Deep-Sequencing Analysis Reveals that the miR-199a2/214 Cluster within DNM3os Represents the Vast Majority of Aberrantly Expressed MicroRNAs in Sézary Syndrome

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TO THE EDITOR

MicroRNA (miR) is a class of non-coding RNA, modulating gene expression through negative regulation of target gene expression. Currently, >1,000 human miRs have been identified which can have crucial roles during (tuning of) T-cell activation and differentiation (Lindsay, 2008; Lodish *et al.*, 2008), and there is increasing evidence for a role of miRs in the pathogenesis of lymphoma and leukemia (Croce, 2009).

Recent miR array studies indicate that a considerable amount of miRs show aberrant expression in cutaneous T-cell lymphoma (CTCL), including Sézary (Sz) syndrome (Ballabio *et al.*, 2010; Narducci *et al.*, 2011; Ralfkiaer *et al.*, 2011; van Kester *et al.*, 2011). However, findings from these array-based analyses are not fully consistent, perhaps as a result from variant technological approaches and disadvantages such as background noise and cross-hybridization problems. These drawbacks can be overcome by next generation (deep) sequencing, which offers increased sensitivity and specificity, and has an unlimited detecting range.

Maybe even more importantly, deep sequencing also reveals the proportion of individual miRs in relation to the complete miRNome, which provides insights into complexity of miRNomes, as well as permits the discovery of previously unreported miRs (Creighton *et al.*, 2009) information that cannot be collected using miR array-based platforms.

Using deep-sequencing technology (Supplementary Information online), we quantitatively and qualitatively analyzed the miRNomes of CD4+ T cells isolated from the peripheral blood of Sz patients (diagnosed according to criteria described in Willemze *et al.* (2005)). For comparison, we not only determined the miRNomes of CD4+ T cells from healthy controls, but also from erythroderma secondary to atopic dermatitis (EAD) patients, as a clinically relevant benign counterpart of Sz. Using the miRBase annotation, 612 known miR sequences were identified in all 20 sequenced samples. Quantitative analysis revealed that the genome-wide miR expression in T cells of Sz patients, EAD patients, and healthy donors was rather similar. We found that a small amount of abundantly expressed miRs contributed to the major part of the each miRNome, e.g., the 20 most abundant miRs contributed to >90% of each miRNome (Figure 1a).

When compared with EAD patients, 11 miRs were found to be statistically significantly differentially expressed (Figure 1b), including upregulation of miR-214/214* and miR-199a/199a* in Sz as the most profound difference. In comparison with healthy donors (used as an additional control), 17 miRs were identified to be significantly differentially expressed in Sz patients (Figure 1c); again, upregulation of miR-214/214* and miR-199a/199a* was the most prominent distinction.

For the upregulated miRs, in comparison to healthy controls, our deep-sequence data are well in agreement with previously published results: four out of six miRs identified via deep sequencing as being upregulated in Sz versus controls were also identified in array studies (miR-214, miR-199a, miR-199a*, and miR-486).

Seven out of the 13 miRs identified via deep sequencing as being downregulated in Sz as compared with healthy controls have also been reported to be lower expressed using array-based analysis (miR-19a, miR-192, miR-140, miR-146b, miR-30e, miR-142, and miR-31). As these miRs are also low abundantly expressed in EAD, an association with the malignant character of Sz is not expected. In comparison with EAD, we identified seven miRs that were significantly lower expressed and also downregulated in many other tumors (Calin and Croce, 2006), thus probably reflecting a general cancer miR signature.

Previous array-based studies showed that the major change in the Sz miRNome in comparison with healthy cells was downregulation of many more miRs, e.g., an additional 25 (Narducci *et al.*, 2011) or 104 miRs (Ballabio *et al.*, 2010). This discrepancy with the current deep-sequencing data may result not only from the different technologies used (including statistical analysis) but also from the selection of reference material. For instance, in our comparison we used CD4+ T cells isolated by negative selection (''untouched''), whereas positively selected CD3+ and CD4+ T cells were used in the studies of Ballabio *et al.* (2010) and Narducci *et al.* (2011).

To discover hitherto previously unreported miRs, we used the miRDeep2.0 pipeline to map and analyze all the reads to the human genome for the three different groups: Sz ($n=12$), EAD ($n=4$), and healthy controls ($n=4$). A list with all miRDeep scores of >1.0 and additional information are available as Supplementary Information online (Supplementary Table S3 online). Although this analysis revealed many previously unreported miRs, most of them were expressed

Abbreviations: CTCL, cutaneous T-cell lymphoma; DNM3os, dynamin 3 opposite strand; EAD, erythroderma secondary to atopic dermatitis; miR, microRNA; Sz, Sézary

