MicroRNA Signatures in Diagnosis and Prognosis of Cutaneous T-Cell Lymphoma

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Cutaneous T cell lymphoma (CTCL) can have clinical and histological features resembling benign inflammatory dermatosis and can be difficult to diagnose. Very limited biomarkers are available for CTCL prognosis. We aimed to identify microRNA (miR) signatures to facilitate diagnostic and prognostic evaluations of CTCL. A cross-platform miR microarray identified 10 miRs that were differentially expressed between CTCL and benign inflammatory dermatosis patients. Subsequent reverse transcription polymerase chain reaction validation was used to generate a 5-miR–based diagnosing classifier, which showed high diagnostic accuracy in CTCL (area under the curve = 0.985 and 0.956 for training and testing set, respectively). Association between miR expressions and patient prognosis was studied. miR-155 and miR-200b were significantly associated with overall survival in CTCL patients, outperformed Ki-67. miR expressions were combined with Ki-67 to create a classifier for 5-year overall survival in CTCL patients. Our work provided miR signatures to facilitate CTCL diagnosis and prognosis with satisfying accuracy.

INTRODUCTION

Cutaneous T cell lymphoma (CTCL) is the most frequent primary lymphoma of skin, with a broad spectrum of clinical presentations, and at times subtle histologic features that can be difficult to distinguish from benign inflammation. Mycosis fungoides (MF) is the most common type of CTCL, comprising more than half of CTCL cases in United States (Willemze et al., 2005). In Asian patients, MF accounts for 45% to 65% of all CTCL (Lee 2003; Liu et al., 2014). Typical MF usually follows an indolent disease course, beginning with erythema, patches, and plaques at early stage and evolving into tumor and disseminating to lymph nodes or visceral organs at advanced stage. Early-stage MF may resemble benign inflammatory dermatosis (BID) clinically and histologically, such as eczema or psoriasis. Other types of CTCL, including cutaneous anaplastic large cell lymphoma (c-ALCL), peripheral T-cell lymphoma (PTCL), and natural killer T-cell lymphoma, also mimic inflammatory dermatosis clinically. Despite various diagnosing options with immunohistochemistry and gene rearrangement, accurate diagnosis of CTCL remains challenging. High false-positive rates are found when determining T-cell clonality by T-cell receptor rearrangement because they may also be detected in elderly patients and BID (Dabiri et al., 2011). Distinction between CTCL and BID is important at initial presentation because early diagnosis prompts better treatment and more favorable clinical outcomes. The identification of effective and reliable biomarkers for discrimination of CTCL from BID has a significant diagnostic value.

Furthermore, long-term follow-up studies are very limited in CTCL. Staging is generally used as the main indicator to predict clinical outcome (Agar et al., 2010; Benton et al., 2013; Scarpisbrick et al., 2015). Patients with early-stage MF have a 10-year survival of 97% to 98%, but the 10-year survival decreases to 42% in patients at tumor stage (Diamandidou et al., 1999; Willemze et al., 2005). The prognosis of other types of CTCL is generally worse than MF and depends on subtypes as well as staging (Benner et al., 2012b; Kohrt and Advani 2009; Liu et al., 2003; McKelvie et al., 2012). The Cutaneous Lymphoma International Consortium, involving multiple established cutaneous lymphoma groups around the world, has identified that tumor staging, age, large-cell transformation and increased lactate dehydrogenase levels are four variables that have significant prognostic value in advanced MF and Sézary syndrome. Two other studies showed that, independent of large-cell transformation, a low CD30 expression predicts worse clinical outcomes (Benner et al., 2012b; Talpur et al., 2016). Other than clinical and pathological features, very few studies were reported regarding biomarkers (Gambichler et al., 2016; Lindahl et al., 2018; Litvinov et al., 2010, 2015, 2017).

Gene profiling studies have identified several candidate genes and mRNA clusters helpful in evaluation of patient clinical outcomes.

