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Correlation Between Toxin-Antitoxin Systems and Persistence States in *Staphylococcus aureus* Isolates

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A B S T R A C T

Introduction: Levels of the human epidermal growth factor receptor 2 (HER2) gene are low in normal breast tissue, but half of the patients with breast cancer exhibit higher levels of this receptor. The differential expression of the HER2 gene in normal and malignant cells makes it an excellent biomarker for therapeutic purposes. In this study, we evaluated the degree of HER2 overexpression in patients and its relationship with age and the occurrence of metastases.

Materials & Methods: In this retrospective, registry-based, two-center cohort study, information on 1500 breast cancer patients recruited from Shahid Mostafa Khomeini Hospital in Ilam Province was collected from 2020 to 2023.

Results: The likelihood of metastasis in cancer patients with HER2 gene expression was three times higher (adjusted OR: 2.82; 95% CI: 1.79–3.29; P=0.001). Additionally, the involvement of lymph nodes (adjusted OR: 2.01; 95% CI: 0.87–3.79; P=0.03) was significantly associated with increased metastasis.

Conclusion: This study demonstrates that HER2 gene expression and the number of involved lymph nodes are significant prognostic factors that increase the risk of metastasis. Therefore, implementing comprehensive breast cancer screenings can play an important role in the treatment and prevention of metastasis in breast cancer patients.

Keywords: Toxin-antitoxin systems, Persister Cells, Recalcitrance, Tolerant, Physiology

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Introduction

Staphylococcus aureus causes a large number of nosocomial and community-acquired illnesses. It causes a variety of diseases, ranging from deadly systemic disorders to localized skin infections (1). Several investigations have demonstrated that *S. aureus* may live an intracellular existence, thriving inside osteoblasts and osteocytes found in human bone cells mostly in the *S. aureus* SCV state (2), but this is also likely to be the case in its L-form cell state. *S. aureus* can live inside these host cells for an extended period without being noticed; it has been demonstrated that these bacteria have a malfunctioning host recognition mechanism (3).

This allows the bacteria to survive for a long time in the environment of bone cells and serves as a reservoir for more infections that may recur. It is widely accepted that microbes use survival mechanisms to deal with outside stimuli (4). One of the most prevalent microbial stress reactions to environmental stressors, such as oxidative stress, temperature changes, food shortages, and high osmolarity, is a viable but nonculturable (VBNC) condition (5). A unique physiological state known as a VBNC state occurs when bacteria fail to grow on a medium but continue carrying out certain metabolic processes (6). However, in a high concentration of antibiotics, some *S. aureus* subpopulations would alter their metabolic process, enter the dormancy state, and become persistent (7). Many studies suggested the implication of Toxin-antitoxins (TAS) in the formation of persistent cells (8). TAS modules, which comprise a toxin and its antitoxin equivalent, are ubiquitous gene loci identified in bacteria. Antitoxin reduces the toxicity of toxins under normal physiological conditions; however, under stress, TA modules are crucial to the physiology of bacteria because they help with post-segregational death, abortive infection, biofilm formation, and persister cell development. While other intracellular molecular targets have also been reported, most toxins are proteinaceous and impact translation or DNA replication. Conversely, antitoxins can be either proteins or RNAs that, through direct interaction or with the aid of other signalling elements, neutralize their cognate toxin and aid in the regulation of the TA module (8). Nearly every species of bacteria has TAS (9). They can range from being absent in certain bacteria to being quite common in others (10). They can be located on plasmids or chromosomes (11). For specifics on TAS system types I through VI (12). Typically, a TAS codes for a stable toxic ingredient and its unstable antidote, or antitoxin, which can be a protein or RNA that, under normal growth conditions, inactivates the harmful action. Lower antidote concentrations are the outcome of stress conditions, and higher toxin activity is caused by an imbalance between toxin and antitoxin (8). An antitoxin works through various mechanisms to stop its cognate toxin from being toxic under optimal development conditions. However, it is either downregulated or quickly broken down by intracellular proteases in response to stress or plasmid loss (13). According to the type of antitoxin and the way the toxin and antitoxin interact to produce their effects, TAS is categorized into six types (13). In summary, the components of type I TAS are non-coding antisense RNA antitoxin, which binds to the mRNA of the toxin to either inhibit its translation into proteins or promote its mRNA degradation (14). The production of toxin mRNA and, in most cases, small membrane-associated pore-forming toxin peptides, also known as cytosolic nucleases, occurs when the labile antitoxin short RNA (sRNA) is broken down. Toxins and antitoxins are both proteins in Type II systems. Since most type II toxins are endoribonucleases, antitoxins feature two domains: one for binding DNA and the other for binding toxins, which inhibits the activity of the toxin (15). Proteolysis breaks down antitoxins under different stress circumstances, releasing the toxins to prevent translation and replication. RNA is the antitoxin type III, just as in type I. The toxin nuclease breaks down the antitoxin precursor mRNA into antitoxin sRNAs, which then bind to the toxin directly to neutralize its active site and create pseudoknots (16). Both components of type IV are proteins, but they do not

