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Original article

Comparative analysis of CpG islands in two genotypes of African swine fever virus

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Abstract

African swine fever (ASF) is an acute, hemorrhagic, and devastating viral infectious disease that causes important economic losses to the swine industry. Currently, there are no effective vaccines or drugs available. Epigenetic mechanisms, especially cytosine methylation of cytosine--phosphate-guanine (CpG) islands, have a significant impact on the life cycle of several viruses. Hence, drugs targeting DNA methylation may potentially be used for the treatment of ASF. Here, we selected the inner core, core shell, inner membrane, capsid, and external envelope membrane, to analyze the characteristics of CpG islands in the ASF virus (ASFV) genomes. Furthermore, we analyzed the promoters and CpG islands in the upstream regions of these genes. Results showed that the CpG islands of seven genes were conserved in the genomes of two genotype of ASFV strains, whereas the CpG islands of other genes were relatively conserved (ASFV strains differed mainly in the quantity of CpG islands). The different distribution of CpG islands in the genomes of different ASFV strains may affect their methylation status, which may in turn affect the regulation of viral gene expression, leading to different clinical outcomes. In addition, the predicted promoter regions based on the upstream sequences of most genes overlapped with CpG island positions. Methylation of the binding sites of the promoter regions inhibits the binding of the transcription factors to the promoters, thus inhibiting the activation of the promoters and limiting the synthesis of viral proteins. The results of this study provide a basis for exploring new antiviral therapeutic strategies from an epigenetic perspective.

Key words: African Swine Fever Virus (ASFV), genotype, DNA methylation, CpG island

Introduction

African swine fever (ASF) is a devastating viral hemorrhagic porcine disease that can kill up to 100% of affected domestic pigs (Schafer et al. 2022). It is a severe infectious disease caused by African swine fever virus (ASFV) infection in pigs that is characterized by high fever, acute and high mortality, hemorrhage, and lymphoid necrosis (Njau et al. 2021, Ata et al. 2022). ASF first appeared in Kenya in 1921 and spread to most parts of central and southern Africa (Costard et al. 2013). In 1957, ASF was first introduced into Europe through Portugal (Sanchez-Cordon et al. 2018, Cwynar et al. 2019). Subsequently, ASF was reported in Spain, France, Belgium, the Netherlands, and Italy (Sanchez-Cordon et al. 2018, Cwynar et al. 2019,

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Gaudreault et al. 2020). In 1971, it was introduced from Western Europe into Cuba, which was followed by outbreaks in Haiti, Dominican Republic, Brazil, and other South American countries (Sanchez-Cordon et al. 2018, Gaudreault et al. 2020). In 2007, it was introduced into the Caucasus region bordering Eurasia (Georgia, Armenia, and Azerbaijan) and the Russian Federation (Sanchez-Cordon et al. 2018, Gaudreault et al. 2020). In 2017, there were several outbreaks of ASF in the Russian Far East (Sanchez-Cordon et al. 2018, Gaudreault et al. 2020). Before 2018, it was mainly prevalent in Africa, Central and Eastern Europe, and the Caucasus (Sanchez-Cordon et al. 2018, Gaudreault et al. 2020). Since 2018, ASF has also rapidly expanded to Asian countries such as China, Mongolia, Vietnam, Cambodia, North Korea, Laos, Myanmar, Timor-Leste, the Philippines, South Korea, and Indonesia, causing severe economic losses to the pig industry (Gaudreault et al. 2020, Kedkovid et al. 2020, Ito et al. 2022).