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Abbreviations: AUC, area under the curve; c-ALCL, cutaneous anaplastic large cell lymphoma; BID, benign inflammatory dermatosis; CTCL, cutaneous T cell lymphoma; FFPE, formalin-fixed paraffin-embedded; MF, mycosis fungoides; miRNA, microRNA; OS, overall survival; PTCL, peripheral T-cell lymphoma

Received 18 December 2017; revised 22 February 2018; accepted 8 March 2018; accepted manuscript published online 17 March 2018; corrected proof published online 26 May 2018
MicroRNAs (miRs) are non-coding RNA molecules of 20 to 25 nucleotides that post-transcriptionally regulate various biological pathways. Many miRs are implicated in the pathogenesis of cancers and can serve as biomarkers, given high correlation between their levels and disease diagnosis, progression, and responsiveness to therapy. Several studies have reported that miR expression profiles are different in CTCL and BID, including miR-155, miR-203, miR-205, miR-92a, miR-93, miR-15a, miR-16, let-7a, let-7d, and let-7f (Benner et al., 2012a; Garaicoa et al., 2016; Narducci et al., 2011; Papadavid et al., 2016; Taylor et al., 2003; van Kester et al., 2011). There is very limited study on global analysis of non-coding RNA in CTCL diagnosis. Odum’s group has developed a miR classifier that achieves an accuracy of >90% in Scandinavians, indicating miR classifiers (consisting of miR-155, miR-203, and miR-205) are feasible and valuable tools in CTCL diagnosis (Marstrand et al., 2014; Ralfkiaer et al., 2011, 2014). The role of racial disparity in CTCL is unclear and studies in additional ethnicities would yield insight into the pathogenesis of CTCL. Additionally, prognostic values of miRs are barely studied in CTCL (Lindahl et al., 2018).

In this study, we used unbiased screening, validation, and modeling to identify miRs as valid predictors in diagnosis of CTCL; and showed 2 significantly altered miRs have strong prognostic value in CTCL.

RESULTS
Study population
Clinical specimens were collected from patients in Cutaneous Lymphoma Clinic, Department of Dermatology, Ruijin Hospital. Formalin-fixed paraffin-embedded (FFPE) tissues were prepared from 158 CTCLs (including MF, c-ALCL, PTCL, and natural killer/T-cell lymphoma) and 70 BIDs (including psoriasis, atopic dermatitis, and prurigo nodularis) patients at initial clinic visit. The patient characteristics are presented in Table 1. Mean age at the time of CTCL diagnosis was 52.3 ± 13.3 years old, which was the youngest cohort compared with other studies (Agar et al., 2010; Benton et al., 2013; Scarisbrick et al., 2015). Similar to other studies, male sex showed a slight predominance over female sex (59.1%).

Candidate miR identification
We sought to define an miR-based diagnostic tool for CTCL and to identify predictors of CTCL prognosis (Supplementary Figure S1 online). To screen for the significant differentially expressed miRs between CTCL patients and BID patients, total RNA from 71 skin biopsy samples (50 CTCL and 21 BID control, Table 1) were hybridized to Agilent (Santa Clara, CA) and Affymetrix (Santa Clara, CA) miR microarrays, respectively. A cross-platform analysis was used in order to increase both sensitivity and specificity of miRs screening (Del Vescovo et al., 2013; Mestdagh et al., 2014). Data were available at National Center for Biotechnology Information’s Gene Expression Omnibus, reference number: GSE106646.

Supplementary Figure S2 (online) illustrates the clustering and top list of differentially expressed miRs between BID and CTCL groups. Among the top hits, nine miRs were shared by both platforms and were considered as candidates for further reverse transcription polymerase chain reaction validation: miR-27b, miR-130b, miR-150, miR-155, miR-200b, miR-200c, miR-203, and miR-342-3p. Additionally, miR-142-3p, the most differentially expressed in Agilent miR microarray was also selected as candidate. The 10 candidate miRs were tested in an independent cohort of 83 FFPE samples with reverse transcription polymerase chain reaction, including 25 BID controls and 58 CTCL patients (Supplementary Table S1 online). Each of the 10 candidate miRs is a decent predictor with area under the curve (AUC) ranging from 64% to 89% (Supplementary Figure S3 online). All candidate miRs showed consistent trend of fold change with microarray result. miR expressions from these 83 FFPE samples were then used to generate miR-based classifier for CTCL diagnosis.