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interact. The antitoxin counteracts the toxin action by binding and maintaining cytoskeletal filament bundling, whereas the toxin component prevents cytoskeletal proteins from polymerizing to inhibit cell division (17,18). There has only been one description of Type V and VI TAS. The ghoT toxin mRNA in Type V TAS is degraded by the RNAse antitoxin GhoS. A TAS being governed by another TAS is a unique instance. SocB toxin and SocA antitoxin are both proteins found in Type VI (12). As of right now, *S. aureus* has been reported to harbor type I SprA1-SprA1AS and SprF1-SprG1, type II MazEF, YefMSa1-YoeBSa1, YefMSa2-YoeBSa2, and PemIK TAS (19). The molecular and biological processes of TAS are being extensively studied because they may provide solutions to common clinical issues such as biofilm-forming infections, pathogenicity, antibiotic resistance, and persistence. However, many of the discovered TAS still have unknown biological roles (20). Further research is still required to advance our knowledge of TAS in Gram-positive bacteria, as the majority of TAS research has been conducted in Gram-negative bacteria (21). The goal of this review is to outline and talk about what is currently known about *S. aureus* TAs.

Persistence of Staphylococcus aureus Infection

Staphylococcus aureus can change into the small-colony variant (SCV) phenotype to live inside host cells. Small colony variations (SCVs) are bacterial variants of *S. aureus* that arise from changes in metabolic genes, leading to the formation of auxotrophic bacterial subpopulations (22). Dormancy, which is defined here as a state in which cells are not engaged in any metabolic activity, is the cause of these persister cells. In 1942, *S. aureus* was used to characterize this trait for the first time and discovered that penicillin did not kill one in a million *S. aureus* cells and that the surviving cells did not undergo any genetic changes. As a result, these cells should not be regarded as resistant but rather as phenotypic varieties that are tolerant to antibiotics (23).

Persistent infections, either recurrent or chronic, develop in tandem with the appearance of SCVs. This slow-growing *S. aureus* subpopulation exhibits a changeable phenotype (stable or unstable), is produced in the host cells, has a non-homogeneous genetic background, and produces tiny colonies on solid-medium agar. The stable SCVs recovered from clinical specimens have been found to carry particular alterations in metabolic pathways, even though almost all SCVs separated from clinical specimens can quickly grow back to their parental condition (24). Certain *S. aureus* subpopulations do not respond to antibiotics, making bacterial removal inefficient. Through changes in its genetic composition, *S. aureus* can develop resistance to antibiotics, and genetic investigations have uncovered a significant percentage of the mechanisms driving the emergence of these antibiotic-resistant species. Persister cells are another subpopulation that is resistant to antibiotics. Growing clinical data indicate that these persister cells play a significant role in antibiotic resistance and persistent infection; nevertheless, a thorough understanding of the mechanisms underlying the development of *S. aureus* persister cells is lacking (25). Bacterial persister cells are known as phenotypic variations that display antibiotic tolerance and a temporary non-growing condition. According to studies, bacteria that survive antibiotic treatment inside host cells are persister cells. They exhibit biphasic death and, when monitored at the single-cell level, eventually achieve a uniformly non-responsive, non-dividing condition. After the antibiotics are stopped, this phenotype is stable yet reversible. While still metabolically active, intracellular *S. aureus* persister cells exhibit a changed transcriptome profile that is in line with the activation of stress responses, such as heat shock, cell wall stress, SOS pathway (an inducible DNA damage repair system), and stringent response (26).

