Comparative Proteomics-Based Identification of Genes Associated with Glycopeptide Resistance in Clinically Derived Heterogeneous Vancomycin-Intermediate \textit{Staphylococcus aureus} Strains

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Abstract

Heterogeneous vancomycin-intermediate \textit{Staphylococcus aureus} (hVISA) is associated with clinical treatment failure. However, the resistance mechanism of hVISA has not been fully clarified. In the present study, comparative proteomics analysis of two pairs of isogenic vancomycin-susceptible \textit{S. aureus} (VSSA) and hVISA strains isolated from two patients identified five differentially expressed proteins, IsaA, MsrA2, Asp23, GpmA, and AhpC, present in both isolate pairs. All the proteins were up-regulated in the hVISA strains. These proteins were analyzed in six pairs of isogenic VSSA and hVISA strains, and unrelated VSSA (n = 30) and hVISA (n = 24) by real-time quantitative reverse transcriptase–PCR (qRT–PCR). Of the six pairs of isogenic strains, IsaA, msrA2 and ahpC were up-regulated in all six hVISA strains; whereas asp23 and gpmA were up-regulated in five hVISA strains compared with the VSSA parental strains. In the unrelated strains, statistical analyses showed that only IsaA was significantly up-regulated in the hVISA strains. Analysis of the five differentially expressed proteins in 15 pairs of persistent VSSA strains by qRT–PCR showed no differences in the expression of the five genes among the persistent strains, suggesting that these genes are not associated with persistence infection. Our results indicate that increased expression of IsaA may be related to hVISA resistance.

Introduction

\textit{Staphylococcus aureus} can cause serious hospital- and community-acquired infections, including skin and soft tissue infections, pneumonia, bacteremia, endocarditis, and even septic shock. The high prevalence of methicillin-resistant \textit{S. aureus} (MRSA) and the extensive use of vancomycin have led to the emergence of reduced vancomycin susceptibility among \textit{S. aureus} strains. Heterogeneous vancomycin-intermediate resistant \textit{S. aureus} (hVISA) [vancomycin minimum inhibitory concentration (MIC) ≤ 2 µg/mL], the precursor of vancomycin-intermediate resistant \textit{S. aureus} (VISA, MIC of 4 – 8 µg/mL), is a strain that contains subpopulations of vancomycin-intermediate daughter cells, but for which the MIC of vancomycin for the parent strain is in the susceptible range. Although vancomycin-resistant \textit{S. aureus} (VRSA) strains are rare, hVISA/VISA are common in the clinical setting, especially in persistent MRSA bacteremia and endocarditis. Our previous studies have shown that the prevalence of hVISA is 13% to 16% in large teaching hospitals in China [1]. Moreover, several studies have indicated that hVISA/VISA infections are associated with vancomycin treatment failure [2,3].

To date, no specific genetic determinants of hVISA/VISA have been universally defined, whereas VRSA strains acquire the \textit{vanA} gene from \textit{Enterococcus}. Several phenotypic features are characteristic of hVISA/VISA strains, among which significant cell wall thickening is a common feature associated with vancomycin resistance [4]. Compared with vancomycin-susceptible \textit{S. aureus} (VSSA), hVISA produces three to five times the amount of penicillin-binding proteins (PBPs) 2 and 2'. The amounts of intracellular murein monomer precursor in hVISA are three to eight times greater than those in VSSA strains [4]. Factors such as the increased synthesis of non-amidated muropeptides and the resultant reduced peptidoglycan cross-linking contribute to the vancomycin resistance of VISA through increased affinity trapping of vancomycin [5]. In addition to thickened cell walls, hVISA/VISA strains exhibit other phenotypic changes, including reduction in autolytic activity [6], reduced growth rate [7], resistance to lysostaphin [8], PBP changes [9], and metabolic changes [10].
Several transcriptional changes have been detected in hVISA/ VISA. DNA microarray analyses have been used to determine changes in the transcriptional profile of hVISA or VISA strains [11–15]. However, the protein profiles of hVISA or VISA are rarely analyzed via comparative proteomics. In a proteomics study that compared VSSA and VISA strains, several differentially expressed proteins were identified [16]. Another study identified 65 significant protein abundance changes by comparing three isogenic strains derived from a clinical VISA isolate [17].

To our knowledge, a comparative proteomics analysis of hVISA strains has not been performed to date. Here, we used comparative proteomics to analyze hVISA and VSSA strains isolated from the same patients treated with vancomycin. The differentially expressed proteins identified in our screen were validated in six pairs of isogenic hVISA and VSSA strains and unrelated hVISA (n = 24) and VSSA (n = 30) strains to identify the potential resistance mechanisms of hVISA. To further analyze the potential association of these differentially expressed genes with persistent infection, their expression was examined in 15 pairs of persistent VSSA strains. The results of our study provide insight into the molecular mechanisms underlying hVISA resistance.

Materials and Methods

The study protocol and written informed consent were approved by the Medical Ethical Committee of Peking University People’s Hospital. Written informed consent was obtained from all patients at the time of enrollment.

Bacterial isolates

A clinical VSSA (CN9) strain with a vancomycin MIC of 0.5 μg/mL and teicoplanin MIC of 2 μg/mL, and hVISA (CN10) with a vancomycin MIC of 2 μg/mL and teicoplanin MIC of 8 μg/mL were isolated in 2008 from the purulent sputum of an 84-year-old man who had MRSA pneumonia with a 20-year history of emphysema. Strain CN9 (VSSA), isolated after approximately 1 year of hospitalization, was the parental (pre-therapy) vancomycin-susceptible isolate; strain CN10 was the hVISA organism recovered during vancomycin treatment. Another clinical VSSA (CN3) strain with vancomycin MIC of 0.5 μg/mL and teicoplanin MIC of 1 μg/mL, and hVISA (CN4) with vancomycin MIC of 0.5 μg/mL and teicoplanin MIC of 2 μg/mL were isolated in 2008 from a 90-year-old woman who had MRSA bacteremia with a 15-year history of diabetes mellitus. Strain CN3 (VSSA), isolated after approximately 1 month of hospitalization, was the parental (pre-therapy) vancomycin-susceptible isolate; strain CN4 was the hVISA organism recovered during vancomycin treatment. The above two pairs of VSSA and hVISA strains were selected for comparative proteomics analysis.