ASFV is the only member of the genus ASFV in the Family ASFV, and the only arbovirus with a DNA genome (Njau et al. 2021). The viral genome is a linear, covalently closed double stranded DNA (dsDNA) molecule (Njau et al. 2021). The length ranges from 170 to 193 kb (Njau et al. 2021). It contains 151-167 open reading frames (ORFs) and can encode more than 200 proteins, of which more than 60 are structural and more than 100 are non-structural (Wang et al. 2021). The virus particle consists of five layers, including the inner core, core shell, inner membrane, capsid, and external envelope membrane (Wang et al. 2021). According to the partial sequence of the B646L gene, which encodes the main capsid protein P72, ASFV can be divided into 24 different genotypes (Gaudreault et al. 2020, Qu et al. 2022). Of the 24 known ASFV genotypes, only genotypes I and II are endemic outside Africa. Genotype I is present mainly in West Africa; ASFV emerged in Europe, the Russian Federation, and South America from the late 1950s to the early 1980s, but was largely eradicated by the mid-1990s (Sanchez-Cordon et al. 2018, Gaudreault et al. 2020, Kedkovid et al. 2020). In 2021, genotype I ASFV emerged in China (Sun et al. 2021). Genotype II is currently prevalent in Europe and Asia (Sanchez-Cordon et al. 2018, Gaudreault et al. 2020, Kedkovid et al. 2020). At present, there are no effective drugs or vaccines for protection against ASFV infection.

Cytosine-phosphate-guanine (CpG) islands are CpG-rich regions (Venter et al. 2001). In eukaryotes, about 1–2% of the entire genome is composed of regions with a higher CpG density than the rest of the genome (Venter et al. 2001). CpG islands often appear in the regulatory regions of coding genes (Venter et al. 2001). For example, approximately 70% of gene promoters are located within CpG islands. DNA methylation alters genetic expression without altering the DNA sequence (Almouzni and Cedar 2016, Galbraith and Snuderl 2022). Both in humans and animals, DNA methylation is involved in gene expression regulation, DNA repair, chromosome stability, and maintenance of normal cell differentiation (Almouzni and Cedar, 2016, Bind et al. 2022, Mattei et al. 2022). CpG island methylation affects the structure of chromatin and is often involved in the regulation of gene expression (Bind et al. 2022, Mattei et al. 2022). Under the action of DNA methyltransferase, a methyl group is covalently bonded to the 5' carbon position of the cytosine in the CpG dinucleotides to generate 5-methyl group cytosine (Almouzni and Cedar 2016, Bind et al. 2022, Mattei et al. 2022).

DNA methylation is widespread in viral genomes and plays an important role in the regulation of viral gene expression and interaction with the host (Xiao et al. 2022). Recently, methylation was suggested as a novel host defense mechanism (Arumugam et al. 2021). For example, DNA methylation leads to the down-regulation of hepatitis B virus (HBV) gene expression, and is closely related to latent and persistent infections of various viruses such as human immunodeficiency virus (HIV-1), Epstein-Barr virus, human papilloma virus, and HBV (von Knebel Doeberitz and Prigge 2019, Arumugam et al. 2021, Zhang et al. 2021, Guo and Gewurz 2022). Therefore, drugs that promote viral DNA methylation may be a viable antiviral treatment strategy. However, there is currently little information available on the characteristics and methylation status of CpG islands within the ASFV genome. A study on CpG island characteristics and methylation status of partial gene sequences in the ASFV genome of the BA71V strain (genotype I) showed that, among the 13 ASFV genes analyzed, seven genes contained at least a CpG island (Weber et al. 2018). At the same time, samples of Vero cells infected with the BA71V strain were analyzed at different time points using bisulfite sequencing technology (Weber et al. 2018). No methylated cytosine residues were found in the selected ASFV genome sequences, but the existence of CpG island methylation sites in the remaining genes of the genome could not be ruled out(Weber et al. 2018). Here, our aim was to analyze and compare the features, including number, distribution, and length, of the CpG islands present in the genomes of genotype I and II ASFV strains.

Table 1. Locations and lengths of CpG islands within gene sequences encoding core shell protein of African swine fever virus (ASFV) strains.