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Importance of Defining Persistence in the Study of Persisters

One subset of bacteria has an epigenetic characteristic known as the persistence phenotype, characterized by slow growth and resistance to antibiotic treatment. The phenotype is obtained through an impulsive, reversible transition between persister and normal cells (Figure 1). These data show that persister cells, whose sluggish rate of division during growth results in lower population fitness, may be used by clonal bacterial populations as a kind of "insurance policy" against drug interactions (27). Persister cells, by definition, can withstand extended exposure to antibiotics before returning to a sensitive, actively developing phenotype in the absence of environmental stress, which underscores their importance. Persister cells that tolerate antimicrobial therapy may serve as a breeding ground for mutants resistant to antibiotics (28).

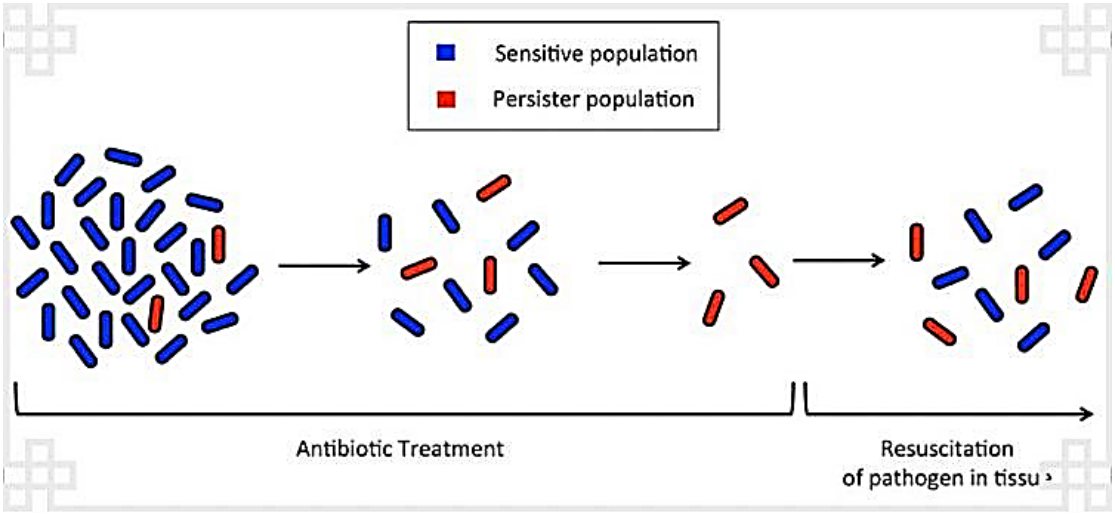


Figure 1. Progression of Persister Cell Formation During Antibiotic Treatment (7)

Pathogenesis of Staphylococcus aureus Associated with Persister Formation

Based on the proteome and metabolites observed during the development of *S. aureus* persister cells exposed to vancomycin and enrofloxacin, the research provided dynamic insights into the molecular physiology of persister cell formation following exposure to two different antibiotics with different mechanisms of action. The information reveals that while cells that are phenotypically categorized as persister cells share a number of molecular traits, they also significantly differ in a large number of other molecular characteristics. These differences shed light on eliminating persister cells, which has important therapeutic implications (29). Another study used CRISPR-dCas9 to knock down tcaA, tcaB, and tcaR. The results demonstrated that while tcaB suppression did not result in persistence, a notable increase in persister cells was observed when tcaA was suppressed by dCas9. The results were further investigated by producing a tcaA mutant, which demonstrated tcaA created a large increase in persister cells in contrast to the wild type. It was concluded that the gene tcaA enhances persister cells and glycopeptide resistance in *S. aureus* and may be a possible target for therapy (30). In many bacterial species, PhoU homologs play a vital role in the control of persister cells production and phosphate metabolism; however, the specific features of their roles vary depending on the species. The development of persister cells and virulence factors are tightly linked to the pathogenesis of *S. aureus*. It is still unknown what two PhoU homologs in *S. aureus*, PhoU1 and PhoU2 (31).