Six pairs of VSSA and hVISA strains, and unrelated 24 hVISA and 30 VSSA strains from our previous study were selected to validate the proteomics results [1]. Fifteen pairs of persistent VSSA strains were selected to determine whether the differentially expressed genes were associated with persistent infection. Pairs of bacterial strains with the same genetic background were isolated from the same patient (Table 1). All hVISA strains were confirmed by the population analysis profile (PAP)-area under the curve (AUC) (PAP-AUC) method. All the strains used in the study were tested from frozen stocks and were not frozen and thawed multiple times. Each strain was stored at −80°C in three separate tubes.

PAP-AUC Method

PAP-AUC was determined as described previously [1]. Briefly, 50 μL of a 0.5 McFarland standard suspension at dilutions of 10−4 and 10−6 was inoculated onto brain heart infusion (BHI) agar plates containing 0, 0.5, 1.0, 2.0, 2.5, 4.0, and 8.0 μg/mL of vancomycin. After 48 h of incubation at 35°C, the colonies were counted and the log CFU/mL was plotted against vancomycin concentration. The ratio of the AUC of the test isolate to the AUC of S. aureus Mu3 was calculated and interpreted as follows: for VSSA, a ratio of <0.9; for hVISA, a ratio of 0.9 to 1.3; and for VISA, a ratio of ≥1.3. S. aureus ATCC 29213 was used as the reference VSSA strain.

Molecular Typing Methods

All isolates were analyzed by SCCmec typing, spa typing, MLST typing, and PFGE. The SCCmec types were determined by the multiplex PCR strategy developed by Kondo et al. [18]. The spa typing was performed as described previously [19]. Purified spa PCR products were sequenced, and short sequence repeats were assigned by using the spa database website (http://www.ridom.de/spaserver). MLST was carried out as described previously [20]. The sequences of the PCR products were compared with the existing sequences available on the MLST website (http://saureus.mlst.net) for S. aureus. DNA extraction and Smal restriction were performed as described previously [21]. The PFGE patterns were visually examined and interpreted according to the criteria of Tenover et al. [22].

Protein Sample Preparation

Overnight cultures of VSSA and hVISA strains were diluted at 1/100 in BHI broth and harvested at similar culture densities (exponential phase, OD600 nm = 0.5). The samples were centrifuged at 7,000 g for 10 min to collect the deposits. The deposits were then washed in 50 mM PBS three times and incubated in 220 μL of 20 mM Tris-HCl, pH 7.3; 50 μL of 1 mg/mL lysozyme; 4 μL of protease inhibitor cocktail; and 6 μL of DNase for 30 min at 37°C. Subsequently, 1.5 mL of 2D lysis buffer (100 μL aceton, 20 mM DTT, 10% TCA) was added, and the samples were vortexed and frozen at −20°C for 2 h. Samples were centrifuged at maximum speed in a microcentrifuge for 2 min to remove insoluble materials, and protein was quantitated using the 2D Quant Kit (GE Healthcare, Arizona, USA).

Two-Dimensional Gel Electrophoresis (2DE)

2DE was performed as described previously [17]. Samples were run in triplicate. In the first dimension, 500 μg of protein was run on 24 cm Immobiline DryStrips (GE Healthcare) at a pH range of 4 to 7 on an IPIgphorII IEF system (GE Healthcare) as recommended by the manufacturer. Strips were equilibrated in equilibration buffer (50 mM Tris-Gly, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, and 0.25% trace of bromophenol blue) containing 10 mg/mL of DTT for 15 min followed by incubation in the same buffer containing 40 mg/mL of iodoacetamide for 15 min. The strips were then applied to 12.5% self-made acrylamide gels using 0.5% agarose in standard Tris-glycine electrophoresis buffer.

Second-dimension sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a Protean II (Bio-Rad, Hercules, CA, USA) at 40 mA/gel and 15°C until the tracking dye ran-off the gel. Proteins were visualized by Coomassie brilliant blue (CBB) staining. Gels were fixed in 20% TCA for 1 h, stained in 0.1% CBB for 2 h, destained in 40% ethanol and 10% acetic acid for 2×30 min, intensified overnight in 1% acetic acid, and then washed in deionized water for 30 min. Gels were imaged using ImageScanner (GE Healthcare) and images were analyzed by PDQuest 6.0.
Table 1. The study isolates, susceptibilities, typing results and clinical aspects.

