ORIGINAL RESEARCH

Molecular Epidemiological Survey of Prophages in MRSA Isolates in Taiwan

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Introduction: The prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) type SCC*mec* IV or V is increasing in Taiwan. It has been suggested that the surface protein SasX is responsible for their transmission. However, the *sasX* gene was not detected in our SCC*mec* IV or V isolates. Since *sasX* was originally found in *S. epidermidis* and believed to be transferred to *S. aureus* by a prophage, studies were conducted to detect and type this prophage in our clinical isolates.

Materials and Methods: A total of 1192 MRSA isolates collected from 2006 to 2014 were examined. Multiplex PCRs were performed to determine SCC*mec, sasX*, and prophage types.

Results: The prevalence of SCC*mec* IV and V isolates was increased in recent years (from 2006 to 2014). The *sasX* gene was present in most SCC*mec* III isolates but was absent in SCC*mec* IV or V isolates. The Sa5 prophage was found only in SCC*mec* IV and SCC*mec* V (or V$_t$) isolates, and the Sa6 prophage was mainly present in SCC*mec* III isolates. MRSA isolates harboring prophage combinations Sa1, Sa2, and Sa3; Sa2 and Sa3; Sa2, Sa3, and Sa7; or Sa2 and Sa7 were mainly of SCC*mec* II, and those that harbored prophage combinations Sa3 and Sa6; Sa3, Sa6, and Sa7; or Sa3 and Sa7 were mostly of SCC*mec* III. The numbers of SCC*mec* II isolates containing prophages Sa2, Sa3, and Sa7 and those of SCC*mec* III isolates containing prophages Sa3 and Sa6 or Sa3, Sa6, and Sa7 were decreased from 2010 to 2014. The number of SCC*mec* IV isolates with prophage Sa3 or prophages Sa3 and Sa5 was decreased, but that of those with prophage Sa6 or prophages Sa2 and Sa3 was increased from 2010 to 2014.

Conclusion: The *sasX* gene was found to play no role in clonal selection of MRSA. The finding that different SCC*mec* types of MRSA harbored different types of prophages suggests that these prophages may affect the survival and clonal expansion of certain types of MRSA.

Keywords: MRSA, SCC*mec*, *sasX*, prophage

Background

Staphylococcus aureus is a common opportunistic pathogen colonized on mucosal or skin surface. Methicillin-resistant *Staphylococcus aureus* (MRSA) strains containing the staphylococcal cassette chromosome mec (SCC*mec*) have become the main cause of nosocomial infections.[1] SCC*mec* is carried by a mobile genetic element, and its *mecA* gene encodes the penicillin-binding protein 2a (PBP2a) responsible for the methicillin resistance phenotype.[2] More than six types of SCC*mec* have been discovered.[3] SCC*mec* type II and type III MRSA strains are mainly found in hospitals and are commonly referred to as hospital-acquired MRSA (HA-MRSA).[4] SCC*mec* type IV and type V strains are mostly distributed in communities and are called CA-MRSA.[5] In addition to SCC*mec* typing, multilocus sequence typing (MLST) is also commonly done to classify MRSA isolates. Previous epidemiological studies have demonstrated that some

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MLST types are closely related to SCC*mec* types. For example, ST239 isolates are mostly SCC*mec* III, and ST59 isolates are mainly SCC*mec* IV or V.[6]

In the past decades, most MRSA isolates belonged to SCC*mec* II or III, but the prevalence of SCC*mec* IV or V isolates was increased in recent years.[7,8] It has been reported that ST239 isolates containing the SasX surface protein are prevalent in China.[9] SasX is one of the LPXTG-motif containing surface proteins in *Staphylococcus aureus*, and the *sasX* gene has been shown to be transmitted by the prophage *ΦSPβ* from *Staphylococcus epidermidis*.[10] SasX is considered a virulence factor as it can enhance MRSA colonization.[9]

It has been shown that most *S. aureus* lysogenic phages belong to the *Siphoviridae* family.[11] Based on the sequences of the integrase gene, seven major prophage types Sa1 to Sa7 have been determined.[11,12]

In order to understand the increase in the prevalence of SCC*mec* IV and V MRSA, we conducted an epidemiological study to investigate the roles of the *sasX* gene and prophages in the transmission of MRSA isolates.

