A nonmuscle myosin light chain kinase–dependent gene signature in peripheral blood mononuclear cells is linked to human asthma severity and exacerbation status

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Abstract: Asthma is increasingly recognized as a heterogeneous disease influenced by complex genetic and environmental contributions. Myosin light chain kinase (MLCK; gene symbol, *MYLK*), especially the nonmuscle isoform nmMLCK, is a cytoskeleton protein known to be related to human asthma susceptibility and severity, findings confirmed in preclinical models of asthmatic inflammation. In this study, we define the central capacity for a nmMLCK-influenced gene signature in human peripheral blood mononuclear cells to predict human asthma severity and exacerbation status. We refined this signature from a list of nmMLCK-influenced genes identified in lung tissues of nmMLCK knockout mice exposed to inflammatory stimuli (ventilator-induced lung injury), with subsequent identification of nmMLCK-influenced genes in a list of human asthma severity–related genes expressed in blood. The enriched nmMLCK-influenced gene signature successfully predicted human asthma severity and exacerbation status in both discovery and validation human asthma cohorts. These findings validate the central role played by nmMLCK in asthma susceptibility, severity, and exacerbation and further provide novel gene signatures as effective asthma biomarkers for severity, exacerbation, and prognosis.

Keywords: asthma exacerbation, asthma severity, nmMLCK, gene expression.

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Asthma is a chronic allergic lung disease characterized by airway hyperresponsiveness and bronchial eosinophilia, with diverse etiology and broad inclusion due to similar symptoms.¹ As a complex disease with genetic and environmental contributions, asthma, especially severe asthma, exhibits increased rates of morbidity and mortality.² To date, numerous risk genes have been reported to be associated with asthma susceptibility in various populations. Our prior work³ identified *MYLK* as a candidate asthma susceptibility gene, with coding polymorphisms associated with severe asthma susceptibility in underrepresented ethnic groups, including African American cohorts. *MYLK* encodes myosin light chain kinase (MLCK), a critical cytoskeletal effector in smooth muscle and nonmuscle cells, including the vascular endothelium.⁴ In addition, the level of expression of the nonmuscle isoform of MLCK (nmMLCK), the major isoform expressed in vascular endothelium, is correlated with levels of inflammation in murine asthmatic models.⁵ Taken together, these data support a potentially key role for nmMLCK/*MYLK* in the pathogenesis of asthma. In this study, using novel bioinformatics approaches, we further validate the central role played by nmMLCK in human asthma susceptibility and severity by implicating the contribution of nmMLCK-influenced genes expressed in blood cells in modulating human asthma severity.

METHODS

Gene expression data on lung tissues from ventilator-induced lung injury (VILI)–exposed wild-type (WT) and VILI-exposed nmMLCK knockout (KO) mice were obtained from the Gene Expression Omnibus (GEO) database (GSE14525)⁶ and used to filter nmMLCK-influenced mouse genes. Similarly, the gene expression data sets representing human asthma in blood were also downloaded from the GEO database. The data set (GSE27011)⁷ from Sweden (SWE) was used as a discovery cohort to filter genes associated with asthma severity (normal control, mild asthma, and severe asthma). The data set (GSE27876) from Korea (KOR) was used to validate the severity-associated gene expression pattern. We also investigated the differential expression between stable human asthma and exacerbation status with the data set (GSE19301)⁸ from the United States (USA), analyzed as a discovery cohort, and the data set (GSE16032)⁹ from Australia (AUS), analyzed as a validation cohort.

The significance analysis of microarrays (SAM)¹⁰ algorithm was used to identify differentially expressed genes between VILI-exposed WT and nmMLCK KO mice. Genes with fold changes of >1.5 and a false discovery rate (FDR) of <5% were deemed to be nmMLCK-influenced genes. We also used the SAM algorithm to identify human genes whose expression significantly correlated with asthma se-

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verity. Genes with an FDR of <5% were deemed to be asthma severity-associated genes. To identify the genes differentially expressed between stable and exacerbation asthma status, we applied analysis of covariance (ANCOVA) with both asthma status (stable or exacerbated) and patient identifier used as covariates, as repeated measures were obtained from a given patient. Genes with an adjusted P value (corrected by the Benjamini-Hochberg procedure) of <0.05 were considered to be differentially expressed between stable and exacerbation asthma status.

