpG motifs in plasmid DNA is inversely correlated with the duration of transgene expression from the plasmid DNA. By using a mouse lung metastasis model, we demonstrated that administration of interferon-gamma (IFNγ)-expressing plasmid DNA with less CpGs by the hydrodynamic injection method was more effective in inhibiting tumor growth in the lung than the conventional IFN-expressing plasmid DNA with many CpGs (41, 55). Moreover, a single administration of IFNγ-expressing plasmid DNA with much fewer CpGs suppressed the onset of atopic dermatitis in Nc/Nga mice while multiple administration of conventional IFNγ-expressing plasmid DNA could not (53). Therefore, our studies suggest the possibility that prolonging the transgene expression period is an effective approach to improve the therapeutic effect after gene therapy.

The effect of CpG elimination from plasmid DNA on transgene expression was observed not only with naked DNA delivery but also with carrier-based DNA delivery. Yew *et al.* intravenously administered a lipoplex of plasmid DNA with few or many CpG motifs (48). They found that administration of lipoplex with less CpG motifs

resulted in higher and more sustained transgene expression in the lung than that of lipoplex with many CpG motifs. The same trend was observed when plasmid DNA complex was administrated into the airways by aerolization (49). Administration of CpG-free plasmid DNA produced higher and more sustained gene expression in the lung. Not only in the case of lipoplex, but also in the case of plasmid DNA/cationic polymer (polyplex), CpGs in plasmid DNA had an effect on the transgene expression. DeWolf *et al.* administered CpG-rich or CpG-free plasmid DNA complexed with liposome or polyethyleneimine and found that the depletion of CpG motifs within the plasmid DNA of lipoplex and polyplex enhances the degree and duration of transgene expression (56).

4.2.2. Factors that link CpG motifs with transgene expression

Compared with the established role of CpG motifs in the induction of an inflammatory response, the mechanism whereby CpG-depleted plasmid DNA generally produces more sustained transgene expression is still unclear. To date, several putative mechanisms have been postulated. Persistence of plasmid DNA within cells is a prerequisite for the long-term transgene expression and, therefore, CpG-reduced plasmid DNA might remain longer than CpG-rich plasmid DNA after administration. However, there has been little evidence to support this hypothesis. Another possible mechanism involves the inflammatory response. As it has been demonstrated that inflammatory cytokines such as TNF-α and IFNs suppress transgene expression, the inflammatory response induced by CpG motifs may reduce and suppress transgene expression (57, 58). Therefore, avoiding the inflammatory response by eliminating CpGs from plasmid DNA can positively affect transgene expression. However, reduced inflammatory response alone cannot be the mechanism whereby CpG depletion has a positive effect on transgene expression because CpG depletion can also improve transgene expression after naked DNA delivery, in which the inflammatory response is induced to a much lower extent than carrier-based DNA delivery. Moreover, the time courses of transgene expression from plasmid DNA administered by hydrodynamic delivery were almost identical between the control mice and those injected with lipoplexes to induce inflammatory responses (55). Another putative mechanism is CpG methylation (Figure 1). As enzymatically methylated plasmid DNA shows lower transgene expression *in vivo* than the unmethylated plasmid DNA (55), CpG methylation has a negative effect on the transgene expression from plasmid DNA. Therefore, eliminating the CpG motifs reduces the number of methylation target sites, which can prevent silencing by methylation and extend the period of transgene expression. The other hypothetical mechanism is the interaction of histone with plasmid DNA, which is related to the methylation of DNA (Figure 1). Endogenous DNA in the nucleus usually interacts with histone. It has been reported that administered plasmid DNA also interacts with histone after it reaches the nucleus (59). The interaction of histone with plasmid DNA, a heterochromatin-like structure, reduces the level of transcription. However, the weak interaction of plasmid DNA with histone, a euchromatin-like structure, transcribes more mRNA. As interaction of DNA with histone is suggested to be regulated by CpG motifs, changing the number of CpG motifs changes the interaction mode of plasmid DNA with histone, which might affect the transgene expression profile.

5. CONCLUSION

Although the number of CpG motifs in plasmid DNA contributes to the safety and effectiveness of nonviral gene therapy remains a matter of debate, CpG-free plasmid DNA is much less likely to induce an unexpected response such as inflammation than CpG-replete plasmid DNA. Especially in performing carrier-based nonviral gene therapy, the response to CpG motifs is boosted compared with that of naked DNA-based, which could interfere with the evaluation of the effect of transgene products not only in animal experiments but in clinical trials. These considerations clearly indicate that the use of CpG-free plasmid DNA is desirable even in animal experiments. As CpG elimination has great advantages and seems to have few disadvantages, a CpG-depleted or CpG-free DNA vector will be the standard DNA for use as nonviral gene therapy in the future.

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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

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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

(13-15), and more importantly, the liver with these nodules might reflect a higher potential for hepato-carcinogenesis or the presence of undetectable precursor lesions in other sites of the liver. Conversely, the absence of these nodules potentially identifies the patients at a low risk for subsequent typical HCC development at any sites. The purpose of this study was to assess the risk of subsequent typical HCC development at any sites of the liver with and without hypovascular hypointense nodules on gadoxetic acid–enhanced MRI.