Materials and Methods
Bacterial Strains
A total of 1192 MRSA isolates from the Chang Gung Memorial Hospital (CGMH) were used. These isolates were collected from 2006 to 2014 and stored at −80°C. All isolates were identified by MALDI-TOF and tested for oxacillin susceptibility as an indication of the presence or absence of SCC*mec* and further characterized by SCC*mec* typing. Three hundred and three randomly selected MRSA isolates from 2006 to 2010 were examined for the presence of the *sasX* gene. All isolates were grown on tryptic soy agar (TSA) plates at 37°C for 16 hrs for the studies.

DNA Preparation and PCR Analysis of *sasX*
MRSA cells of overnight cultures were pelleted and then resuspended in the lysis buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCL, pH 8.0) containing lysozyme (50 mg/mL), lysostaphin (5 mg/mL), and 10% SDS. Genomic DNA was isolated using the QIAGEN™ DNeasy extraction kit and stored at −20°C until used. The *sasX* gene was detected by PCR as described previously.[13,14]

SCC*mec* and Prophage Typing
SCC*mec* typing was achieved by multiplex PCR using two sets of primers to identify *mecA* and eight sets of primers to differentiate the five *ccr* genes as described previously.[15] Prophage typing was also done by multiplex PCR as described.[11] All primers used are listed in Table 1. Student *t*-test was performed to determine the significant difference between data from two different years.

Results
Lack of *sasX* in SCC*mec* IV and V MRSA Isolates
To determine the prevalence of various types of MRSA in northern Taiwan, a total of 602 clinical isolates collected from 2006 to 2009 were SCC*mec* typed (Table 2). Results showed that the prevalence of SCC*mec* II MRSA was about the same from 2006 to 2009 (41% in 2006 and 42.1% in 2009), but that of SCC*mec* III MRSA was decreased from 44.6% in 2006 to 31.2% in 2009. However, the prevalence of SCC*mec* IV MRSA was increased from 8.6% in 2006 to 16.4% in 2009. The prevalence of SCC*mec* V MRSA was slightly increased, from 5% in 2006 to 7% in 2009. Since these 602 isolates were randomly selected from our collections from 2006 to 2009, there may be analytical bias. Therefore, a second study was performed on all isolates collected from 2010 (347 isolates) and 2014 (243 isolates). Results showed that the prevalence of SCC*mec* II MRSA was decreased from 14.4% in 2010 to 13.6% in 2014, and that of SCC*mec* III MRSA was decreased from 40.6% in 2010 to 33% in 2014. In contrast, the prevalence of SCC*mec* IV MRSA was increased from 22.5% in 2010 to 26.7% in 2014, and that of SCC*mec* V MRSA was increased from 15.3% to 22.2%. These results indicated that the prevalence of SCC*mec* II and III MRSA was decreasing and that of SCC*mec* IV and V MRSA was increasing from 2010 to 2014.

To determine whether the *sasX* gene plays any role in MRSA transmission, its presence in all MRSA isolates from 2006 to 2010 was determined (Table 3). Results showed that all SCC*mec* III and some SCC*mec* II isolates harbored *sasX*, but none of the SCC*mec* IV and SCC*mec* V isolates contained *sasX*.

Types of Prophages in Various SCC*mec* Isolates
To understand the impact of prophages on the epidemiology of various types of MRSA, the prophages in all SCC*mec* isolates collected in 2010 (322 isolates) and 2014 (232 isolates) were typed by multiplex PCR of the integrase gene (Tables 4 and 5). Results showed that type Sa3 prophage was predominate and

Table 1 Primers Used in This Study

Name		Sequence	Reference
sasX	sasX-pp-F sasX-sa-R sasX-F sasX-R	AAGTCCATTCCTATTTCTCC CTATCCCCGTTATAACAACC AGAATTAGAAGTACGTCTAAATGC GCTGATTATGTAAATGACTCAAATG	[13]
Sa1int	Sa1-F Sa1-R	AAGCTAAGTTC GGGCACA GTAATGTTTGGGAGCCAT	[11]
Sa2int	Sa2-F Sa2-R	TCAAGTAACCCGTCAACTC ATGTCTAAATG TGTGCGTG	
Sa3int	Sa3-F Sa3-R	GAAAAACAAACGGTGC TAT TTATTGACTCTACAGGCTGA	
Sa4int	Sa4-F Sa4-R	ATTGATATTAACGGAACTC TAAACTTATATG CGTGTGT	
Sa5int	Sa5-F Sa5-R	AAAGATGCCAAACTA GCTG CTTGTGGTTTTGTTCTGG	
Sa6int	Sa6-F Sa6-R	GCCATCAATTCAAGGATAG TCTGCAGCTGAGGAC AAT	
Sa7int	Sa7-F Sa7-R	GTCCGGTAGCTAGAGGTC GGCGTATGCTTGACTGTGT	