| Isolate * | Specimen Type | Phenotype b | MIC (μg/ml) for c | PAP-AUC | SCCmec Typ b | Spa Type | MLST | PFGE | Age, years | Diagnosis | Duration of VAN exposure, days d | Therapy *          | Outcome                        |
|-----------|---------------|-------------|-------------------|----------|--------------|----------|------|------|-------------|-----------|--------------------------------|---------------------|--------------------------------|
| Pair 1 (19d) |               |             |                   |          |              |          |      |      |             |           |                                 |                     |                                |
| CN1       | sputum        | VSSA        | 0.5               | 4        | >256         | 0.6      | III  | t030 | ST239 A    | 67        | Severe pneumonia               | 6                   | LEV, TEC Infection cleared    |
| CN2       | sputum        | hVISA       | 1                 | 4        | 256          | 0.9      | III  | t030 | ST239 A    | 69        | Diabetes mellitus, bacteremia, pneumonia | 10                  | MFX, IPM VAN Infection cleared |
| CN3       | blood         | VSSA        | 0.5               | 1        | 256          | 0.5      | III  | t030 | ST239 B    | 90        | Diabetes mellitus, bacteremia, pneumonia | 10                  | MFX, IPM VAN Infection cleared |
| CN4       | sputum        | hVISA       | 0.5               | 2        | 256          | 0.9      | III  | t030 | ST239 B    | 90        | Diabetes mellitus, bacteremia, pneumonia | 10                  | MFX, IPM VAN Infection cleared |
| Pair 2 (25d) |               |             |                   |          |              |          |      |      |             |           |                                 |                     |                                |
| Pair 3 (31d) |               |             |                   |          |              |          |      |      |             |           |                                 |                     |                                |
| CN5       | sputum        | VSSA        | 1                 | 2        | 256          | 0.5      | III  | t030 | ST239 A    | 54        | Coronary heart disease, diabetes mellitus, pneumonia | 21                  | IPM, TZP VAN Infection persisted, patient died of respiratory failure |
| CN6       | sputum        | hVISA       | 1                 | 2        | 256          | 0.9      | III  | t030 | ST239 A    | 54        | Coronary heart disease, diabetes mellitus, pneumonia | 21                  | IPM, TZP VAN Infection persisted, patient died of respiratory failure |
| Pair 4 (20d) |               |             |                   |          |              |          |      |      |             |           |                                 |                     |                                |
| Pair 5 (95d) |               |             |                   |          |              |          |      |      |             |           |                                 |                     |                                |
| CN7       | blood         | VSSA        | 1                 | 1        | 256          | 0.6      | III  | t030 | ST239 C    | 18        | Peumonia, bacteremia            | 7                   | LEV, AMK, IPM, Infection cleared VAN |
| CN8       | sputum        | hVISA       | 1                 | 2        | 256          | 0.9      | III  | t030 | ST239 C    | 18        | Peumonia, bacteremia            | 7                   | LEV, AMK, IPM, Infection cleared VAN |
| Pair 6 (29d) |               |             |                   |          |              |          |      |      |             |           |                                 |                     |                                |
| Pair 7 (29d) |               |             |                   |          |              |          |      |      |             |           |                                 |                     |                                |
| CN11      | blood         | VSSA        | 1                 | 2        | >256         | 0.6      | III  | t030 | ST239 B    | 22        | Multiple endocrine neoplasia syndrome, abdominal infection | 9                   | CKM, MFX, SCF, Infection cleared VAN |
| CN12      | abdominal fluid | hVISA   | 1                 | 2        | >256         | 0.9      | III  | t030 | ST239 B    | 22        | Multiple endocrine neoplasia syndrome, abdominal infection | 9                   | CKM, MFX, SCF, Infection cleared VAN |
| VSSA-pair 1 (45d) |         |             |                   |          |              |          |      |      |             |           |                                 |                     |                                |
| V-F-1     | sputum        | VSSA        | 1                 | 2        | 256          | 0.7      | III  | t030 | ST239 B    | 43        | Coronary heart disease, pneumonia | 11                  | TEC, IPM, SCF, Infection cleared CAZ |
| V-R-1     | sputum        | VSSA        | 1                 | 2        | 256          | 0.7      | III  | t030 | ST239 B    | 43        | Coronary heart disease, pneumonia | 11                  | TEC, IPM, SCF, Infection cleared CAZ |
| VSSA-pair 2 (19d) |         |             |                   |          |              |          |      |      |             |           |                                 |                     |                                |
| V-F-2     | sputum        | VSSA        | 1                 | 2        | 256          | 0.7      | III  | t030 | ST239 B    | 73        | Severe pneumonia                | 16                  | VAN, LEV, MEM, Infection cleared RIF |
| V-R-2     | sputum        | VSSA        | 1                 | 2        | 256          | 0.7      | III  | t030 | ST239 B    | 73        | Severe pneumonia                | 16                  | VAN, LEV, MEM, Infection cleared RIF |
| VSSA-pair 3 (20d) |         |             |                   |          |              |          |      |      |             |           |                                 |                     |                                |
| V-F-3     | sputum        | VSSA        | 1                 | 2        | 256          | 0.7      | III  | t030 | ST239 B    | 49        | Chronic renal failure, pneumonia | 6                   | VAN, CRO, CAZ, Infection cleared LEV |