Diagnostic classifier
Generation of classifier. We chose the top 5 candidate miRs to generate a classifier for CTCL diagnosis (Figure 1). Reverse transcription polymerase chain reaction showed that the expression levels of miR-200b and miR-203 were decreased in CTCL lesions compared with BID lesions (fold changes = 0.74 and 0.06, respectively). Reverse transcription polymerase chain reaction also showed increased expression levels are observed for miR-130b, miR-142-3p, and miR-155 (fold changes = 1.22, 1.66, and 1.47, respectively). In the training set, a risk of CTCL diagnosis was calculated using generalized linear model. Relative contributions of each candidate miRs were calculated; using logistic regression model based on all five candidate miRs to generate receiver operating characteristic curve. The diagnostic performance of the classifier was evaluated in the training cohort: the AUC was 98.5% (95% confidence interval = 96.5%–100%; sensitivity = 98.3%; specificity = 96.0%; Figure 2a).

Validation. We used another independent set of 75 FFPE samples (25 BID patients and 50 CTCL patients) to validate the established miR classifier for CTCL diagnosis. With our 5-miR-based classifier, AUC of receiver operating characteristic curve was 95.6% (95% confidence interval = 91%–100%; sensitivity = 96%; specificity = 72%; Figure 2b). We further showed that the performance of our 5-miR-based classifier was desirable in all three subtypes of CTCLs: early-stage MF (stage I and IIa), advanced-stage MF (stage IIb, III, and IV) and other types of CTCLs (sensitivity = 0.93, 0.96, 0.975 respectively, specificity = 0.8).

CTCL subtype prediction. Based on the expression of all 5 candidate miRs in the training group, we used multi-level logistic model to generate a new classifier predicting each of CTCL subtypes: early-stage MF (stage I and IIa), advanced-stage MF (stage IIb, III, and IV) and other types of CTCL including ALC, PTCL, and natural killer/T-cell lymphoma. The model was validated in test group: 64% patients were classified correctly (48 of 75 patients: 18 BID patients, 16 early MF, 3 advanced MF, and 11 other type CTCL patients; Supplementary Table 2 online). Furthermore, the new classifier showed excellent performance in differentiating advanced diseases from benign disorders: no advanced MF and other types CTCL case was misclassified as BID and early MF.

Prognostic miRNA signature
Patients in the training and test groups were regularly followed-up in the cutaneous lymphoma clinic of Ruijin Hospital. The median follow-up duration is 90 months until
April 2017. Unfortunately, 50% (54/108) of our patients succumbed to disease.

We applied Cox proportional hazards regression model to identify miR expression signature predictive of patient overall survival (OS). Two of 10 candidate miRs identified in previous screen were significantly associated with OS among patients with CTCL. miR-155 and miR-200b showed significant, yet opposite directions of association (coefficient $\hat{\beta} = 0.0098$ and $e^{1.034}$; $P = 0.019$ and $P = 0.0005$, respectively, Supplementary Table 3 online).

The median expression value of each miR was used to categorize patients into two groups of equivalent numbers. As demonstrated by Kaplan-Meier curve, patients with high expression of miR-155 had significantly worse clinical outcomes (log-rank $\chi^2 = 13.16$, $P = 0.0001$; Figure 3a). The hazard ratio was 2.706 in patients with high miR-155 expression. The median OS for miR-155 high-expressers was 96 months compared with 384 months in low-expressers. Comparatively, patients with high miR-200b expression showed more favorable prognosis (log-rank $\chi^2 = 17.57$, $P < 0.0001$; Figure 3b) with a hazard ratio being 3.367 in patients with low miR-200b expression. The median OS for miR-200b low-expressers is 96 months compared with 336 months in miR-200b high-expressers. We have further analyzed the positivity of lesional Ki-67 expression in all patient tissue biopsies at initial presentation. The median Ki-67 positivity in 108 patients was 30%. Similar with other types of lymphomas, higher Ki-67 expression correlated with a poor prognosis in our cohorts (log-rank $\chi^2 = 21.8$, $P < 0.0001$; Figure 3c). The hazard ratio was 3.854 in patients with higher Ki-67 positivity. The median OS for high-Ki-67 was 84 months compared with 384 months in low Ki-67 group.