Strains		BA71V strain of ASFV			Liaoning strain of ASFV		
Protein (Layer)	Gene	Islands	Location (bps) in Figure	Length (bps)	Islands	Location (bps) in Figure	Length (bps)
			229 - 328	100		457 - 570	114
	CP530R	3	457 - 563	107	2	1080 - 1187	108
			1008 - 1187	180			
			2109 - 2271	163		2106 - 2314	209
			2572 - 2720	149		4678 - 4786	109
			4657 - 4774	118		4855 - 4992	138
Core shell	CP2475L	7	4857 - 4989	133	6	5114 - 5410	297
			5155 - 5410	256		5516 - 5686	171
			5584 - 5683	100		5785 - 5888	104
			5782 - 5915	134			
			125 - 269	145		125 - 262	138
	S273R	2	441 - 751	311	3	447 - 578	132
						603 - 751	149

Materials and Methods

Sequence information

The nucleotide sequence of the genome of the ASFV strains BA71V (GenBank: NC_001659.2; genotype I) and Liaoning (GenBank: MK333181.1; genotype II) from GenBank of National Biotechnology Information Center was searched using "African swine fever virus," "complete genome," and "LN" or "BA71V" as search terms. The gene regions encoding the proteins of the inner core (including A104R, A224L, B475L, C147L, C962R, D205R, D339L, D1133L, E165R, EP296R, EP424R, EP1242L, G1340L, H359L, K78R, NP419L, NP868R, NP1450, O174L, and Q706L), core shell (including CP530R, CP2475L, and S273R), inner envelope membrane (including B119L, D117L, E183L, E248R, H108R, KP177R, and O61R), capsid (including B438L, B646L, and E120R), and external envelope membrane (including EP402R) were selected from the whole genome sequence. Furthermore, the 2000 bp upstream sequences of the above selected genes were extracted and used as the promoter prediction regions.

Promoter prediction

Promoter sites were predicted using the online prediction software Berkeley Drosophila Genome Project (http://www.fruitfly.org/seq_tools/promoter.html).

CpG island analysis

CpG island analysis was performed on genes encoding the proteins of the inner core, core shell, inner membrane, capsid, and external envelope membrane, and on the upstream 2000 bp sequences of the above genes. The online software Meth Primer was used to predict the CpG islands of the above genes from two ASFV strains (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi). This method predicts CpG islands by detecting the GC content and determining the observation/expected value (observation-to-expected CpG dinucleotide ratio) of a sequence window. The standard setting of the CpG island is: sequence window length not less than 100 bases; GC content not less than 50%; observation/expected value not less than 60%.

Results

Analysis of the CpG Islands in the Genotype I ASFV Strain

First, we predicted the CpG islands in the genes of the ASFV strains BA71V (Genotype I), and their number, location, length, and distribution characteristics were analyzed. The results showed that, among the analyzed genes encoding the ASFV core shell proteins, three genes contained at least two CpG islands (Table 1). Among them, CP530R contained three CpG islands with lengths of 100, 107, and 180 bp (Table 1). S273R contained two CpG islands, with lengths of 145 and 311 bp. CP2475L contained seven CpG islands, with lengths of 163, 149, 118, 133, 256, 100 and 134 bp (Table 1). Of the analyzed genes encoding ASFV inner core proteins, 12 genes contained at least one CpG

island (Table 2). Among them, C147L, D205R, EP296R, NP419L, and NP868R had one CpG island, with lengths of 186, 141, 137, 153 and 222 bp, respectively; H359L had two CpG islands with lengths of 118 and 120 bp (Table 2). Q706L, C962R, and D1133L contained four CpG islands each; the lengths of the Q706L CpG islands were 111, 162, 213, and 127 bp, those of the C962R CpG islands were 174, 148, 358, and 143 bp, and those of the D1133L CpG islands were 319, 182, 100, and 189 bp (Table 2). G1340L had five CpG islands with lengths of 140, 206, 123, 110, and 106 bp (Table 2). EP1242L had seven CpG islands with lengths of 546, 288, 134, 111, 217, 121, and 276 bp (Table 2). There were 10 CpG islands in NP1450L, with lengths of 112, 197, 149, 123, 285, 171, 222, 108, 192, and 129 bp (Table 2). Among the analyzed genes encoding ASFV inner envelope proteins, E183L contained one CpG island with a length of 173 bp (Table 3). Among the analyzed genes encoding ASFV capsid proteins, B438L and B646L contained one CpG island each, with lengths of 102 and 154 bp, respectively (Table 3). Among the analyzed genes encoding ASFV external envelope membrane proteins, EP402R contained one CpG island with a length of 165 bp (Table 3).

