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Short Communication

Genetic stability of *Mycobacterium abscessus* during antibiotic treatment

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ABSTRACT

Objectives: Genetic changes in *Mycobacterium abscessus* during antibiotic treatment are not fully understood. This study aimed to investigate the genetic changes in *M. abscessus* in patients receiving antibiotic treatment, and their clinical implications.

Methods: Pretreatment and 12-month post-treatment *M. abscessus* isolates were obtained from patients with *M. abscessus* pulmonary disease. Isolates from each time point were separated into six groups based on their distinctive morphological characteristics. Twenty-four isolates, comprising 12 from patient A exhibiting progressive disease and 12 from patient B demonstrating stable disease, underwent sequencing. Subsequently, minimal inhibitory concentrations (MICs) for the administered antibiotics were measured.

Results: Persistent infection with a single strain was observed in patients A and B. During 12 months of treatment, MICs for administered drugs did not generally change over time in either patient and single nucleotide variations (SNV) associated with antimicrobial resistance (*rrl, rrs, erm*(41), gyr*A, gyrB, whiB7* and *hflX*) were not mutated. Although not significant, 47 and 52 non-synonymous SNVs occurred in *M. abscessus* from patients A and B, respectively, and the accumulation of these SNVs differed in patients A and B, except for five SNVs. The most variable positions were within a probable NADH-dependent glutamate synthase gene and a putative YrbE family protein gene in patients A and B, respectively.

Conclusions: Persistent infections by a single strain of *M. abscessus* were observed in two patients with different clinical courses. Genetic changes in *M. abscessus* during antibiotic treatment were relatively stable in these patients.

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1. Introduction

Nontuberculous mycobacteria (NTM), which comprise more than 200 species, are ubiquitous organisms isolated from municipal water, dust, and soil. NTM can cause chronic infections in humans, and the most common manifestation is pulmonary disease (PD) [1]. During the last decade, the clinical importance of NTM infections has rapidly increased globally. In South Korea, between 2010 and 2021, the prevalence of NTM infection increased from 11.4 to 56.7 cases per 100,000 population [2]. Eventually, the

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burden of NTM-PD in South Korea is expected to exceed that of tuberculosis in the near future [2].

Despite the epidemiological importance of NTM-PD, its treatment remains challenging [1]. Treatment requires the combined use of multiple antibiotics for >12 months after achieving culture conversion [1]. However, this strategy can lead to adverse reactions. Moreover, treatment outcomes are unsatisfactory, especially in patients with *Mycobacterium abscessus*-PD [3]. *M. abscessus*, which is the second-most common pathogen causing NTM-PD in East Asia, exerts its virulence with distinctive cell wall properties as well as type IV secretion systems [4]. Moreover, *M. abscessus* has an intrinsic, adaptive, and acquired resistance to commonly used antibiotics [4]. Consequently, more than two-thirds of patients with *M. abscessus*-PD fail to be cured and continue to have positive sputum cultures during treatment [3].

During the course of chronic infection, bacteria can survive using evolutionary strategies to adapt to the host microenvironment and promote antimicrobial resistance [5]. In patients with cystic fibrosis (CF), in which *M. abscessus* maintains a persistent infection, stepwise evolution of *M. abscessus* can be observed. These evolutionary changes have led to the genetic divergence of *M. abscessus* and the development of sub-clones over time [6]. However, whether intra-patient microevolution of *M. abscessus* occurs in patients without CF remains unknown. Furthermore, the relationship between genetic variant acquisition, antibiotic treatment, and resultant clinical implications is not fully understood.

For these reasons, this study investigated sputum isolates from two non-CF patients infected with *M. abscessus* subspecies *abscessus* (hereinafter referred to as *M. abscessus*), which were collected at the time of treatment initiation and 12 months thereafter. Although these patients could not be cured, the radiographic responses during antibiotic treatment were different: improved in one patient and progressively aggravated in the other. By identifying inter- and intra-patient genetic variations over time, we aimed to demonstrate the presence of microevolution and its association with the clinical course.