was present in isolates of all SCC*mec* groups. The Sa5 prophage was only found in SCC*mec* IV and SCC*mec* V (or V_t) isolates, and the Sa4 prophage was not present in any isolates (Table 4). Type Sa6 prophage was found in only 4–9.1% of SCC*mec* II and 1.9–5.6% of SCC*mec* V (or V_t) isolates but was present in 86.3–88.6% of SCC*mec* III isolates. Type Sa1

prophage was present in 1.4% of SCC*mec* III isolates, and type Sa7 was present in 1.5–2.6% of SCC*mec* IV and 5.7–9.3% SCC*mec* V (or V_t) isolates (Table 4).

Some MRSA isolates were found to harbor multiple prophages (Table 5). The combination of prophages Sa1, Sa2, and Sa3; Sa2 and Sa3; Sa2, Sa3, and Sa7; or Sa2 and Sa7 was found only in SCC*mec* II isolates. Isolates that harbored prophages Sa3 and Sa6; Sa3, Sa6, and Sa7; or Sa3 and Sa7 were mainly SCC*mec* III. The number of SCC*mec* II isolates containing prophages Sa2, Sa3, and Sa7 was decreased from 15 in 2010 to 9 in 2014. The number of SCC*mec* III isolates with prophages Sa3 and Sa6 was decreased from 56 in 2010 to 22 in 2014, and that of those with prophages Sa3, Sa6, and Sa was decreased from 51 in 2010 to 25 in 2014. The number of SCC*mec* IV isolates with the Sa3 prophage was decreased from 25 in 2010 to 16 in 2014, and that of those with prophages Sa3 and Sa5 was decreased from 13 in 2010 to 7 in 2014. The number of SCC*mec* IV isolates with the Sa6 prophage was increased from 1 in 2010 to 9 in 2014, and that of those with prophages Sa2 and Sa3 was increased from 1 in 2010 to 11 in 2014. There were no significant changes in prophage types in SCC*mec* V isolates between 2010 and 2014.

Discussion

The change in the prevalence of various SCC*mec* types of MRSA has been reported.[16–18] In this study, we found that

Table 2 Number and Percentage of MRSA Isolates of Various SCC*mec* Types from 2006 to 2014

Year		2006	2007	2008	2009	2010	2014
SCC*mec*	II	57 (41.0%)	81 (42.8%)	62 (42.4%)	54 (42.1%)	50 (14.4%)	33 (13.6%)
	III	62 (44.6%)	72 (38.1%)	53 (36.3%)	40 (31.2%)	141 (40.6%)	80 (33%)
	IV	13 (8.6%)	26 (13.7%)	23 (15.0%)	21 (16.4%)	78 (22.5%)	65 (26.7%)
	V or Vt	7 (5.0%)	10 (5.4%)	9 (6.1%)	9 (7.0%)	53 (15.3%)	54 (22.2%)*
	Others#	–	–	–	–	25 (7.2%)	11 (4.5%)
	Total	139	189	146	128	347	243

Notes: #*mecA* or unknown type. –none selected. *Significant difference between 2010 and 2014 ($p < 0.05$).

Table 3 The Prevalence of MRSA Isolates of Various SCC*mec* Types with the *sasX* Gene from 2006 to 2010

Year		Number of *sasX* Positive Isolates/Total Number of Isolates				
		2006	2007	2008	2009	2010
SCC*mec*	II	9/24 (37.5%)	0/20	3/23 (13%)	1/22 (4.5%)	0/20
	III	12/12 (100%)	21/22 (95.5%)	8/8 (100%)	17/18 (94.4%)	45/46 (97.8%)
	IV	0/7	0/8	0/16	0/9	0/15
	V	0/6	0/2	0/5	0/7	0/13
	Total	49	52	52	56	94

Table 4 Percentage of Various Types of SCC*mec* Isolates Harboring Prophages in 2010 and 2014