Two scoring systems were developed on the basis of gene expression level. First, a severity score was assigned to each patient on the basis of the expression of genes associated with asthma severity (eq. [1]). Next, we calculated an exacerbation score for each patient on the basis of the genes differentially expressed between stable and exacerbation status (eq. [2]).

$$S_{SE} = \sum_{i=1}^{N_{SE}} W_{SEi} (e_i - \mu_i) / \tau_i, \quad (1)$$

$$S_{EX} = \sum_{i=1}^{N_{EX}} W_{EXi} (e_i - \mu_i) / \tau_i. \quad (2)$$

In equation (1), S_{SE} is the severity score of the patient, N_{SE} is the number of asthma severity-associated genes, and W_{SEi} denotes the severity weight of gene i , which is equal to 1 or -1 if the expression of gene i is positively or negatively correlated with severity, respectively. In equation (2), S_{EX} is the patient's exacerbation score, N_{EX} is the number of genes differentially expressed between stable and exacerbation status, and W_{EXi} denotes the exacerbation weight of gene i , which is equal to 1 or -1 if gene i is up- or downregulated in exacerbation status, respectively. In both equations, e_i denotes the expression level of gene i , and μ_i and τ_i are the mean and standard deviation, respectively, of the gene expression values for gene i across all samples. Both severity and exacerbation weights were fixed on the basis of the discovery cohorts and then evaluated in the validation cohorts.

RESULTS

At the specified significance level (fold change of >1.5 and FDR of <5%), 638 genes were found to be differentially expressed between VILI-exposed WT and nmMLCK KO mice, with 314 upregulated and 324 downregulated genes in nmMLCK KO mice (Fig. S1 and Table S1; Figs. S1–S4 and Tables S1–S5 are available online). We searched the pathways associated with these genes in the BioCarta, KEGG, PANTHER, and Reactome pathway databases and identified several pathway terms significantly enriched among these differentially expressed genes ($P < 0.05$), such as alpha adrenergic receptor signaling pathway, histamine H1 receptor-mediated signaling pathway, vascular smooth muscle contraction, oxytocin receptor-mediated signaling pathway, and inflammation mediated by chemokine and cytokine signaling pathway (Fig. S2). To determine whether nmMLCK-influenced genes derived from the nmMLCK KO mouse model were relevant to human asthma, we matched the 638 nmMLCK-influenced mouse genes to 551 distinct human orthologs according to the definition of BioMart.¹¹ We

deemed these human orthologs to be nmMLCK-influenced human genes.

For human asthma patients, we first investigated the correlation between blood gene expression and asthma severity in the SWE cohort. At the specified significance level (FDR of <5%), 604 human genes were found to be significantly correlated with asthma severity (Table S2). Among these genes, 30 were found to overlap with the nmMLCK-influenced genes, which is statistically significant ($P = 2.4 \times 10^{-4}$ by cumulative hypergeometric distribution function). This overlap suggests that nmMLCK-influenced genes are significantly enriched among the asthma severity-associated gene set. We deemed these 30 genes to be an asthma severity-associated 30-gene signature (Table S3). A severity score was assigned to each patient on the basis of the expression of the 30-gene signature. A higher severity score implies more severe asthma, and in the SWE cohort the patient severity score was significantly and positively correlated with asthma severity ($\rho = 0.82$, $P = 2.0 \times 10^{-14}$ by Spearman's rank correlation test; Fig. 1A). Next, we tested the relationship between severity score and asthma severity in the validation cohort (KOR). A significant and positive correlation between these two indexes was also observed in the KOR cohort ($\rho = 0.70$, $P = 3.7 \times 10^{-3}$ by Spearman's rank correlation test; Fig. 1A), suggesting the predictive power of the nmMLCK-influenced 30-gene signature for asthma severity. We also performed a resampling test to check whether the predictive power of the 30-gene signature occurred by chance and generated 1,000 random gene signatures of identical size (30 genes). Spearman's rank correlation test was conducted for each resampled gene signature. The association between each random gene signature and asthma severity was measured by the correlation coefficient. In both the SWE and the KOR cohort, the null hypothesis that the association between the 30-gene signature and severity occurs by chance was rejected. The correlation coefficient of the 30-gene signature was significantly larger than that of randomized gene signatures ($P < 10^{-3}$ for the SWE cohort, $P = 4.6 \times 10^{-2}$ for the KOR cohort; Fig. S3).