Analysis of CpG Islands in the Genotype II ASFV Strain

Next, we analyzed the number, location, length, and distribution characteristics of the CpG islands present in the genes of the ASFV strain Liaoning (genotype II). The results showed that, among the genes encoding ASFV core shell proteins, three contained at least two CpG islands (Table 1). Among them, CP530R had two CpG islands, with lengths of 108 and 114 bp (Table 1). S273R contained three CpG islands, with lengths of 132, 138, and 149 bp. CP2475L contained six CpG islands, with lengths of 104b, 109, 138, 171, 209, and 297 bp (Table 1). Among the genes encoding ASFV inner core proteins, 12 contained at least one CpG island (Table 2). The lengths of the CpG islands in C147L, D205R, EP296R, NP419L, and NP868R were 183, 136, 137, 130, and 215 bp. H359L had two CpG islands, with lengths of 115 and 126 bp (Table 2). G1340L and Q706L contained three CpG islands each, with lengths of 118, 124, and 206, and 148, 213, and 124 bp, respectively. C962R contained four CpG islands, with lengths of 141, 151, 176, and 384 bp (Table 2). D1133L had five CpG islands, with lengths of 315, 175, 183, 102, and 189 bp (Table 2). EP1242L had eight CpG islands, with lengths of 474, 279, 119, 292, 181, 102, 130, and 165 bp (Table 2). NP1450L contained eleven CpG islands, with lengths of 111, 191, 147, 125, 242, 226, 104, 222, 126, 192, and 127 bp (Table 2). Among the analyzed genes encoding ASFV inner envelope proteins, E183L contained one CpG island with a length of 165 bp (Table 3). Of the analyzed genes encoding ASFV capsid proteins, B646L contained one CpG island with a length of 150 bp (Table 3). Among the analyzed genes encoding ASFV external envelope membrane proteins, none of the genes contained CpG islands (Table 3).

Comparison of the CpG islands in genotype I and II ASFV strains

We further compared the CpG islands present in the genomes of BA71V (genotype I) and Liaoning (genotype II) ASFV strains. The results showed that, of the genes encoding ASFV core shell proteins, CP530R and CP2475L from the BA71V strain had one more CpG island than those from the Liaoning strain (Table 1). In contrast, S273R had one less CpG island in the BA71V strain than in the Liaoning strain (Table 1). Among the analyzed genes encoding ASFV inner core proteins, D1133L, EP1242L, NP1450L, and Q706L had one less CpG island each in the BA71V strain than in the Liaoning strain. In contrast, G1340L had two more CpG islands in the BA71V strain than in the Liaoning strain (Table 2). Among the analyzed genes encoding ASFV capsid and external envelope membrane proteins, B438L and EP402R had one CpG island each in the BA71V strain, while those in the Liaoning strain did not have CpG islands (Table 3).

Promoter activity of the genes

After the software was run, the predicted results were output. The reliability of the predicted results increased with increasing their score. The score value ranged from 0.8 to 1.0, indicating that the predicted results were reliable and the predicted promoter activity was credible. In addition, the larger font base in each predicted result of the promoter sequence represents the transcription start site. Meanwhile, MethPrimer software was used to predict CpG islands in the 2000 bp upstream sequence of the genes, and the number, location, length, and distribution characteristics of the CpG islands were analyzed. The 2000 bp upstream sequence of CP204L, S183L, CP2475L, S273R, C147L, D339L, D1133, G1340L, H359L, NP1450L, O174L, Q706L, C962R, D205R, E165R, E296R, EP424R, NP868R, B119L, D117L, E183L, E248R, H108R, B438L, B646L, and E120R in the ASFV strains BA71V (Genotype I) contained at least one promoter active region and one CpG island. In the ASFV strain Liaoning (genotype II), those sequences also contained at least one promoter active region and one CpG island, except for the sequence upstream of B438L. The promoter

Table 2. Locations and lengths of CpG islands within gene sequences encoding inner core protein of ASFV strains.