| V-R-3     | sputum        | VSSA        | 1                 | 2        | 256          | 0.7      | III  | t030 | ST239 B    | 49        | Chronic renal failure, pneumonia | 6                   | VAN, CRO, CAZ, Infection cleared LEV |
| Isolate * | Specimen Type | Pheno-type b | MIC (µg/ml) for c | PAP-AUC | SCCmec Typb | Spa Type | MLST | PFGE | Age, years | Diagnosis | Duration of VAN exposure, days | Therapy * | Outcome |
|-----------|---------------|--------------|------------------|---------|-------------|---------|------|------|------------|-----------|-------------------------------|-----------|---------|
| V-F-3     | sputum        | VSSA         | 0.5              | 2       | >256        | 0.5     | II   | t002 | ST5      | D                     | 16       | VAN, CRO, IPM, Infection cleared | RIF, SXT |         |
| V-R-3     | sputum        | VSSA         | 0.5              | 2       | >256        | 0.6     | II   | t002 | ST5      | D                     | 16       | VAN, CRO, IPM, Infection cleared | RIF, SXT |         |
| VSSA-pair (33d) |           | VSSA         | 0.5              | 2       | >256        | 0.5     | II   | t002 | ST5      | D                     | 16       | VAN, CRO, IPM, Infection cleared | RIF, SXT |         |
| V-F-4     | pus           | VSSA         | 0.5              | 0.5     | 0.25        | 0.3     | NA   | t034 | ST398    | E                     | 11       | VAN, CRO, SCF, Infection cleared | LEV, SXT |         |
| V-R-4     | pus           | VSSA         | 0.5              | 0.5     | 0.25        | 0.3     | NA   | t034 | ST398    | E                     | 11       | VAN, CRO, SCF, Infection cleared | LEV, SXT |         |
| VSSA-pair (76d) |           | VSSA         | 0.5              | 1       | >256        | 0.5     | III  | t030 | ST239    | A                     | 23       | VAN, CRO, LEV, Infection cleared | MFX, IPM, TZP |         |
| V-F-5     | sputum        | VSSA         | 0.5              | 1       | >256        | 0.4     | III  | t030 | ST239    | A                     | 23       | VAN, CRO, LEV, Infection cleared | MFX, IPM, TZP |         |
| V-R-5     | sputum        | VSSA         | 0.5              | 1       | >256        | 0.5     | II   | t030 | ST239    | A                     | 23       | VAN, CRO, LEV, Infection cleared | MFX, IPM, TZP |         |
| VSSA-pair (25d) |           | VSSA         | 0.5              | 2       | >256        | 0.5     | III  | t030 | ST239    | B                     | 23       | VAN, CRO, LEV, Infection cleared | MFX, IPM, TZP |         |
| V-F-6     | sputum        | VSSA         | 0.5              | 2       | >256        | 0.5     | III  | t030 | ST239    | B                     | 23       | VAN, CRO, LEV, Infection cleared | MFX, IPM, TZP |         |
| V-R-6     | sputum        | VSSA         | 0.5              | 2       | >256        | 0.5     | III  | t030 | ST239    | B                     | 23       | VAN, CRO, LEV, Infection cleared | MFX, IPM, TZP |         |
| VSSA-pair (76d) |           | VSSA         | 0.5              | 1       | >256        | 0.4     | II   | t002 | ST5      | D                     | 84       | VAN, LZD, IPM, Infection cleared | CIP, CAZ, SCF, TZP |         |
| V-F-7     | sputum        | VSSA         | 0.5              | 1       | >256        | 0.5     | II   | t002 | ST5      | D                     | 84       | VAN, LZD, IPM, Infection cleared | CIP, CAZ, SCF, TZP |         |
| V-R-7     | sputum        | VSSA         | 0.5              | 2       | >256        | 0.5     | II   | t002 | ST5      | D                     | 84       | VAN, LZD, IPM, Infection cleared | CIP, CAZ, SCF, TZP |         |
| VSSA-pair (18d) |           | VSSA         | 0.5              | 2       | >256        | 0.6     | II   | t030 | ST239    | C                     | 6        | VAN, LZD, IPM, Infection cleared | CIP, CAZ, SCF, TZP |         |
| V-F-8     | sputum        | VSSA         | 0.5              | 2       | >256        | 0.6     | II   | t030 | ST239    | C                     | 6        | VAN, LZD, IPM, Infection cleared | CIP, CAZ, SCF, TZP |         |
| V-R-8     | sputum        | VSSA         | 0.5              | 2       | >256        | 0.6     | II   | t030 | ST239    | C                     | 6        | VAN, LZD, IPM, Infection cleared | CIP, CAZ, SCF, TZP |         |
| VSSA-pair (21d) |           | VSSA         | 0.5              | 1       | >256        | 0.6     | II   | t030 | ST239    | B                     | 16       | TEC, IPM, TZP, Infection cleared | SCF |         |
| V-F-9     | sputum        | VSSA         | 0.5              | 1       | >256        | 0.6     | II   | t030 | ST239    | B                     | 16       | TEC, IPM, TZP, Infection cleared | SCF |         |
| V-R-9     | sputum        | VSSA         | 0.5              | 1       | >256        | 0.6     | II   | t030 | ST239    | B                     | 16       | TEC, IPM, TZP, Infection cleared | SCF |         |
| VSSA-pair (77d) |           | VSSA         | 0.5              | 2       | >256        | 0.5     | II   | t030 | ST239    | B                     | 75       | Chronic obstructive pneumonia, | 6 |         |
| Isolate * | Specimen Type | Phenotype b | MIC (µg/ml) for Vocabulary | PAP-AUC | SCCmec Typb | Spa Type | MLST | PFGE | Age, years | Diagnosis | Duration of VAN exposure, days d | Therapy e | Outcome |
|----------|---------------|-------------|-----------------------------|---------|-------------|----------|------|------|------------|-----------|--------------------------------|-----------|---------|
| VSSA-pair11 (104d) | | | | | | | | | | | | | |
| V-F-11 | sputum | VSSA | 0.25 | 2 | >256 | 0.5 | III | t030 | ST239 | A | | | |
| V-R-11 | sputum | VSSA | 0.5 | 2 | >256 | 0.7 | III | t030 | ST239 | A | | | |
| VSSA-pair12 (18d) | | | | | | | | | | | | | |
| V-F-12 | sputum | VSSA | 0.5 | 2 | 256 | 0.5 | III | t030 | ST239 | C | | | |
| V-R-12 | sputum | VSSA | 0.5 | 2 | 256 | 0.5 | III | t030 | ST239 | C | | | |
| VSSA-pair13 (143d) | | | | | | | | | | | | | |
| V-F-13 | sputum | VSSA | 0.5 | 1 | 256 | 0.5 | III | t030 | ST239 | B | | | |
| V-R-13 | sputum | VSSA | 0.5 | 2 | 256 | 0.7 | III | t030 | ST239 | B | | | |
| VSSA-pair14 (15d) | | | | | | | | | | | | | |
| V-F-14 | abdominal fluid | VSSA | 0.5 | 2 | >256 | 0.6 | III | t030 | ST239 | A | | | |
| V-R-14 | abdominal fluid | VSSA | 0.5 | 2 | >256 | 0.6 | III | t030 | ST239 | A | | | |
| VSSA-pair15 (20d) | | | | | | | | | | | | | |
| V-F-15 | sputum | VSSA | 0.5 | 2 | >256 | 0.4 | III | t037 | ST239 | F | | | |
| V-R-15 | sputum | VSSA | 0.5 | 2 | >256 | 0.6 | III | t037 | ST239 | F | | | |