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Toxin-Antitoxin Modules That Affect the Proportion of Staphylococcus aureus Persisters

Most prokaryotes have tiny genetic components called toxin-antitoxin (TA) systems. They are countered by antitoxins and encode toxin proteins that impede essential cellular activities (32). The TA types I, II, and III genes have been found in *S. aureus*. A sequence-specific RNase called MazF, the toxin of the mazEF locus, cleaves several transcripts, including those encoding pathogenicity factors. Two distinct, but auto-regulated TA systems that result in ribosome-dependent RNases are represented by two yefM-yoeB paralogs (19)

Furthermore, a tripartite TA system consisting of omega, epsilon, and beta contributes to the stability of resistance factors (19). RNA antitoxins post-transcriptionally regulate the SprA1/SprA1AS and SprF1/SprG1 systems, which encode tiny membrane-damaging proteins (figure 2). Although they have not yet been validated experimentally, TA systems in *S. aureus* that are regulated by the interaction between toxin protein and antitoxin RNA have been discovered in silico (33). If these genetic loci are druggable targets, a deeper examination of potential connections between TA systems and *S. aureus* pathophysiology will clarify this (20,34). The conversion of a cyclopeptide antibiotic from a staphylococcal TA toxin emphasizes the promise of TA systems as relatively unexplored sources of drug development (19).

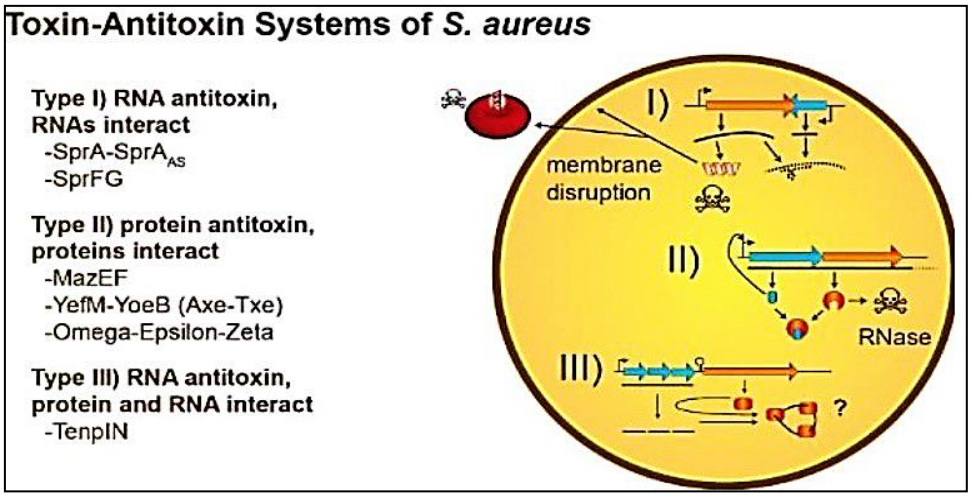


Figure 2. Staphylococcus aureus Toxin-Antitoxin Systems (19).

Regulation of Toxin and Antitoxin Modules in Promoting Staphylococcus aureus Persisters Formation

The development of persister cells has been connected to toxin-antitoxin systems; nonetheless, the molecular pathways underlying bacterial persistence remain mostly unclear. Schuster et al. demonstrate how the important human pathogen *S. aureus* binds to translating ribosomes of SprF1, a type I antitoxin, reducing the pathogen's total protein synthesis during growth. Because of its increased stability under hyperosmotic stress, SprF1 levels rise, collect on polysomes, and reduce protein production (19). Through the use of an internal 6-nucleotide sequence on its 5′ end, SprF1 binds to ribosomes and inhibits initiator transfer RNA binding, hence reducing translation initiation. The ribosome-bound antitoxin is displaced by an excess of messenger RNA, which permits the ribosomes to go through extra translation cycles even though this RNA antitoxin can also displace ribosome-bound mRNA (35).