2. Material and methods

2.1. Patient selection

A prospective cohort of NTM-PD patients was initiated on 1 July 2011, at the Seoul National University Hospital (NCT01616745). Among the enrolled patients in the cohort, isolates from two patients (patients A and B), who were infected with *M. abscessus* and whose sputum cultures were positive despite antibiotic treatment for more than 12 months, were collected according to the radiographic responses. Demographic variables and antibiotic prescriptions were also assessed. Chest computed tomography (CT) was performed at the time of treatment initiation and 12 months thereafter, and radiographic findings were interpreted by two chest radiologists. The patients provided informed consent for this study and the Institutional Review Board of Seoul National University Hospital approved the protocol (IRB No. 2208–052–1349). The study was performed in accordance with the principles of the Declaration of Helsinki.

2.2. Sample collection

M. abscessus isolates obtained immediately before treatment (A and B, respectively) and 12 months after treatment (A' and B', respectively) were used in this study. The identification of each isolate was confirmed by 16S rRNA and *rpo*B gene sequencing [7,8]. Isolates were plated on Middlebrook 7H10 agar (Difco Laboratories, Detroit, MI, USA) supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC), and five distinct colonies from each isolate, in terms of colony size and morphotype, were purified (Supplementary Figure 1). First, we selected several colonies that represented the majority. In addition, we selected the colonies for sequencing if any differences from major representatives were observed in plates in terms of size, shape, elevation, and margin. Finally, *M. abscessus* isolates at each time point were separated into six samples: one from directly cultured sputum samples (A-1 and B-1) and the others, based on distinctive morphological characteristics (A-2–A-6 and B-2–B-6).

2.3. MICs measurement

The isolated colonies were sent to the Korea Institute of Tuberculosis to measure the minimal inhibitory concentration (MIC) of azithromycin (USP No.1046056, MD, USA), amikacin (USP No.1019508), clarithromycin (USP No.1134379), clofazimine (Sigma-Aldrich No.C8895, MO, USA), imipenem (USP No.1337809), and moxifloxacin (USP No.1448606) that has been administered to the patients. The MICs for each drug were determined by a broth microdilution system using antimicrobial concentrations derived from serial 2-fold dilutions [9]. The incubation time was 5 d. Exceptionally, to detect inducible resistance, the incubation time for clarithromycin was extended, and MICs were measured at 14 and 5 d. The MIC breakpoints for *M. abscessus* were obtained from the Clinical and Laboratory Standards Institute M62 [9].

2.4. Whole-genome sequencing

A total of 24 samples were cultivated in Middlebrook 7H9 liquid broth (Difco Laboratories) supplemented with 10% OADC at 37 °C for whole-genome sequencing. Genomic DNA was extracted using the MGTM Genomic DNA Purification Kit (Macrogen Inc., Seoul, Korea), following the manufacturer's instructions, and quality control was performed. Libraries were constructed using a TruSeq Nano DNA Library Prep Kit (Illumina, San Diego, CA, USA). Sequencing was performed on an Illumina HiSeq X with a paired-end read length of 151 bp by Macrogen Inc. (Seoul, Korea). FastQ was cleaned by trimming adapters and removing low-quality reads using Trimmomatic (v0.38) and FastQC (v0.11.6), respectively. Qualified reads were mapped using BWA (v0.7.17) onto the reference genome, *M. abscessus* ATCC 19977 and duplicated reads were removed with Sambamba (v0.6.7).

2.5. Sequence data analysis

Raw sequence data were assembled using SPAdes (v3.15.3), and the assembled contigs were used to search for the most probable reference genome for each strain based on the sum of coverage and similarity. The reads were aligned to the whole-genome sequence of *M. abscessus* ATCC 19,977 (NC_010397.1) and whole genomes, GD05 (NZ_CP065287.1) and FDAARGOS_1605 (NZ_CP085918.1), respectively, for strains from patients A and B to identify single nucleotide variations (SNVs) using Snippy (v4.6.0). The selected genomes were reannotated using Protein BLAST (v2.13.0) against the protein sequences of *M. abscessus* ATCC 19,977. IQ-TREE (v2.1.2) was used to find the best-fit substitution model and maximum likelihood estimation with a thousand bootstraps was applied to construct a phylogenetic tree using whole-genome multilocus sequence typing sequences. Gubbins (v3.1.6) were used to predict recombination. Phandango (v1.3.0) was used to visualise the phylogenetic tree, metadata of the isolates, and recombination prediction over the chromosome of *M. abscessus* ATCC 19,977.