Interval (day) of the two isolates.

Defined by population analysis profile (PAP)–area under the curve (AUC) method (PAP-AUC) (see Materials and Methods).

NA, did not carry SCCmec.

The duration of VAN exposure represents the total length of time that the patient received vancomycin. The VSSA isolate of each pair obtained prior to beginning vancomycin / teicoplanin therapy.

VAN, vancomycin; TEC, teicoplanin; OXA, oxacillin; LEV, levofloxacin; MFX, moxifloxacin; CIP, ciprofloxacin; IPM, imipenem; MEM, meropenem; AMK, amikacin; FEP, cefepime; CRO, ceftazidime; CAZ, ceftazidime; OX, cefuroxime; SCF, cefoperazone-sulbactam; TAZ, piperacillin-tazobactam; RIF, rifampin; SXT, sulfamethoxazole-trimethoprim; LZD, linezolid.

doi:10.1371/journal.pone.0066880.t001
Mass Spectrometry for 2D Gel Protein Identification

Gel plugs containing differentially expressed proteins were excised using a ProXcision robot (Perkin Elmer Inc., Wellesley, MA, US) and subjected to matrix-assisted laser desorption ionization-time of flight/time of flight (MALDI-TOF/TOF) analysis (Bruker Daltonics, Leipzig, GER). Gel plugs were placed in 96-well plates, and then washed with 25 mM NH₄HCO₃ (pH 8.0). Gel plugs were pre-frozen at –80°C for 1 h, and then digested with trypsin (Promega, WI, USA). After extraction from the gel into 50% acetonitrile/0.1% formic acid, peptides were lyophilized in a speed vacuum and resuspended in 10 μL of 0.1% formic acid solution. Peptide MS/MS spectra were obtained by MALDI-TOF/TOF (Bruker Daltonics). The resulting MS/MS spectra were interpreted using Mascot and searched against eubacterial proteins in the National Center for Biotechnology Information protein database. Results showing MASCOT score ≥75 and confidence level ≥95% were considered reliable [23].

Real-Time Quantitative Reverse Transcription–PCR (qRT–PCR)

Total RNA was extracted from each sample using the RNaseasy Kit (Qiagen, CA, USA). Complementary DNA (cDNA) was generated from total RNA using a random primer hexamer. Gene-specific primers were designed using Primer Express 3.0 (Applied Biosystems) and are shown in Table 2. Samples were run in triplicate and quantified by qRT-PCR following the protocol for SYBR® Premix Ex Taq™ II (TaKaRa, Tokyo, Japan). The mixture was incubated at 95°C for 30 s, and then cycled at 95°C for 5 s and at 60°C for 20 s 40 times using the LightCycler® 480 (Roche, Mannheim, GER). Amplification efficiencies were validated and normalized to the expression of the 16 S rRNA gene as a standard. The quantities of the target and standard genes were calculated according to a standard curve.

### Table 2. List of primers for real-time quantitative reverse transcriptase – PCR.

| Primer Name | Sequence | Length (bp) | NCBI Accession No. |
|-------------|----------|-------------|--------------------|
| 16S rRNA-F  | GGCAAGCGTTATCCGGAATT | 20 | NC_002745          |
| 16S rRNA-R  | GTTCCCAAGCCTTCCACG  | 20 |                   |
| isaA-F      | TGCCCTAAGCTGTCGTTT  | 20 | NP_375681.1        |
| isaA-R      | GACCCCCACGCGATGTTT  | 20 |                   |
| mbrA2-F     | TGACAAATAGCGAGGATTG  | 21 | NP_374474.1        |
| mbrA2-R     | GCATGACGCACTGATAAC  | 20 |                   |
| asp23-F     | CTTTACGGAAAGTATTAGGTGTA | 25 | NP_375295.1        |
| asp23-R     | CAAGGCGTTACTGCTGAGGTGTG | 25 |                   |
| gpmA-F      | TCAGACTTTCGAAATAAGCATC | 23 | NP_375527.1        |
| gpmA-R      | ACCCTGCTAAACCGGAACAA | 21 |                   |
| ahpC-F      | CACGGCCAATTCGCTCA  | 17 | NP_373615.1        |
| ahpC-R      | TGACCCCCATCAAAAACACTC | 23 |                |

Mass Spectrometry for 2D Gel Protein Identification

Gel plugs containing differentially expressed proteins were excised using a ProXcision robot (Perkin Elmer Inc., Wellesley, MA, US) and subjected to matrix-assisted laser desorption ionization-time of flight/time of flight (MALDI-TOF/TOF) analysis (Bruker Daltonics, Leipzig, GER). Gel plugs were placed in 96-well plates, and then washed with 25 mM NH₄HCO₃ (pH 8.0). Gel plugs were pre-frozen at –80°C for 1 h, and then digested with trypsin (Promega, WI, USA). After extraction from the gel into 50% acetonitrile/0.1% formic acid, peptides were lyophilized in a speed vacuum and resuspended in 10 μL of 0.1% formic acid solution. Peptide MS/MS spectra were obtained by MALDI-TOF/TOF (Bruker Daltonics). The resulting MS/MS spectra were interpreted using Mascot and searched against eubacterial proteins in the National Center for Biotechnology Information protein database. Results showing MASCOT score ≥75 and confidence level ≥95% were considered reliable [23].