Strains		BA71V strain of ASFV			Liaoning strain of ASFV		
Protein (Layer)	Gene	Islands	Location (bps) in Figure	Length (bps)	Islands	Location (bps) in Figure	Length (bps)
	C147L	1	200 - 385	186	1	204 - 386	183
			502 - 675	174		500 - 675	176
	C962R	4	1247 - 1394	148	4	1291 - 1441	151
			1990 - 2347	358		1963 - 2346	384
			2486 - 2628	143		2486 - 2628	141
	D205R	1	419 - 559	141	1	24 - 559	136
			1406 - 1724	319		1138 - 1452	315
			2332 - 2513	182		1596 - 1770	175
	D1133L	4	2826 - 2925	100	5	2061 - 2243	183
			3155 - 3343	189		2554 - 2655	102
						2885 - 3073	189
	EP296R	1	538 - 674	137	1	538 - 674	137
			51 - 596	546		59 - 532	474
			677 - 964	288		676 - 954	279
			1054 - 1187	134		1054 - 1172	119
	EP1242L	7	1351 - 1461	111	8	1255 - 1546	292
			1728 - 1944	217		1816 - 1996	181
			2014 - 2134	121		2423 - 2524	102
			3088 - 3363	276		3088 - 3217	130
						3233 - 3397	165
Inner core			1423 - 1562	140		1960 - 2165	206
			1960 - 2165	206		2326 - 2443	118
	G1340L	5	2321 - 2443	123	3	2509 - 2632	124
			2525 - 2634	110			
			3456 - 3561	106			
	H359L	2	356 - 473	118	2	353 - 478	126
			855 - 974	120		855 - 969	115
	NP419L	1	274 - 426	153	1	297 - 426	130
	NP868R	1	1867 - 2088	222	1	1871 - 2085	215
			90 - 201	112		90 - 200	111
			1071 - 1267	197		1071 - 1261	191
			1705 - 1853	149		1710 - 1856	147
			2032 - 2154	123		2030 - 2154	125
			2333 - 2617	285		2336 - 2577	242
	NP1450L	10	2815 - 2985	171	11	2765 - 2990	226
			3317 - 3538	222		3064 - 3167	104
			3741 - 3848	108		3317 - 3538	222
			3945 - 4136	192		3741 - 3866	126
			4154 - 4282	129		3945 - 4136	192
						4154 - 4280	127
			230 - 340	111		445 - 592	148
			442 - 603	162		1045 - 1257	213
	Q706L	4	1045 - 1257	213	3	1593 - 1716	124
			1590 - 1716	127			

Table 3. Locations and lengths of CpG islands within following gene sequences of ASFV strains.

Strains		BA71V strain of ASFV			Liaoning strain of ASFV		
Protein (Layer)	Gene	Islands	Location (bps) in Figure	Length (bps)	Islands	Location (bps) in Figure	Length (bps)
Inner envelope membrane	E183L	1	80 - 252	173	1	80-244	165
Capsid	B438L	1	475 - 576	102			
	B646L	1	1418 - 1571	154	1	1418-1567	150
External envelope membrane	EP402R	1	876 - 1040	165			

active region and the CpG island in the 2000 bp upstream region overlapped in some of the genes in both the BA71V and Liaoning ASFV strains. The genes with such characteristic included CP204L, S183L, CP2475L, D1133L, G1340L, NP1450L, Q706L, D205R, E165R, E296R, EP424R, NP868R, B119L, D117L, E183L, E248R, H108R, B646L, and E120R in the BA71V ASFV strain, and S183L, D1133L, G1340L, NP1450L, O174L, Q706L, D205R, E165R, E296R, EP424R, NP868R, B119L, D117L, E183L, E248R, H108R, B646L, and E120R in the Liaoning ASFV strain.

Discussion

ASFV is a devastating viral hemorrhagic porcine disease that causes severe economic loses to the swine industry and for which there are currently no effective treatments or vaccines. Epigenetic mechanisms, particularly cytosine methylation of CpG dinucleotides, affect the life cycle of viruses by regulating the expression of viral genes, allowing viruses such as herpesviruses to establish latent states, or others such as retroviruses to maintain persistent infection (Arumugam et al. 2021, Guo and Gewurz 2022). In general, there is a correlation between CpG dinucleotide methylation in the viral genome and viral latency (von Knebel Doeberitz and Prigge 2019, Arumugam et al. 2021, Zhang et al. 2021, Guo and Gewurz 2022). Since methylation may act as a host defense mechanism, drugs promoting viral DNA methylation may be a viable antiviral treatment strategy for ASFV. However, very little is known about the characteristics and methylation status of CpG islands within the ASFV genome. Here, we sought to analyze and compare the features, including number, distribution, and length, of CpG islands in the genomes of ASFV strains BA71V (Genotype I) and Liaoning (genotype II).