We next evaluated the blood gene expression pattern related to asthma stable/exacerbation status using the USA asthma cohort with multiple samples from a given asthma patient obtained during stable periods and during exacerbation.⁸ Therefore, with these control comparators for samples obtained from the same patient, we applied ANCOVA to compare gene expression between stable and exacerbation status. We identified 1,245 genes differentially expressed between stable and asthma exacerbation status (Table S4), with 52 genes overlapping with nmMLCK-influenced genes ($P = 3.7 \times 10^{-2}$ by cumulative hypergeometric distribution function). These results suggest that nmMLCK-influenced genes are significantly enriched among the stable/exacerbation status-associated gene set. We deemed the 52 genes to be an asthma exacerbation-associated 52-gene signature (Table S5). An exacerbation score was assigned to each patient on the basis of the expression of the 52-gene signature. In the USA cohort, the patient exacerbation score was significantly higher for exacerbation status than for stable status ($P = 1.6 \times 10^{-19}$ by ANCOVA; Fig. 1B). We next tested the

Figure 1. Nonmuscle isoform of myosin light chain kinase (nmMLCK)-influenced genes differentiate asthma severity and exacerbation status. A, The asthma severity score of the 30-gene signature positively correlated with asthma severity in both the discovery (Sweden [SWE]) and the validation (Korea [KOR]) cohort. Correlation coefficients (ρ) and P values were computed by Spearman's rank correlation. HC: healthy control; MA: mild asthma; SA: severe asthma. B, The exacerbation score of a 52-gene signature is significantly higher for exacerbation status than for stable status in both the discovery (United States [USA]) and the validation (Australia [AUS]) cohort. In the USA cohort, multiple samples obtained from a given asthma patient during stable periods served as control comparators for samples obtained from the same patient during exacerbations. The AUS cohort consisted of 25 asthma patients divided into five different pools, with each pool containing equal amounts of RNA from five patients. P values were computed by analysis of covariance. ST: stable status; EX: exacerbation status.

exacerbation score in the validation cohort (AUS). The scores for exacerbation status were significantly higher than those for stable status ($P = 3.2 \times 10^{-5}$ by ANCOVA; Fig. 1B), suggesting the differentiation power of the nmMLCK-influenced 52-gene signature for asthma status. We performed a resampling test following procedures similar to those mentioned above. We generated 1,000 random gene signatures with 52 genes. ANCOVA was conducted for each resampled gene signature. The association between each random gene signature and asthma status was measured by the F statistic. We found that, in both the USA and the AUS cohort, the F statistic for the 52-gene signature was significantly larger than that for randomized gene signatures ($P < 10^{-3}$ for the USA cohort, $P = 4.0 \times 10^{-3}$ for the AUS cohort; Fig. S4).

DISCUSSION

Single-nucleotide polymorphisms of the calcium/calmodulin-dependent kinase gene *MYLK* are known to be strongly associated with severe asthma, especially in African American patients.³ *MYLK* encodes the smooth muscle MLCK isoform (smMLCK), a key regulatory molecule for airway smooth muscle contraction, a feature of asthma exacerbation. *MYLK* also encodes nmMLCK, an isoform that is less well studied in the context of human asthma but whose role and function in asthma severity is supported by studies using preclinical asthma models.⁵ Using preclinical and clinical data sets, the current study further confirms the central role played by nmMLCK-influenced gene signatures in asthma. We demonstrate that a nmMLCK-dependent gene signature differentiates patients with varying degrees of asthma severity (control, mild asthma, severe asthma) as well as asthma stability (stable, exacerbation). This study provides the first evidence to define a central role played by nmMLCK in asthma generated using genomic approaches.

The current study exhibits three areas of significance and novelty. First, our results indicate that a nmMLCK-dependent gene signature is effective in differentiation of asthma severity and exacerbation status. Together with our prior findings that nmMLCK protein levels are correlated with asthma severity and susceptibility,⁵ this study confirms that nmMLCK plays a central role in asthmatic inflammation. Second, the established genomic signatures (30-gene signature for severity, 52-gene signature for exacerbation status) are derived from human blood mononuclear cells, therefore providing a novel diagnostic tool that has high feasibility for clinical asthma severity and exacerbation status with the established scoring system. Third, the translational genomic strategy used in this study will be valuable to examine and validate novel asthma genetic markers or potential therapeutic targets.

In conclusion, our translational genomic studies validate the central role played by nmMLCK in asthma inflammation, with clear evidence for differentiation of both asthma severity and exacerbation status by nmMLCK-influenced gene signatures in blood.

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Conflict of Interest: None declared.

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