SCC*mec*	Year (No.)	Integrase Type [% (No.)]						
		Sa1	Sa2	Sa3	Sa4	Sa5	Sa6	Sa7
II	2010 (50)*	30% (15)	82% (41)	70% (35)	0	0	4% (2)	46% (23)
	2014 (33)	24.2% (8)	93.9% (31)	75.8% (25)			9.1% (3)	54.5% (18)
III	2010 (141)	1.4% (2)	9.3% (13)	93.6% (131)	0	0	88.6% (124)	54.3% (76)
	2014 (80)	0	18.8% (15)	88.8% (71)			86.3% (69)	63.8% (51)
IV	2010 (78)	19.2% (15)	15.4% (12)	74.4% (58)	0	27% (21)	15.4% (12)	2.6% (2)
	2014 (65)	23.1% (15)	24.6% (16)	75.4% (49)		18.5% (12)	24.6% (16)	1.5% (1)
V or V$_t$	2010 (53)	30.2% (16)	51% (27)	34% (18)	0	3.8% (2)	1.9% (1)	5.7% (3)
	2014 (54)	16.7% (9)	59.3% (32)	29.6% (16)		1.9% (1)	5.6% (3)	9.3% (5)

Note: *Number of isolates.

both the prevalence (from 44.6% to 31.2%) and number (from 62 to 40) of SCC*mec* III isolates were decreased and those of SCC*mec* IV (prevalence from 8.6% to 16.4%, number from 13 to 21) and V (or V$_t$) (prevalence from 5% to 7%, number from 7 to 9) isolates were increased from 2006 to 2009. This trend is similar to that seen in other countries.[19] Although the prevalence of SCC*mec* IV, V, or Vt isolates was increased from 2010 to 2014 (SCC*mec* IV, from 22.5% to 26.7%; SCC*mec* V or V$_t$, from 15.3% to 22.2%), there was a general decrease in the number of isolates of all SCC*mec* groups, except SCC*mec* V (or V$_t$) (53 in 2010 and 54 in 2014). The decrease in isolate number was most apparent for SCC*mec* III MRSA (from 141 in 2010 to 80 in 2014).

It has been speculated that the surface antigen gene *sasX* is responsible for the spreading of MRSA.[13] However, we found that the *sasX* gene was not present in SCC*mec* IV and V isolates. This result suggests that the *sasX* gene plays no significant role in clonal selection or prevalence of certain types of MRSA in Taiwan.

Previous studies have shown that the *sasX* gene is transmitted from *S. epidermidis* to *S. aureus* by the $\phi SP\beta$ phage.[10] This bacteriophage has been shown to carry genes encoding toxins and virulence factors.[11,12] The integration of a bacteriophage into the genome of MRSA to become a prophage may affect its survival or virulence by affecting the expression of genes near the insertion sites.[20,21] A significant finding was that 56 in 2010 and 22 in 2014 of SCC*mec* III isolates harbored prophages Sa3 and Sa6 and that 51 in 2010 and 25 in 2014 of SCC*mec* III isolates harbored prophages Sa3, Sa6, and Sa7. These results suggest an adverse effect of these prophages on the survival of SCC*mec* III isolates.

The mechanisms by which prophages affect the survival of MRSA isolates are unknown. It has been postulated that the bacterial immune system and phage integration affect MRSA clonal selection.[11] The type I restriction-modification (R-M) system is a bacterial immune system, which may restrict the uptake of foreign DNA from other *S. aureus* strains.[22,23] Since each MRSA clonal lineage has its own specific R-M system, it may allow intake of specific types of prophages. Our observation that prophage Sa5 existed only in SCC*mec* IV or V isolates and that Sa6 and Sa7 prophages were mainly present in SCC*mec* III isolates supports this hypothesis. It is possible that these prophages confer survival advantage to certain types of MRSA, e.g., SCC*mec* III, IV, and V.

The integration of phage genome into MRSA genome is another mechanism that may affect clonal selection.[24–26] Prophage integration may mutate or affect the expression of host genes as evidenced by the observation that Sa3 and Sa6 prophage integration adversely affected the survival of *S. aureus*.[20,21]

The Sa3 prophage usually integrates into the *hlb* gene, which encodes the beta-toxin.[20,21] The beta-toxin has been shown to play a role in colonization,[27] biofilm formation,[28,29] and virulence of MRSA.[30] The inactivation of the *hlb* gene by Sa3 integration has been shown to weaken the biofilm formation ability.[21,28,29] In conjunction with the delta-toxin, beta-toxin can enhance the pathogenicity of MRSA by making it resistant to degradation by phago-endosomes.[30] It has been demonstrated that the beta-toxin null strain SA113 failed in phagosomal escape, while complementation to recover beta-toxin production in SA113 enabled its escape from phagosomal degradation.[30] The beta-toxin has also been shown to enhance the activities of sphingomyelinase