We utilized recursive partitioning analysis to combine miR-155 and Ki-67 expressions to generate a new prognostic model (log-rank $\chi^2 = 35.15$, $P < 0.0001$; Figure 3d). New cut-offs levels for miR-155 and Ki-67 categorized patients into three groups with different prognosis. A good prognostic group was defined by a low positivity of Ki-67 expression <$15%$. In this group, only 3 of 37 patients in this group succumbed to disease. High expression of miR-155 more than 52 defined a poor prognosis group with a median OS of 58 months (log-rank hazard ratio = 16.95). The remaining patients showed Ki-67 >$15%$ and miR-155 expression <$52$. This group of patients carried an intermediate risk with a median OS of 96 months (log-rank hazard ratio = 11.21).

### Prognostic classifier for 5-year OS in CTCL

Based on expression of two prognostic miRs, we generated a classifier to predict the 5-year OS; 71.8% (76 of 108) of CTCL patients were alive at 5 years after diagnosis, resulting in an imbalanced sample size. The number of deceased patients was outweighed by 2.55-fold by random resampling. The

### Table 1. Characteristics of Study Participants in the Discovery, Training, and Testing Sets

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Discovery</th>
<th>Training</th>
<th>Testing</th>
<th>Total</th>
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</thead>
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<tr>
<td>CTL, n</td>
<td>50</td>
<td>58</td>
<td>50</td>
<td>158</td>
</tr>
<tr>
<td>Age at diagnosis, y, mean ± SEM</td>
<td>50.6 ± 12.6</td>
<td>53.4 ± 13.2</td>
<td>52.8 ± 13.8</td>
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<tr>
<td>Sex, n (%)</td>
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<td></td>
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<tr>
<td>Male</td>
<td>29 (58.0)</td>
<td>34 (58.6)</td>
<td>30 (60.0)</td>
<td>93 (58.9)</td>
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<tr>
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<td>24 (41.4)</td>
<td>20 (40.0)</td>
<td>65 (41.1)</td>
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<tr>
<td>MF stage, n (%)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>20 (40.0)</td>
<td>14 (24.1)</td>
<td>17 (34.0)</td>
<td>51 (32.3)</td>
</tr>
<tr>
<td>IIa</td>
<td>4 (8.0)</td>
<td>9 (15.5)</td>
<td>3 (6.0)</td>
<td>16 (10.1)</td>
</tr>
<tr>
<td>IIb</td>
<td>4 (8.0)</td>
<td>4 (6.9)</td>
<td>0 (0)</td>
<td>8 (5.1)</td>
</tr>
<tr>
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<td>0 (0)</td>
<td>1 (1.7)</td>
<td>0 (0)</td>
<td>1 (0.6)</td>
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<tr>
<td>IV</td>
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<td>10 (17.2)</td>
<td>10 (20.0)</td>
<td>30 (19.0)</td>
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<td>Other type, n (%)</td>
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<tr>
<td>c-ALCL</td>
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<td>6 (10.3)</td>
<td>6 (12.0)</td>
<td>17 (10.8)</td>
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<td>10 (20.0)</td>
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<td>Mortality, n (%)</td>
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<td></td>
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<tr>
<td>Surviving</td>
<td>—</td>
<td>28 (48.3)</td>
<td>26 (52.0)</td>
<td>54 (50.0)</td>
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<tr>
<td>Death</td>
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<td>30 (51.7)</td>
<td>24 (48.0)</td>
<td>54 (50.0)</td>
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<tr>
<td>Follow-up time, mo, mean ± SEM</td>
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<td>—</td>
<td>—</td>
<td>90 ± 48.5</td>
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<tr>
<td>BID, n</td>
<td>20</td>
<td>25</td>
<td>25</td>
<td>70</td>
</tr>
<tr>
<td>Age at diagnosis, y</td>
<td>53.3</td>
<td>51.6</td>
<td>47.5</td>
<td>50.1</td>
</tr>
<tr>
<td>Sex, n (%)</td>
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<td></td>
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<td></td>
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<tr>
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<td>18 (72.0)</td>
<td>17 (68.0)</td>
<td>38 (54.3)</td>
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<tr>
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<td>7 (28.0)</td>
<td>8 (32.0)</td>
<td>22 (45.7)</td>
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<tr>
<td>Type, n (%)</td>
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<td></td>
<td></td>
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<tr>
<td>PsV</td>
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<td>13 (52.0)</td>
<td>13 (52.0)</td>
<td>40 (57.1)</td>
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<td>5 (20.0)</td>
<td>5 (20.0)</td>
<td>13 (18.6)</td>
</tr>
<tr>
<td>PN</td>
<td>3 (15.0)</td>
<td>7 (28.0)</td>
<td>7 (28.0)</td>
<td>17 (24.3)</td>
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</table>