We have found that the CpG islands of C147L, EP296R, H359L, NP419L, NP868R, E183L, and B646L are completely conserved in the genomes of the ASFV strains BA71V (Genotype I) and Liaoning (genotype II), while the CpG islands of other genes are relatively conserved, with differences mainly in the number. These differences were mainly caused by mutations and deletions in these regions (Chapman et al. 2008, Wang et al. 2022). The different distribution of CpG islands in the different genotypes may affect the methylation status of the CpG islands, and consequently, the regulation of gene expression, leading to different clinical outcomes.

In human and animal tissues, gene expression can be regulated by DNA methylation, which often occurs in CpG islands (Venter et al. 2001, Angeloni and Bogdanovic 2021). CpG island methylation usually down regulates mRNA and protein production (Vivekanandan et al. 2009, Almouzni and Cedar 2016, Mattei et al. 2022). DNA methylation can directly inhibit the binding of transcription initiation factors to promoters; Alternatively, the transcription inhibition complex recruited by DNA methylation competes with transcription factors for binding to the binding sites on promoters, thus preventing transcription initiation and leading to gene silencing (Kitazawa et al. 2022). Viral gene expression is thought to be partially regulated by DNA methylation, which contributes to transcriptional silencing and gene suppression through multiple mechanisms in host tissues (LaMere et al. 2019). Several studies found CpG islands in the HBV genomes of different strains, some of which were located at transcription start sites or upstream and downstream of promoters (Vivekanandan et al. 2008, Chen et al. 2018). In human liver tissues, HBV DNA can be methylated, which plays an important role in the regulation of HBV gene expression (Vivekanandan et al. 2008, 2009). In this study, the promoter and methylation status of the 2000 bp region upstream of the gene was examined. The results showed that the promoter sites overlapped with CpG island locations within the 2000 bp promoter region. Methylation of these binding sites may inhibit the binding of transcription factors to promoters and ultimately inhibit the activation of gene promoters to limit the synthesis of viral proteins.

Currently, there are few studies on ASFV CpG island methylation and its inhibitory effect on viral protein expression. Future studies will be needed to con-

firm the location and methylation status of CpG islands in these genes. A previous study used bisulfite sequencing (the gold standard for DNA methylation detection) and found that the genome of the BA71V strain does not present methylated cytidine residues upon infection of Vero cells (Weber et al. 2018). At present, most studies have focused on CpG island methylation (Weber et al. 2018). Detection methods such as polymerase chain reaction can only detect methylation in CpG islands (Weber et al. 2018). Non-CpG methylation has been reported in other exogenous retroviral infections, such as mouse leukemia virus infection (Lorincz et al. 2000, Dodge et al. 2002). Moreover, the methylation of the host genome after viral infection needs to be clarified. Genome-wide disulfide sequencing allows the detection of methylation in both host and viral CpG islands as well as non-CpG islands (LaMere et al. 2019, Gu et al. 2022).

The control of viral infectious diseases is mainly based on prevention, such as biosafety measures and available vaccination. When disease develops, controlling viral replication is the main therapeutic goal to reduce the morbidity and mortality (e.g., chronic HBV and HIV infections) (Vivekanandan et al. 2010, Schinazi et al. 2022). At present, there are no effective drugs or vaccines against ASFV infection (Liu et al. 2021). Thus, drugs promoting viral DNA methylation may be a viable antiviral treatment strategy. Here, we have identified CpG islands in crucial viral genes, and determined their methylation status. The results of this study may provide a basis for exploring the potential of DNA methylation in the treatment of ASF and identifying the specific biology of different ASFV genotypes.

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