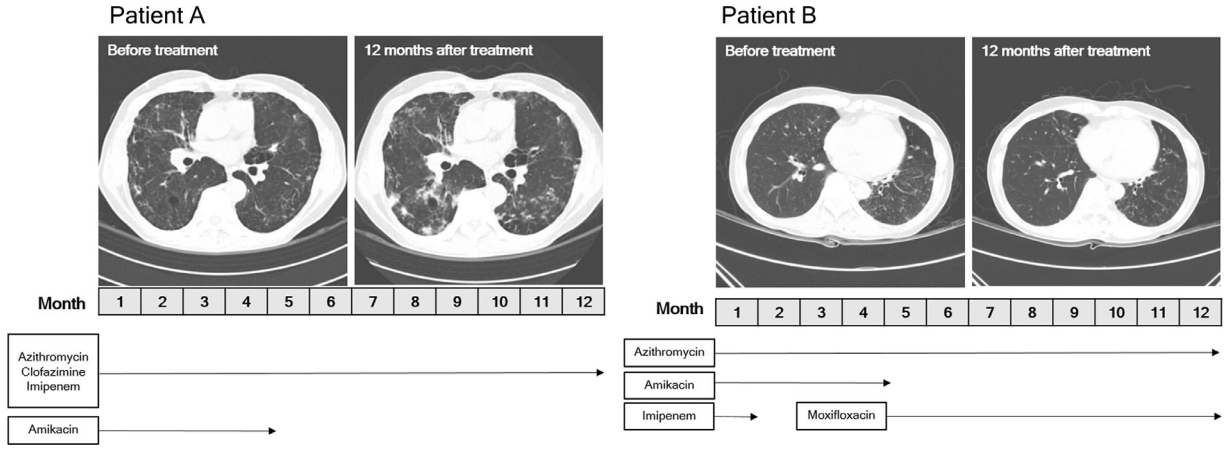

Fig. 1. Overview of antibiotic administration and radiographic changes during 12 months of treatment.

3. Results

3.1. Clinical courses of two patients

Patient A: A 69-y-old man without any underlying disease presented with sputum for several years. *M. abscessus* was repeatedly isolated from the sputum, and chest CT scans showed multiple nodules and bronchiectasis in both lungs. During the 6-months observation period after *M. abscessus*-PD diagnosis, the radiographic lesions worsened over time, and antibiotic treatment with azithromycin, amikacin, imipenem, and clofazimine was administered. After 4-months of treatment, amikacin was discontinued because of tinnitus. Subsequently, azithromycin, imipenem, and clofazimine were administered. At 12 months of treatment, the patient failed to achieve culture conversion, and his radiographic lesions aggravated further (Fig. 1).

Patient B: A 54-y-old woman without any underlying disease visited the clinic for sputum production for a year. Centrilobular nodules were detected in the right middle and left lower lobes, and *M. abscessus* was identified in the sputum. Because of radiographic aggravation on the chest CT scan, treatment with azithromycin, amikacin, and imipenem was initiated. Amikacin was replaced with moxifloxacin because of hearing impairment after 4-months of treatment. Although culture conversion failed after 12 months of treatment, the amount of sputum decreased and radiographic infiltrations improved (Fig. 1).

3.2. In vitro *mic values of clinical isolates*

The MIC values of the 24 isolates from patients A and B are summarised in Supplementary Table 1. In patient A, the MICs of the administered drugs (azithromycin, amikacin, clofazimine,