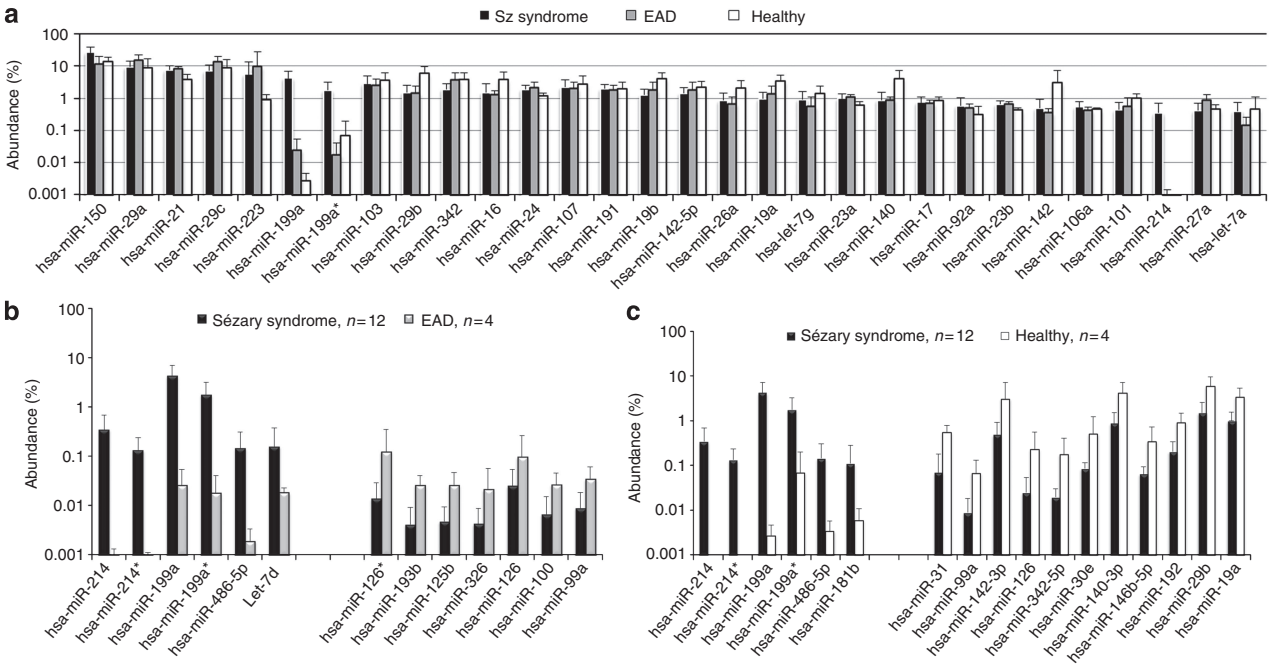

Figure 1. Deep-sequence analysis of expressed microRNAs (miRs) in Sézary (Sz) syndrome, erythroderma secondary to atopic dermatitis (EAD), and healthy controls. The expression of each miR was normalized within samples, and miRs per group are given as percentage (% of mean ± SD). (**a**) Top 30 of most abundantly expressed miRs in CD4+ T cells of Sz syndrome, EAD, and healthy donors. (**b**) MiRs statistically significantly differentially expressed in Sz syndrome versus EAD at an adjusted $P<0.05$. Individual adjusted *P*-values are listed in Supplementary Table S1 online. (**c**) MiRs statistically significantly differentially expressed in Sézary syndrome versus healthy controls at an adjusted $P<0.05$. Individual adjusted *P*-values are listed in Supplementary Table S2 online.

at very low levels (<100 copies per million read counts). The most abundant miR is found to be located on the positive strand in chr12:130494576–130494683. However, this miR is also expressed in benign controls (EAD and healthy donors) at comparable levels.

In the course of analyses, we observed many sequence variations in comparison with sequences deposited in miRBase. These deviancies consisted of single-nucleotide substitutions, 3′ and/or 5′ deletions and extensions, as well as non-template additions (tailing). This is in particular noteworthy for miR-21, which is proposed and disputed as diagnostic marker for CTCL (Narducci *et al.*, 2011; Ralfkiaer *et al.*, 2011). Deep sequencing shows that the major form of this miR consists of 23 nucleotides (5′-UAGCUUAUCAGA CUGAUGUUGAC-3′) in all samples, whereas arrays and commercially available PCR assays are designed for the detection of a 22-nucleotide-long, less prevalent, and variant miR.

As mature miR-199a and 199a* can arise from different precursors, we measured precursor expression of pre-miR-199a1, pre-miR-199a2, and pre-miR-199b, and compared these data with pre-miR-214 expression. Quantitative PCR analysis demonstrated upregulation and high expression of pre-miR-199a2 and pre-miR-214 in Sz, while expression levels of pre-miR-199a1 and pre-miR-199b did not differ significantly between Sz, EAD, and healthy donors (Figure 2). Pre-miR-199a2 and pre-miR-214 are located as a tandem in a single transcript, so-called dynamin 3 opposite strand (DNM3os), which is thought to be transcriptionally activated by TWIST1 (Lee *et al.*, 2009), a transcription factor which is known to be overexpressed in Sz (van Doorn *et al.*, 2004).

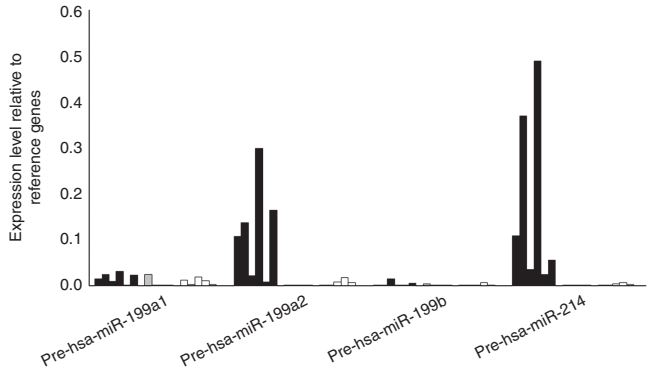

Figure 2. MicroRNA (miR) precursor analysis. Result of SYBR Green-based precursor quantitative PCR (Q-PCR). Expression levels are relative to reference genes. Each bar represents one sample (duplicate determination). Black bars: Sézary, grey bars: atopic dermatitis, open bars: healthy donors.

In sum, our deep-sequence analysis shows that the major changes in the miR repertoire of Sz cells converge to a single determinant, i.e., expression of DNM3os. This transcript harbors four

of the most differentially expressed miRs when comparing Sz with controls, contributing to >5% of all miRs in the Sz miRNome. The clinical use of these miRs and others identified in this study as diagnostic/prognostic classifiers or therapeutic targets warrants additional validation in a larger patient cohort and relevant control samples.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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