Abbreviations: AD, atopic dermatitis; BID, benign inflammatory dermatosis; c-ALCL, cutaneous anaplastic large cell lymphoma; CTCL, cutaneous T-cell lymphoma; MF, mycosis fungoides; NKT, natural killer/T-cell; PN, prurigo nodularis; PsV, psoriasis vulgaris; PTCL, peripheral T-cell lymphoma; SEM, standard error of mean.
prognostic performance for the miR classifier was evaluated by receiver operating characteristic curve analysis. AUC for the 2-miR panel was 0.796 (95% confidence interval = 0.725 to 0.866; sensitivity = 68.9%; specificity = 71.6%; Figure 4a), which outperformed Ki-67 (AUC = 0.741; 95% confidence interval = 0.661 to 0.821; sensitivity = 71.6%; specificity = 62.2%; Figure 4b). We then combined two miRs and Ki-67 to generate a new model. It showed a modest improvement in prediction accuracy (AUC = 0.833; 95% confidence interval = 0.767 to 0.898; sensitivity = 77%; specificity = 81.1%; Figure 4c).

miR-200b as prognostic biomarker for CTCL
Staging is one of the key components in predicting overall survival in CTCL patients. To address the potential role of CTCL staging in miRs’ association with patient OS, we utilized four-step mediation method using three separate models (Baron and Kenny, 1986). Because staging criteria in MF and other CTCLs are different, we chose to focus exclusively on MF patients to study the potential influence from early-stage (I and llb) versus advanced-stage MF (llb, III, and IV). As mentioned previously, miR-155 significantly correlates with OS in CTCL patients, including MF and other types of CTCL (P = 0.019). When patients with other types of CTCL were excluded, the miR-155 expression on patient OS was no longer statistically significant in MF patients (P = 0.302), suggesting miR-155’s effect on OS was mainly among patients with other types of CTCL (c-ALCL, PTCL, and natural killer/T-cell lymphoma). As for miR-200b, it presented a significant association with patient OS in MF patients (Figure 5). To test whether the relationship between miR-200b expression and patient OS was mediated by different stages of MF, a four-step mediation method was conducted (see Method section). All regression coefficients—α, β, and τ—showed statistical significance (P = 0.003, P = 0.00017, and P = 0.002 respectively).

Furthermore, partial regression coefficient τ turned out non-significant (P = 0.303), indicating that contribution of miR-200b to patient OS was washed out by addition of MF staging as covariant. To conclude, in MF, disease staging serves as a mediator in miR-200b’s association with patient survival.

DISCUSSION
In clinical practice, CTCL remains challenging to differentiate from BIDs, especially during early stages. Here, we developed an accurate miR classifier for CTCL diagnosis. Furthermore, our study investigated biomarkers for CTCL prognosis and we found miR-155 and miR-200b to have significant associations with patient overall survival and can serve as valid predictors for patient clinical outcomes.

Recently, Ralfkiaer and colleagues have developed a miR classifier for the differentiation of CTCL and BID that achieved satisfying accuracy. The miR classifier included miR-155, miR-203 and miR-205 (Marstrand et al., 2014; Ralfkiaer et al., 2011, 2014). However, in our cross-platform miR microarray screening, expression level of miR-205 is not significantly altered in our cross-platform microarray screening compared to the previous study. Furthermore, our cohort has a much earlier onset of disease compared with other studies from Europe and the United States (Scarisbrick et al., 2015) (Table 1). The difference may be that patients from different ethnicities possess different disease spectrums, various environmental factors, and potentially distinct underlying biological pathways in CTCL (Wilson et al., 2012). A recent study showed a more favorable clinical outcome in Asian patients compared with African-American and Caucasian patients, with a longer OS (hazard ratio = 0.562) (Su et al., 2017). There is no study yet to investigate the causative molecular variations underlying the racial disparity of
CTCL. Genome-wide comparison among CTCL patients of different ethnicities can be informative.