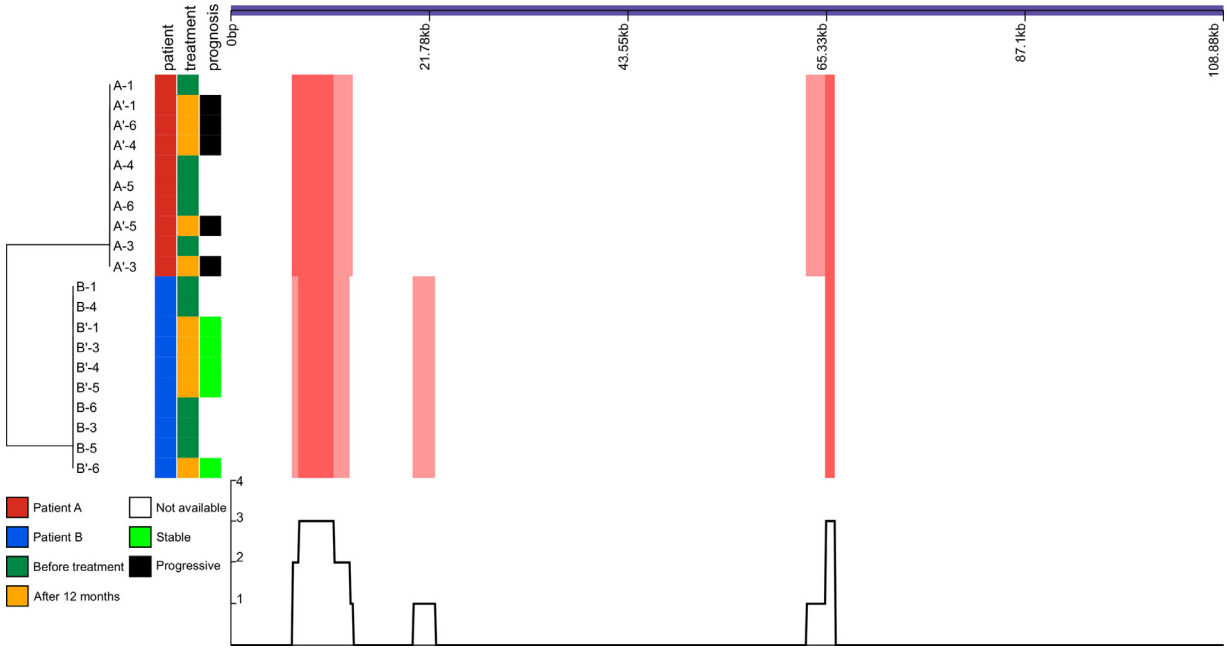

Fig. 2. Genomic profiling by recombination analysis reveals that the patients were infected by distinctive strains**.** The phylogenetic tree of isolates was built using core SNPs which were found by aligning the sequence reads to the chromosome of *Mycobacteria abscessus* ATCC 19,977. Each tip in the clades was labelled by the isolate name. In the phylogenetic tree, the isolates after 12 months of treatment from patient A were shown in pink while the isolates after 12 months of treatment from patient B were shown in blue. The X-axis represents the genomic position of *M. abscessus* ATCC 19,977. The hit map shows the result of recombination prediction, and the bottom graph shows profiling presented by the sum of combination events at each genomic site. The prediction results were highly correlated with the saturation of the recombination block.