Real-Time Quantitative Reverse Transcription–PCR (qRT–PCR)

Total RNA was extracted from each sample using the RNaseasy Kit (Qiagen, CA, USA). Complementary DNA (cDNA) was generated from total RNA using a random primer hexamer. Gene-specific primers were designed using Primer Express 3.0 (Applied Biosystems) and are shown in Table 2. Samples were run in triplicate and quantified by qRT-PCR following the protocol for SYBR® Premix Ex Taq™ II (TaKaRa, Tokyo, Japan). The mixture was incubated at 95°C for 30 s, and then cycled at 95°C for 5 s and at 60°C for 20 s 40 times using the LightCycler® 480 (Roche, Mannheim, GER). Amplification efficiencies were validated and normalized to the expression of the 16 S rRNA gene as a standard. The quantities of the target and standard genes were calculated according to a standard curve.

### Table 3. Proteins differentially expressed between hVISA and VSSA identified in this study.

| Accession Numbera | Protein Name       | Gene   | Protein PIb | Protein MWc (Da) | Protein Score C.I.%d | Protein Function/Pathway                                                                 |
|-------------------|--------------------|--------|-------------|------------------|----------------------|-------------------------------------------------------------------------------------------|
| Q6GDN1            | Probable transglycosylase isaA precursor | isaA   | 6.11        | 24198.5          | 100                  | CLEAVES PEPTIDOGLYCAN                                                                      |
| Q6GGY3            | Peptide methionine sulfoxide reductase 2 | mbrA2  | 6.64        | 20545.9          | 100                  | A REPAIR ENZYME FOR PROTEINS THAT HAVE BEEN INACTIVATED BY OXIDATION, CATALYZES THE REVERSIBLE OXIDATION-REDUCTION OF METHIONINE SULFOXIDE IN PROTEINS TO METHIONINE |
| SHE23             | Alkaline shock protein 23               | asp23  | 5.13        | 19179.7          | 100                  | PLAYING A KEY ROLE IN ALKALINE pH TOLERANCE                                              |
| Q2YZ6             | 2,3-Bisphosphoglycerate-dependent phosphoglycerate mutase | gpmA   | 5.23        | 26663.5          | 100                  | CATALYZING THE INTERCONVERSION OF 2-PHOSPHOGlycerate AND 3-PHOSPHOGlycerate/ CARBONATE DEGRADATION; GLYCOC LS; PYRUVATE FROM D-GYcERALDEHYDE 3-PHOSPHATE: STEP 3/5 |
| QSHI5             | Alkyl hydroperoxide reductase subunit C | ahpC   | 4.88        | 20963.5          | 98.842               | DIRECTLY REDUCING ORGANIC HYDROPEROXIDES IN ITS REDUCED DITHIOL FORM                     |

aAccession numbers are from Swiss-Prot or non-redundant NCBI (gi prefix) protein databases.
bProtein PI list experimentally determined isoelectric point values derived from 2-DE spot positions.
cProtein MW list experimentally determined molecular weight values derived from 2-DE spot positions.
dProtein C.I.% indicates confidence interval that the program assigns to the peptide protein matches. >95% score was considered statistically significant.
doi:10.1371/journal.pone.0066880.t003
Statistical Analysis

Statistical analysis was performed using the Wilcoxon rank sum test or One-Way ANOVA test in SPSS (17.0). A \( p \)-value of \( \leq 0.05 \) was considered statistically significant.

Results

Genotypic Characterization of the Isolate Set

Two pairs of isogenic VSSA and hVISA isolates that belonged to SCC\(^{\text{mec}}\)III-ST239-Spa\(^{\text{t030}}\) were selected for the comparative proteomics analysis to minimize variation unrelated to the vancomycin resistance phenotype. Six pairs of isogenic VSSA and hVISA strains, and unrelated VSSA (\( n = 30 \)) and hVISA (\( n = 24 \)) strains were selected to validate the differentially expressed genes identified by comparative proteomics screening. Fifteen pairs of persistent VSSA isolates were selected to determine whether the differentially expressed genes were associated with persistent infection.

As shown in Table 1, six pairs of VSSA and hVISA isolates that belonged to the SCC\(^{\text{mec}}\)III-ST239-Spa\(^{\text{t030}}\) type were classified into three PFGE patterns. Of the 15 pairs of persistent VSSA isolates, 11 pairs were SCC\(^{\text{mec}}\)III-ST239-Spa\(^{\text{t030}}\), 2 pairs were SCC\(^{\text{mec}}\)III-ST5-Spa\(^{\text{t002}}\), 1 pair was SCC\(^{\text{mec}}\)III-ST239-Spa\(^{\text{t037}}\), and 1 pair was identified as methicillin-susceptible \( S. \) aureus (MSSA)-ST398-Spa\(^{\text{t034}}\) type. The 15 pairs of VSSA isolates were classified into 6 PFGE patterns, with each pair of isolates possessing the same PFGE profile.