Treatment of CTCL remains one of the most difficult questions in dermatology. Given the vast diversity of treatment modalities, ranging from topical corticosteroids to chemotherapies, it is critical to stratify patients to receive treatments most suitable for their circumstances. In recent decades, several studies involving multiple established cutaneous lymphoma groups have investigated clinical factors that have significant prognostic values (Agar et al., 2010; Benton et al., 2013; Nikolaou et al., 2017; Scarisbrick et al., 2015). However, beyond clinical and pathological factors, there are very limited biomarkers available for diagnosis and prognosis of CTCL (Litvinov et al., 2010, 2017; Scarisbrick et al., 2014). Regarding miRs in prognosis, only scarce evidence was reported. All current studies focused on miR expression changes in different stages of CTCL, instead of strict survival analysis (Maj et al., 2012; Marosvári et al., 2015). Our study is hypothesis-driven and has independent training and test set to identify a miR-based classifier for CTCL early diagnosis and prognosis prediction. Furthermore, our CTCL patient cohort is the largest in similar studies and our follow-up time is desirable (median 90 months).

The diagnostic miR classifier includes miR-130b, miR-142-3p, miR-155, miR-200b, and miR-203. With the recent progress in miR research, three of the five miRs are associated with CTCLs and all five miRs are involved in cancer progression as well as T-cell regulation. The reproducibility of these miRs in various studies and different ethnic backgrounds further stresses their biological relevance in CTCL.

miR-155 is one of the first identified and most well-studied miRs in oncology. Numerous studies have found increased expression of miR-155 in CTCLs and other hematological malignancies (Kopp et al., 2013; Saito et al., 2012). Our result that high miR-155 expression has a strong association with worse prognosis in CTCL further supports its key function in disease pathogenesis. Indeed, its STAT-5-mediated up-regulation promotes proliferation and survival of malignant cells, including MF cell lines (Cordeiro et al., 2014; Moyal et al., 2017). Recently, silencing miR-155 showed a potential therapeutic effect in low grade B-cell lymphoma and CTCL (Zhang et al., 2012); medications targeting miR-155 are currently being tested in clinical trials (ClinicalTrials.gov ID NCT02580552). In this study, we confirmed increased miR-155 expression in CTCL and showed a significant correlation between miR-155 expression and patient OS. Our results provide clinical evidence that miR-155 dysregulation plays a critical role in CTCL.

Here, we identified miR-200b to be involved in cutaneous lymphomas. Its down-regulation has been observed in various solid tumors, including esophageal, gastric, colorectal, lung, prostate and brain malignancies (Cantini et al., 2015). The diagnostic miR classifier includes miR-130b, miR-142-3p, miR-155, miR-200b, and miR-203. With the recent progress in miR research, three of the five miRs are associated with CTCLs and all five miRs are involved in cancer progression as well as T-cell regulation. The reproducibility of these miRs in various studies and different ethnic backgrounds further stresses their biological relevance in CTCL.

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2015; Zhang et al., 2016). Plethora of literatures suggested that miR-200b functions to stabilize epithelial phenotype by inhibiting zinc finger E-box-binding factors ZEB1 and ZEB2 (Ungewiss et al., 2016). Loss of miR-200b results in epithelial–mesenchymal transition and enhances tumor metastasis. However, epithelial–mesenchymal transition is hardly the driving mechanism of tumorgenesis in mesoderm-derived CTCL. Other functions of miR-200b are likely to be involved. In our study, miR-200b expression was measured in whole-slice biopsy samples, which included tumorous cells and surrounding microenvironment. As miR-200b is one of the most abundant miRNAs in epithelial tissues (Yi et al., 2006), it is likely that alteration of miR-200b in CTCL derives from surrounding keratinocytes, which could be equally, if not more informative. High-throughput sequencing, such as Ago-HIT-CLIPS, revealed that miR-200b targets are enriched in cell motility pathways and promote tumor invasion (Bracken et al., 2014). Malignant T cells are known to disrupt integrity of epidermis and cause detachment between epithelial and mesenchymal compartments (Thode et al., 2015). It is likely that decreased miR-200b in surrounding keratinocytes promotes malignant T cells to escape from epidermis, thus results in more advanced disease stages. To support this hypothesis, we have shown that MF staging is a potential mediator in miR-200b’s effect on OS, suggesting a causative role of miR-200b in MF progression. Further studies of miR-200b and its targets are highly yielded to elucidate molecular mechanisms of CTCL.