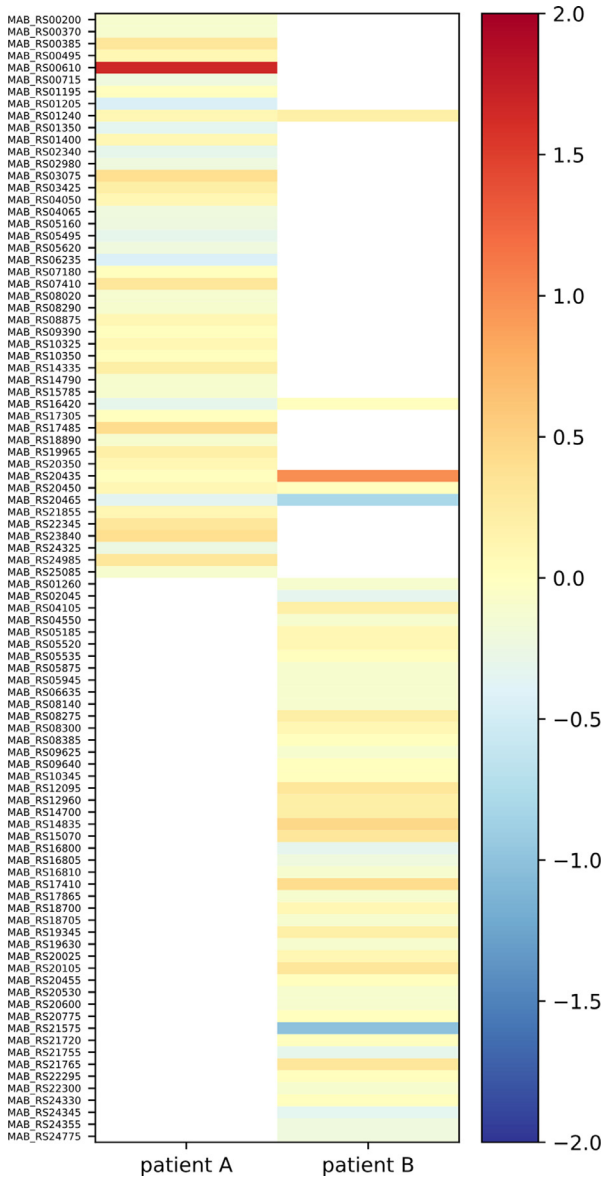

Fig. 3. The accumulation of SNPs shows distinguishable patterns between isolates from each patient. The accumulation of SNPs was calculated by an unweighted sum of SNP sites resulting in a non-synonymous mutation on each gene. The SNPs were normalised by dividing them with the highest absolute value.

and imipenem) were generally unchanged over time. In patient B, while the MICs for amikacin and moxifloxacin were similarly maintained before and after treatment, the MICs for clarithromycin measured 5 d after incubation increased from 0.5 to 4 μg/mL to 32 μg/mL during the treatment period.

3.3. Phylogenetic analysis of clinical isolates

The mean difference in SNVs between clinical isolates from patient A and patient B was 40.11 (range, 45–147), while the within-patient difference for patient A and patient B was 107.57 ± 18.57 and 67.46 ± 11.72, respectively. The generated phylogenetic tree showed two distinct clusters of *M. abscessus* separated by patients A and B (Fig. 2). This result is in line with the finding that isolates from each patient collected at an interval of 12 months were closely related, at least within each isolate. These results showed that patients A and B were infected by different strains without additional strain infections, and that these strains solely caused persistent infections in these patients despite antibiotic treatment.

3.4. Genetic changes over time

To evaluate genetic variations during antibiotic treatment within the isolates from each patient, we compared the genomic information of the clinical isolates based on the collection time. During 12 months of treatment, 47 and 52 non-synonymous SNVs occurred in *M. abscessus* from patients A and B, respectively. All SNVs are provided in Supplementary Appendix 1. The accumulation of novel SNVs in patient A differed from that in patient B over time, with the exception of five SNVs. An overview of the chronological changes is shown in Fig. 3. Although multidrug regimens were administered to both patients, SNVs associated with the known drug-resistance mechanisms (MAB_RS07515 [*rrl*], MAB_RS07510 [*rrs*], MAB_RS11715 [*erm*(41)], MAB_RS00245 [*gyrA*], MAB_RS00180 [*gyrB*], MAB_RS17805 [*whiB7*], MAB_RS15430 [*hflX*]) were not found. Because mutations in *whiB7* and *hflX* have been implicated in antibiotic resistance [10], we further analysed the locus near these genes. We found additional SNVs in both *whiB7* and *hflX* isolates from patient B, which may drive differential responses to antibiotics. However, no SNVs were accumulated during the antibiotic treatment (Supplementary Figure 2). The most variable position in patient A was within the probable NADH-dependent glutamate synthase gene (MAB_RS00610), which is involved in ammonium assimilation in mycobacteria [11]. In patient B, the variation in the putative YrbE family protein gene (MAB_RS20435), which contributes to the virulence of *M. abscessus* [12], was most prominent over time. We also compared the depth of the sequence reads of isolates from each patient to determine whether the multifork features of genome replication changed after antibiotic treatment [13]. As shown in Supplementary Figure 3, no significant change was detected in isolates from both patients, suggesting that genome replication of the isolates was not altered during antibiotic treatment.