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This translation-attenuation mechanism encourages antibiotic persister cell production, which is mediated by an RNA antitoxin. With its dual role of repressing toxin expression via its 3′-end and fine-tuning total bacterial translation via its 5′-end, the untranslated SprF1 is a dual-function RNA antitoxin. SprF1 also interacts with a fraction of polysomes and ribosomes that may facilitate the creation of antibiotic persister cells and translation retardation, owing to a purine-rich region at its 5′ terminus (figure 3). Type II toxins target bacterial translation, but not type I toxins3. The situation is different in our case because the RNA antitoxin, rather than the toxin, binds to a subset of translating ribosomes. In bacteria, two ribosome-associated regulatory RNAs (ribosome-associated non-coding RNAs) were identified: signal-recognition particle RNA27 and transfer-messegner RNA 26 (35,36).

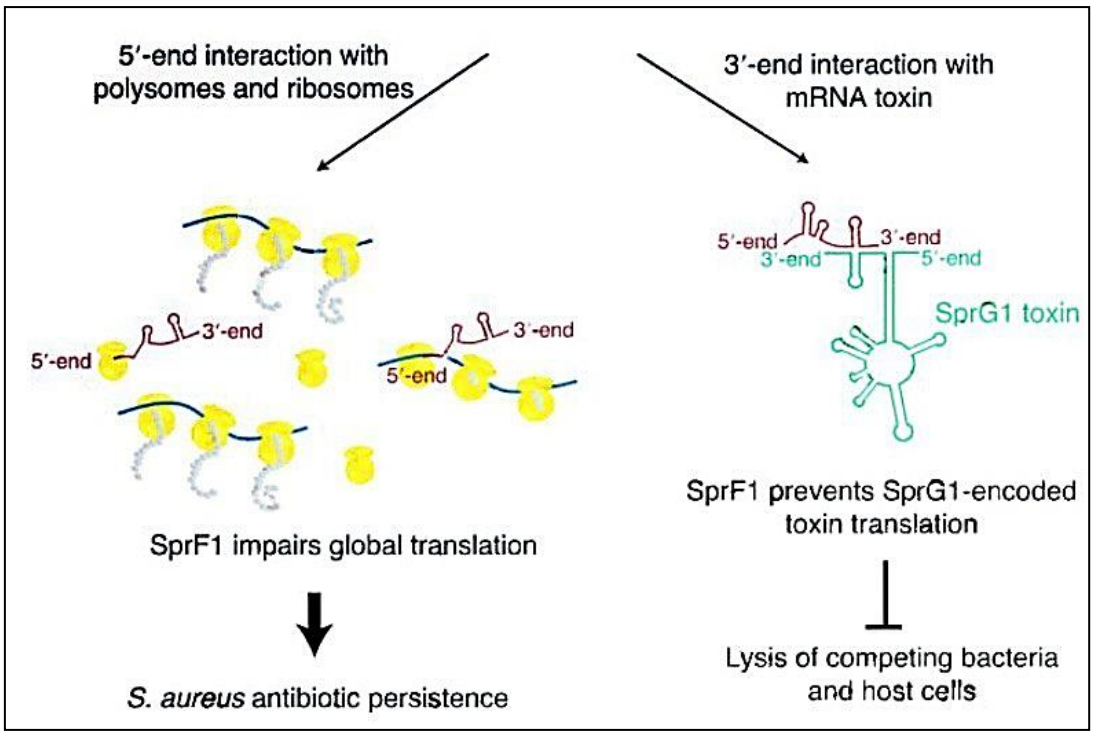


Figure 3. In *S. aureus*, the RNA antitoxin SprF1 binds to ribosomes to inhibit translation, promoting the formation of persister cells (35).

Conclusion

As outlined here, a small portion of the population develops into persister cells making it difficult to treatment. The increasingly evident that the TA systems play a role in bacterial activity and are more than just genetic debris. Therefore, TA systems could serve as targets for the development of anti-persistence model in *S. aureus*.

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Conflict of interest

The authors report no conflict of interest in this study.

Authors' contributions

FAF, and LBA: conceptualization. FAF, and LBA: investigation and writing—original draft preparation. All authors have read and agreed to the published version of the manuscript.

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