In summary, our work adds great value to early diagnosis and prognosis in CTCL from analysis of a large patient population. Recent studies have also achieved significant advances in CTCL diagnosis and prognosis, such as T-cell “fingerprinting” with next-generation sequencing (Kirsch et al., 2015; Sufficool et al., 2015). Those advances would prove useful in determining whether an observation-alone approach in patients with a predicted favorable prognosis, or whether more aggressive therapies, such as chemotherapy should be pursued in patients with poor prognosis.

During the preparation of this article, Lindahl et al. (2018) also reported a prognostic classifier for MF based on expressions of miR-106-5p, miR-148-3p, and miR-338-3p. Compared with the Danish population-based study, our study included Chinese patients and identified different miRs as prognostic markers; we further investigated the mediating factors of miR-200b.

We believe that these miRs will also be very useful adjuncts to current clinical prognostic indicators. If validated in prospective trials, it will stratify patients to facilitate tailoring individualized treatments.

MATERIALS AND METHODS
Study design and patients
FFPE specimens, including 228 CTCL (including MF, c-ALCL, PTCL, and natural killer/T-cell lymphoma) and BID patients were obtained from the archives at the cutaneous lymphoma clinic and general dermatology clinic, Department of Dermatology, Ruijin Hospital, between 2003 and 2015. The diagnosis of CTCL was based on the criteria of the 2005 World Health Organization-European Organization of Research and Treatment of Cancer classifications (Willemze et al., 2005) and staging of MF was based on 2007 International Society for Cutaneous Lymphomas/European Organization of Research and Treatment of Cancer recommendations (Olsen et al., 2007). The cases were then confirmed by the consideration of the clinical presentations and careful examinations of histology, immunohistochemistry (including CD2, CD3, CD4, CD5, CD7, CD8, CD20, CD30, CD56, TIA-1, MUM, perforin, Granzyme-B, Ki-67, and EBER) and T-cell receptor gene rearrangement. All samples were pathologically examined by two dermatopathologists and followed-up by two dermatologists. None of the patients had received treatment before biopsy. Biopsy samples were attributed into three non-overlapping categories (discovery, training, and
Confirmed CTCL patients are then classified into early stage MF (stages I and IIa), advanced stage MF (stages IIb, III, and IV) and other types of CTCL (including c-ALCL, PTCL, and natural killer/T-cell lymphoma). There was no significant difference in the distribution of age and sex between BID and CTCL group as well as among discovery, training, and validation sets of patients (Table 1). The investigational protocol was approved by review boards of Shanghai Jiao Tong University and written informed patient consent was obtained from each participant or each participant’s guardian.

Four-step mediation test

miR-200 expression levels, MF staging (early or advanced) and patient OS were set as three covariates. The hypothetical “mediation” model (Baron and Kenny 1986) was examined by the following four-step regression model (Figure 5):

1. We fitted a Cox survival model where the OS was the response and the miR-200 expression level was explanatory covariate, adjusted for age and sex. This is to check whether there is any significant marginal effect of miR-200 expression on OS. Regression coefficient $\tau$ was estimated for comparison.

2. We fitted a logistic model to check the association between MF staging (binary response) and miR-200 expression (explanatory), adjusted for age and sex. Regression coefficient $\alpha$ was estimated.

3. and 4. A Cox model was fitted with OS as response; both miR-200 and MF staging were included as explanatory covariates adjusted for age and sex. In these models, because two regression coefficients $\beta$ and $\tau'$ were estimated, it was considered as two steps. If steps 1 and 2 models give significant associations of the explanatory factors with their responses, and in steps 3 and 4 model, the conditional

![Diagram illustrating the relationship among miR-200b, MF staging, and patient overall survival. Coefficients with standard errors (SE) and $p$ values of four-step mediation test are presented in the Table. Solid arrows: $P \leq 0.05$. MF, mycosis fungoides.](image-url)
effect of miR-200 (τ) diminishes in the presence of MF staging while MF staging retains a significant association with OS, we can conclude that there potentially exists some mediation effect from MF staging on the regulation path between miR-200 expression and OS.

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CONFLICTS OF INTERESTS
The authors state no conflict of interest.

ACKNOWLEDGMENTS
This study was supported in part by research funding from the National Key Clinical Specialty (grant No. 2012649) to JZ and National Natural Science Foundation of China (grant No. 30901293) to XS.

SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2018.03.1300.

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