4. Discussion

In this study, we performed a whole-genome analysis of clinical isolates of *M. abscessus*, sequentially collected from two patients with different radiographic responses to antibiotic treatment. During antibiotic treatment, persistent infection identified by a single strain was observed regardless of the clinical course. The expression of common genes associated with antimicrobial resistance did not change over time. Although the genetic changes over an interval of 12 months were not significant, the accumulation pattern of SNVs differed between the infected and individual patients.

In patients with CF, *M. abscessus* can be transmitted from person to person and causes a life-threatening disease. *M. abscessus* adapts to the host environment and increases its virulence through stepwise genetic evolution [6]. However, data on genetic changes in patients without CF are scarce. In our study, *M. abscessus* maintained its genetic stability under multiple antibiotic pressures, and this persistent infection was observed in both clinically progressive and stable patients, even after treatment. According to a study from Thailand, the rate of mutations in *M. abscessus* has dramatically reduced at later stages of the clinical course [14]. Further studies are needed to confirm the maintenance of genetic stability in non-CF patients.

While four antibiotics were administered, both patients A and B failed to cure and the mycobacterial culture was persistently positive. However, the MICs did not change over time. For instance, the MICs for clofazimine were almost the same in patient A (0.5–1.0 μg/mL) (who was treated with clofazimine) and patient B (0.5

μg/mL) (who was treated without clofazimine). This finding suggests that the administered antibiotics were not sufficiently delivered to the mycobacterial niche and had no effect on MIC values. In fact, in chronic airway diseases, such as CF or non-CF bronchiectasis, concerns have been raised regarding the poor penetration of antibiotics into target lesions [15].

Nevertheless, the MICs for clarithromycin at 5 d after incubation were significantly increased from 0.5 to 4 mg/mL to 32 mg/mL in patient B, suggesting the development of acquired resistance to macrolide during treatment in patient B. In the case of macrolides, favourable penetration of lung lesions has recently been proven in patients with NTM-PD [16]. In patients with CF, variation in *rrl* gene encoding 23S rRNA, which confers acquired resistance to macrolides, developed over time [17]. However, in patient B, mutations in *rrl* gene were not observed. This discrepancy can be explained in terms of sequence gaps in the draft genome or unknown resistance mechanisms involving the post-transcription level or beyond [18].

Although genetic changes related to drug resistance did not develop, non-synonymous SNVs were identified in 94 genes, with 67 of them previously reported in the literature [19]. Notably, the accumulation of SNVs showed different patterns between the two patients. Although the degree of fraction changes over time was not significant in most SNVs, this separation suggests a host-pathogen interaction. The different clinical responses of the two patients further emphasise the relevance of host factors, although we could not determine whether the changes in SNVs were the cause or the effect of different outcomes.

The most notable alterations observed in patient A were identified within the *gltD* gene, which encodes the NADH-dependent glutamate synthase. *GltD*, in conjunction with the upstream nitrogen regulator *glnR*, is hypothesised to play a role in host adaptation by aiding in ammonium assimilation and facilitating biofilm formation [11]. These factors may have partly contributed to the progressive worsening observed in patient A. In patient B, the fraction of SNVs in a putative YrbE family protein gene, which is associated with virulence in mycobacteria [12], increased the most. Bacteria can adapt to the host environment by attenuating virulence to form a commensal relationship with the host [20]. Therefore, changes in a putative YrbE family protein gene in patients with favourable responses can be explained in terms of bacterial fitness.

This study had several limitations. First, the number of included patients was insufficient to generalise our data. Second, the interval between sample collections was relatively short. However, we analyzed demographic factors, drugs administered, *in vitro* susceptibility, and radiographic response during the course of treatment in a more detailed manner. Additionally, we sequenced several isolates with diverse morphological characteristics at each time point. It can detect the presence of sub-clones and mixed infections, if any.

In conclusion, the genetic changes in *M. abscessus* in patients without CF were relatively stable over time during antibiotic treatment.

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Competing interests: None declared.

Ethics approval: The study was approved by the Institutional Review Board of Seoul National University Hospital approved the protocol (IRB No. 2208–052–1349).

Data availability: All data are available from the corresponding authors upon request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jgar.2023.12.004.

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