Table 5 Prophage Types in Different SCCmec Isolates in 2010 and 2014

SCCmec	II		III		IV		V or V$_t$	
Year	2010	2014	2010	2014	2010	2014	2010	2014
Prophage Type	Number of Isolates Containing Prophage							
No prophage	0	0	0	0	4	1	3	5
Sa1 only	0	0	0	0	6	2	4	2
Sa1-Sa2	3	0	0	0	0	1	5	5
Sa1-Sa2-Sa3	7	5	0	0	0	0	0	0
Sa1-Sa2-Sa3-Sa7	0	1	0	0	0	0	0	0
Sa1-Sa2-Sa7	1	2	0	0	0	0	0	0
Sa1-Sa3	2	0	1	0	7	8	6	2
Sa1-Sa3-Sa5	0	0	0	0	1	1	0	0
Sa1-Sa3-Sa5-Sa6	0	0	0	0	0	1	1	0
Sa1-Sa3-Sa6	0	0	0	0	0	2	0	0
Sa1-Sa3-Sa7	0	0	0	0	1	0	0	0
Sa1-Sa6-Sa7	0	0	1	0	0	0	0	0
Sa1-Sa7	1	0	0	0	0	0	0	0
Sa2 only	5	1	0	0	5	0	21	24
Sa2-Sa3	4	6	0	1	1	11*	0	1
Sa2-Sa3-Sa5	0	0	0	0	3	2	0	0
Sa2-Sa3-Sa5-Sa6	0	0	0	0	0	0	0	0
Sa2-Sa3-Sa6	1	1	1	1	0	0	0	0
Sa2-Sa3-Sa6-Sa7	0	1	7	9	0	0	0	0
Sa2-Sa3-Sa7	15	9	5	3	0	0	0	0
Sa2-Sa5	0	0	0	0	0	0	1	1
Sa2-Sa5-Sa6	0	0	0	0	1	0	0	0
Sa2-Sa6	0	0	0	1	2	2	0	0
Sa2-Sa6-Sa7	0	0	0	0	0	0	0	1
Sa2-Sa7	5	5	0	0	0	0	0	0
Sa3 only	4	1	0	2	25	16	9	8
Sa3-Sa5	0	0	0	0	13	7	0	0
Sa3-Sa5-Sa6	0	0	0	0	2	0	0	0
Sa3-Sa6	0	1	56	22	5	1	0	1
Sa3-Sa6-Sa7	0	0	51	25	0	0	0	1
Sa3-Sa7	1	0	11	8	0	0	2	3
Sa5 only	0	0	0	0	0	0	0	0
Sa6 only	1	0	7	5	1	9	0	0
Sa6-Sa7	0	0	1	3	0	1	0	0
Sa7 only	0	0	0	0	1	0	1	0
Total	50	33	141	80	78	65	53	54

Notes: *Significant difference ($p<0.05$). Bolded data indicates the T-test value is 0.002, which is compared with Sa2-Sa3 of SCCmec IV between 2010 and 2014.

and biofilm ligase, thus inhibiting IL-8 production by host cells.[31] As IL-8 recruits neutrophils, the integration of Sa3 into the *hlb* gene would benefit the survival of MRSA.

The Sa6 prophage is usually inserted into the *geh* gene, which encodes a lipase. Inactivation of host lipase production would also benefit the survival of MRSA as lipase has been shown to degrade the antimicrobial fatty acid triglycerides produced by human skin.[32] Mice immunized with human lipase have been shown to have an enhanced ability to defend *S. aureus* infection.[33]

Conclusions

Our data showed that the *sasX* surface antigen gene plays no role in clonal selection of a certain lineage of MRSA. The decrease in the number of SCCmec III isolates harboring both Sa3 and Sa6 prophages (from 56 in 2010 to 22 in

2014) and of those harboring Sa3, Sa6, and Sa7 prophages (from 51 in 2010 to 25 in 2014) suggests that the presence of these prophages make the isolates less advantageous in survival. The observation that prophage Sa5 existed only in SCC*mec* IV or V isolates and that the combination of Sa6 and Sa7 prophages was mainly found in SCC*mec* III isolates suggests that these prophages confer survival advantage to these types of MRSA. The mechanisms by which prophages affect the survival of MRSA isolates remain to be investigated. Results of this study would provide a guide for further epidemiological studies of the relationship between prophages and specific lineages of MRSA.

Ethics Approval and Consent to Participate

All MRSA isolates used in this study were obtained from Chang Gung Memorial Hospital bacterial bank. No ethics approval was required for the use of the isolates.

Data Sharing Statement

All data are included in this article.

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Disclosure

The authors declare no conflict of interest.

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