様式第１９号

学位論文審査の結果の要旨

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論文題目

Synergistic effects of novel penicillin-binding protein 1A amino acid substitutions contribute to high-level amoxicillin resistance of *Helicobacter pylori*
（ペニシリン結合蛋白質 1A の新規アミノ酸置換の相乗効果は、*Helicobacter pylori* における高いアモキシシリン耐性に寄与する）

論文掲載雑誌名

mSphere

論文要旨

The growing resistance to amoxicillin (AMX) – one of the main antibiotics used in *Helicobacter pylori* eradication therapy—is an increasing health concern. Several mutations of penicillin-binding protein 1A (PBP1A) are suspected of causing AMX resistance; however, only a limited set of these mutations have been experimentally explored. This study aimed to investigate four PBP1A mutations (i.e., T558S, N562H, T593A, and G595S) carried by strain KIN76, a high-level AMX-resistant clinical *H. pylori* isolate with an AMX minimal inhibition concentration (MIC) of 2 μg/mL. We transformed a recipient strain 26695 with the DNA containing one to four mutation allele combinations of the pbp1 gene from strain KIN76. Transformants were subjected to genomic exploration and antimicrobial susceptibility testing. The resistance was transformable, and the presence of two to four PBP1A mutations (T558S and N562H, or T593A and G595S), rather than separate single mutations, was necessary to synergistically increase the AMX MIC up to 16-fold compared with the wild-type (WT) strain 26695. An AMX binding assay of PBP1A was performed using these strains, and binding was visualized by chasing Bocillin, a fluorescent penicillin analog. This revealed that all four-mutation allele-transformed strains exhibited decreased affinity to AMX on PBP1A than the WT. Protein structure modeling indicated that functional modifications occur as a result of these amino acid substitutions. This study highlights a new synergistic AMX resistance mechanism and established new markers of AMX resistance in *H. pylori*.

本研究は，非常に高いアモキシシリン（AMX)耐性を示すピロリ菌 KIN76 株の penicillin-binding protein 1A (PBP1A)に見られた４ヶ所のアミノ酸の置換変異に着目し、AMX 感受性株である 26695 株に変異配列断片を形質転換により導入して、PBP1A 変異体発現株を作製し、PBP1A アミノ酸変異導入による AMX 結合能低下と AMX 耐性能増大の相関を明らかにした。さらにタンパク質構造モデリングにより、構造活性相関を見出し、薬剤耐性機構を明らかにした意義深い研究と考えられた。このため，審査員の合議により本論文は学位論文に値するものと判定した。

様式第２０号

最終試験
の結果の要旨
~~学力の確認~~

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学位申請者は本論文の公開発表を行い，各審査委員から研究の目的，方法，結果，考察について以下の質問を受けた。

１．アモキシシリンの耐性誘導機序について、水平伝播ならどういう環境下で*H. pylori*は、耐性遺伝子を取り込むのか。plasmid, phage, 点突然変異など、どのような機序か。
２．これまで*H. pylori*で報告された最強のアモキシシリンの耐性濃度はどの程度かのものが知られているのか。
３．コンゴ民主共和国の*H. pylori*感染者における高レベルのアモキシシリン耐性を示す臨床*H. pylori*株を過去に報告したと述べているが、同国における*H. pylori*感染の状況は臨床的影響の観点からどの程度深刻なのか。
４．どのような仮説に基づき本研究を開始したか。
５．Fig.4で陽性コントロールとしてのKIN76を用いたCompetitive assay実験は行っていないのか？
６．本研究で実施された実験によって抵抗性を獲得した株はドナー株よりもアモキシシリン耐性レベルが低かったと述べられているが、今後臨床への外挿を考慮する際に、この耐性レベルの乖離が本研究で得られた結果の解釈や妥当性にどのような影響を与えると考えられるか。
７．アモキシシリン耐性能を誘導するために、アモキシシリン存在下でAMXの変異圧力のみをかけた状態で、*in vitro*でpbp1遺伝子に点変異が生じるかどうかを実験的に試してみたことはあるか。
８．実際に生体内環境下で、どのようにして菌はpbp1変異を獲得すると考えられるか。

これらの質疑に対して，申請者は概ね適切に回答した。よって審査委員の合議の結果，申請者は学位取得有資格者と認定した。

（注）不要の文字は２本線で抹消すること。

様式第８号 No. 1

学　位　論　文　要　旨

氏名 Cimuanga Mukanya Alain

論　文　題　目

Synergistic effects of novel penicillin-binding protein 1A amino acid substitutions contribute to high-level amoxicillin resistance of *Helicobacter pylori*

(ペニシリン結合蛋白質 1A の新規アミノ酸置換の相乗効果は、*Helicobacter pylori* における高いアモキシシリン耐性に寄与する)

要　　旨

Background: The emergence of amoxicillin (AMX) resistance in *Helicobacter pylori* (*H. pylori*) poses a significant challenge in current first-line and rescue eradication treatments. Although this resistance is primarily linked to penicillin-binding protein 1A (PBP1A) mutations, experimental evidence has only been obtained for a limited set of them. We aimed to experimentally assess the effects of a panel of PBP1A substitutions, including T558S, N562H, T593A and G595S associated with high-level AMX resistance in clinical isolates. **Method**: Site-directed mutagenesis by natural transformation was used to generate mutant strains harboring different combinations of T558S, N562H, T593A, and G595S substitutions. Therefore, the wild-type *H. pylori* laboratory strain 26695 and the clinical isolate KIN76 (encoding the four mutations) were respectively used as recipient and donor strains for natural transformation.

Mutant and wild-type strains were assessed by whole genome and Sanger sequencing and AMX susceptibility testing by agar dilution method. Additionally, we compared the growth rates of the mutant strains with those of the wild-type strains to evaluate the potential impact of growth on the resistance phenotype. Then a PBP1A-AMX binding assay was performed on membrane protein extracts from different strains, using Bocillin as labeling agent. Finally, 3D structural analyses were conducted to compare mutated and wild-type PBP1A proteins. **Results**: The AMX minimal inhibitory concentrations (MICs) at baseline were 0.0625 and 2 µg/mL for the recipient, respectively. All experimentally generated mutants cumulating T558S and N562H, T593A and G595S, or all the four mutations were AMX resistant (i.e., MIC > 0.125 µg/mL). Mutants with dual mutations exhibited a MIC 4 to 8-fold higher than the MIC of the wild-type recipient strain and those with quadruple mutations showed up to 16-fold higher MIC. In contrast, transforming the wild-type recipient strain with a single T558S, N562H, T593A or G595S mutation did not generate any AMX resistance. Sequencing confirmed site-directed mutagenesis and ruled out any possible concomitant change into genes encoding other penicillin-binding proteins or other proteins interacting with PBPs and involved in AMX resistance. The growth rate analysis showed no significant difference between the resistant mutants and the parental strain 26695, ruling out any substantial effect of growth on the newly acquired resistance phenotype. The Bocillin binding assay showed that the PBP1A affinity for AMX decreased gradually with the accumulation of 2 and 4 mutations compared to the wild-type strain. Protein 3D structural analyses revealed that mutations led to conformational alterations of known AMX binding sites. **Conclusion:** Cumulated PBP1A mutations synergistically increased the MICs of AMX likely due decreased drug affinity by binding site alterations. While establishing new markers of drug resistance, these findings contribute to our understanding of the mechanisms underlying AMX resistance in *H. pylori*.