### Table 4. Relative \( \text{isaA} \), \( \text{msrA2} \), \( \text{asp23} \), \( \text{gpmA} \) and \( \text{ahpC} \) gene expression of hVISA strains compared with that of the parent strains, as determined by quantitative real-time–PCR and normalized to 16S rRNA expression.

| Differentially expressed genes | Relative gene expression (arbitrary unit) | \( p \)-value \(^{b}\) |
|-------------------------------|-----------------------------------------|------------------|
|                              | CN2/CN1       | CN4/CN3       | CN6/CN5       | CN8/CN7       | CN10/CN9      | CN12/CN11     |
| \( \text{isaA} \)           | 10.2          | 72.8          | 674.2         | 6.7           | 12.8          | 5.8           | 0.028         | 0.028         |
| \( \text{msrA2} \)          | 1.4           | 77.2          | 2.3           | 1.6           | 1.2           | 52.5          | 0.028         | 0.028         |
| \( \text{asp23} \)          | 0.6           | 231.1         | 44.3          | 1.7           | 18.6          | 11.4          | 0.075         | 0.075         |
| \( \text{gpmA} \)           | 0.8           | 215.7         | 114.2         | 13.5          | 1.8           | 11.0          | 0.046         | 0.046         |
| \( \text{ahpC} \)           | 1.7           | 12.5          | 29.0          | 1.1           | 1.4           | 2.7           | 0.046         |

\(^{a}\)The value of relative gene expression was the averages of triplicate samples. 
\(^{b}\)\( p \)-value as determined by Wilcoxon rank sum test.

The value of relative gene expression was the averages of triplicate samples. \( p \)-value as determined by One-Way ANOVA test.

Figure 1. Relative \( \text{isaA} \), \( \text{msrA2} \), \( \text{asp23} \), \( \text{gpmA} \), and \( \text{ahpC} \) gene expression of hVISA strains (\( n = 24 \)) compared with VSSA (\( n = 30 \)), as determined by quantitative real-time PCR and normalized to 16S rRNA expression. Bar means the mean of relative gene expression. Error bar: 95% CI. The value of relative gene expression was the averages of triplicate samples. \( p \)-value as determined by One-Way ANOVA test.

doi:10.1371/journal.pone.0066880.g001
Proteins in Clinical VSSA and hVISA Isolates

Relative Expression of the five Differentially Expressed Proteins

The results showed that the expression of these genes, except for \( \text{asp23} \), was significantly up-regulated in the hVISA strains. The expression of these five genes was also evaluated in unrelated VSSA \((n = 30)\) and hVISA \((n = 24)\) strains by qRT-PCR, which showed that only \( \text{isaA} \) was significantly up-regulated in the hVISA strains (Figure 1).

To determine whether the differentially expressed genes were associated with persistent infection, their expression was assessed in 15 pairs of persistent VSSA strains by qRT-PCR. The results showed no significant differences in the expression level of the five genes among the 15 pairs of persistent VSSA strains (Table 5).

### Discussion

Although the clinical importance of hVISA strains has been well-established, the resistance mechanism of hVISA remains unclear. In the present study, the potential mechanism of low-level vancomycin resistance was assessed in two pairs of isogenic VSSA and hVISA isolates by comparative proteomics analysis, which identified five differentially expressed proteins that were up-regulated in both hVISA strains (Table 3).

Among the identified proteins, AphC, Asp23, and MsrA2 are involved in defense mechanisms. AphC directly reduces organic hydroperoxides to their dithiol forms [24]. The hVISA/VISA strains showed thickened cell walls, increased peptidoglycan crosslinking, and a high positive charge [25], which could have caused changes in oxidation and osmotic pressure inside and outside the cell, and further induced AphC expression. The stress response gene \( \text{asp23} \), which encodes the Asp23 protein, is a possible target gene of the key global regulator, SigB [26]. Asp23 has a key role in the alkaline pH tolerance of \( S. \ aureus \) [27]. A previous microarray-based study also showed that Asp23 is up-regulated in the hVISA strain [28]. The results of our comparative proteomics analysis showed that MsrA2 was enhanced in both

#### Table 5. Relative \( \text{isaA}, \text{msrA2}, \text{asp23}, \text{gpmA} \) and \( \text{ahpC} \) gene expression of persistent \( S. \ aureus \) strains, as determined by quantitative real-time–PCR and normalized to 16S rRNA expression.

| Isolate      | Relative gene expression (arbitrary unit) |
|--------------|------------------------------------------|
|              | \( \text{isaA} \) (VSSA-R/VSSA-F) a | \( \text{msrA2} \) (VSSA-R/VSSA-F) | \( \text{asp23} \) (VSSA-R/VSSA-F) | \( \text{gpmA} \) (VSSA-R/VSSA-F) | \( \text{ahpC} \) (VSSA-R/VSSA-F) |
| VSSA-pair1   | 2.62 | 2.18 | 1.74 | 1.36 | 2.48 |
| VSSA-pair2   | 1.75 | 0.85 | 1.03 | 0.51 | 1.60 |
| VSSA-pair3   | 0.87 | 0.90 | 0.70 | 0.31 | 1.37 |
| VSSA-pair4   | 1.23 | 0.95 | 1.70 | 0.86 | 0.91 |
| VSSA-pair5   | 0.52 | 0.22 | 0.22 | 0.61 | 0.42 |
| VSSA-pair6   | 1.65 | 0.49 | 0.20 | 0.65 | 0.65 |
| VSSA-pair7   | 1.66 | 1.64 | 0.45 | 0.44 | 0.42 |
| VSSA-pair8   | 16.03 | 1.97 | 3.21 | 1.45 | 1.07 |
| VSSA-pair9   | 0.27 | 0.75 | 0.30 | 0.31 | 0.67 |
| VSSA-pair10  | 0.22 | 0.91 | 0.95 | 0.74 | 0.79 |
| VSSA-pair11  | 0.88 | 0.70 | 0.86 | 1.16 | 1.99 |
| VSSA-pair12  | 3.53 | 1.13 | 0.24 | 1.40 | 0.31 |
| VSSA-pair13  | 0.24 | 0.83 | 0.78 | 0.79 | 1.15 |
| VSSA-pair14  | 0.66 | 0.38 | 0.93 | 0.34 | 0.75 |
| VSSA-pair15  | 1.65 | 0.57 | 0.04 | 0.19 | 0.74 |

\( \text{p-value} = 0.100 \) \( \text{p} = 0.069 \) \( \text{p} = 0.088 \) \( \text{p} = 0.053 \) \( \text{p} = 0.164 \)

\( \text{VSSA-F} \) means vancomycin-susceptible \( S. \ aureus \) (VSSA) isolated from patient prior to vancomycin therapy; \( \text{VSSA-R} \) means vancomycin-susceptible \( S. \ aureus \) (VSSA) isolated from patient after vancomycin therapy. The value of relative gene expression was the averages of triplicate samples.

\( \text{p-value} \) as determined by Wilcoxon rank sum test.

*VSSA-F means vancomycin-susceptible \( S. \ aureus \) (VSSA) isolated from patient after vancomycin therapy; VSSA-R means vancomycin-susceptible \( S. \ aureus \) (VSSA) isolated from patient prior to vancomycin therapy. The value of relative gene expression was the averages of triplicate samples.

\( \text{p-value} \) as determined by Wilcoxon rank sum test.

doi:10.1371/journal.pone.0066880.t005
hVISA strains. MsrA2, which catalyzes the reversible oxidation-reduction of methionine sulfoxide to methionine, has a key function as a repair enzyme for proteins inactivated by oxidation. *S. aureus* possesses three MsrA enzymes (MsrA1, MsrA2, MsrA3) [29]. The msrA gene is highly induced member gene of the cell wall stress stimulus (CWSS), which can be induced by cell wall-active antibiotics, such as oxacillin and vancomycin. The up-regulation of msrA can lead to an increased rate of peptidoglycan biosynthesis, which results in cell wall thickening [11]. In addition, Msr proteins regulate virulence in several bacteria [29,30]. In a cDNA microarray study [11], msrA2 was over-expressed in VISA strains, which coincided with our results. The study also demonstrated that msrA2 contributes to vancomycin resistance by gene knockout and trans-complementation assay [11]. In addition, cell morphology experiments showed that msrA2 over-expression increases the cell wall thickness of *S. aureus* [11]. Collectively, these observations are consistent with a previous report showing that vancomycin affects the expression of CWSS-associated genes [12].

Another differentially expressed protein, GpmA, functions in cellular metabolism. GpmA catalyzes the interconversion of 2-phosphoglycerate and 3-phosphoglycerate and is therefore involved in the glycolytic pathway [31]. As a key enzyme in glycolysis and energy metabolism, GpmA is a potential target for novel antibiotics [31]. This study is the first to report that GpmA is up-regulated in hVISA.

IsaA, which is involved in cell wall biogenesis, was also over-expressed in both hVISA isolates, as shown in our comparative proteomics results. IsaA cleaves peptidoglycan and thus plays a significant role in peptidoglycan turnover, cell wall crosslinking, and cell division [32]. Therefore, IsaA over-expression could be associated with the thickened cell walls of hVISA strains, which may be related to hVISA resistance. Another comparative proteomics study found that IsaA is up-regulated in the VISA strain Mu50, which is similar to our result [16]. The lack of RNAIII can lead to the over-expression of IsaA [33]. Several studies have indicated that VISA is characterized by *agr* dysfunction or RNAIII down-regulation [6,34,35]. A cDNA microarray study showed that IsaA is up-regulated in VRSA strains [36]. Therefore, the *isaA* gene may have an important function in *S. aureus* resistance to vancomycin.

To validate the accuracy of the results of our comparative proteomics analysis, 6 pairs of isogenic VSSA and hVISA strains isolated from the same patient, unrelated VSSA (n = 30) and hVISA (n = 24) strains, and 15 pairs of persistent VSSA strains were selected for confirmation by qRT–PCR. Analysis of the isogenic strains showed that *isaA, msrA2, gpmA*, and *ahpC* were significantly up-regulated in most of the hVISA strains compared with the VSSA strains, which was partly consistent with the results of comparative proteomics. However, only *isaA* was significantly up-regulated in hVISA strains compared with the unrelated VSSA strains. Therefore, the over-expression of *isaA* may be related to hVISA resistance. Analysis of the 15 pairs of persistent VSSA strains showed no differences in the expression of the identified genes, which indicates that these genes are not associated with persistent infection.

The present study has several limitations. First, the functionality of the identified genes could not be assigned in the absence of gene knockout experiments or further studies. Furthermore, the gene expression changes observed may be a consequence of vancomycin resistance and not causal of this phenotype. For example, these changes may be necessary to compensate for increased cell wall thickness or a consequence of reduced growth rate.

In summary, five differentially expressed proteins, IsaA, MsrA2, Asp23, GpmA, and AhpC, were identified in two pairs of isogenic VSSA and hVISA strains via comparative proteomics analysis. The results of qRT–PCR showed that the *isaA* gene was significantly up-regulated in most of the clinical hVISA isolates, suggesting a relation between increased expression of *isaA* and hVISA resistance.

**Acknowledgments**

The authors would like to thank International Science Editing for critically revising the manuscript.

**Author Contributions**

Conceived and designed the experiments: HW MC. Performed the experiments: HC YL CZ FZ. Analyzed the data: HC YL HW. Contributed reagents/materials/analysis tools: DX JZ. Wrote the paper: HC HW.
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