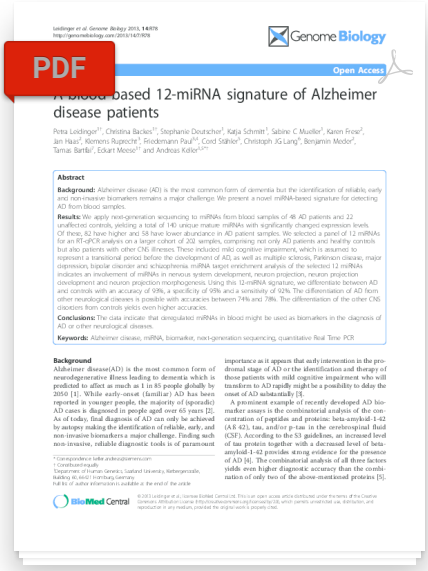

A blood based 12-miRNA signature of Alzheimer disease patients

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A blood based 12-miRNA signature of Alzheime

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Abstract

Background: Alzheimer disease (AD) is the most common form of dementia but the identification of reliable, and non-invasive biomarkers remains a major challenge. We present a novel miRNA-based signature for detect AD from blood samples.

Results: We apply next-generation sequencing to miRNAs from blood samples of 48 AD patients and 22 unaffected controls, yielding a total of 140 unique mature miRNAs with significantly changed expression levels. Of these, 82 have higher and 58 have lower abundance in AD patient samples. We selected a panel of 12 miRI for an RT-qPCR analysis on a larger cohort of 202 samples, comprising not only AD patients and healthy contr but also patients with other CNS illnesses. These included mild cognitive impairment, which is assumed to represent a transitional period before the development of AD, as well as multiple sclerosis, Parkinson disease, n depression, bipolar disorder and schizophrenia. miRNA target enrichment analysis of the selected 12 miRNAs indicates an involvement of miRNAs in nervous system development, neuron projection, neuron projection development and neuron projection morphogenesis. Using this 12-miRNA signature, we differentiate between and controls with an accuracy of 93%, a specificity of 95% and a sensitivity of 92%. The differentiation of AD fr other neurological diseases is possible with accuracies between 74% and 78%. The differentiation of the other disorders from controls yields even higher accuracies.

Conclusions: The data indicate that deregulated miRNAs in blood might be used as biomarkers in the diagnos AD or other neurological diseases.

Keywords: Alzheimer disease, miRNA, biomarker, next-generation sequencing, quantitative Real Time PCR

Background

Alzheimer disease(AD) is the most common form of neurodegenerative illness leading to dementia which is predicted to affect as much as 1 in 85 people globally by 2050 [1]. While early-onset (familiar) AD has been reported in younger people, the majority of (sporadic) AD cases is diagnosed in people aged over 65 years [2]. As of today, final diagnosis of AD can only be achieved by autopsy making the identification of reliable, early, and non-invasive biomarkers a major challenge. Finding such non-invasive, reliable diagnostic tools is of paramount importance as it appears that early intervention in th dromal stage of AD or the identification and ther those patients with mild cognitive impairment wh transform to AD rapidly might be a possibility to de onset of AD substantially [3].

A prominent example of recently developed A marker assays is the combinatorial analysis of th centration of peptides and proteins: beta-amyloi (Aß 42), tau, and/or p-tau in the cerebrospina (CSF). According to the S3 guidelines, an increase of tau protein together with a decreased level of amyloid-1-42 provides strong evidence for the pr of AD [4]. The combinatorial analysis of all three yields even higher diagnostic accuracy than the nation of only two of the above-mentioned protei

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Furthermore, combinatorial analysis of Aß levels and tau levels can discriminate between patients with stable mild cognitive impairment (MCI) and patients with progressive MCI into AD or other types of dementia with a sufficient diagnostic accuracy [6]. Nevertheless, according to the S3 guidelines, the analysis of CSF biomarker is only indicated to confirm the diagnosis if other clinical symptoms give evidence for the presence of neurodegenerative dementia or for the differential diagnostics of other forms of diseases that can cause symptoms like dementia (encephalitis, neuroborreliosis, multiple sclerosis, Lues, brain abscess, metastases).

The use of peripheral markers, like Aß and tau in easily accessible peripheral cells (in particular platelets and skin fibroblasts), as a diagnostic tool has been under investigation for more than 10 years [7,8]. Molecular genetics analyses of common single nucleotide polymorphisms (SNPs) in genes such as presenilin or ApoE4 did not significantly improve risk estimation for the susceptibility of AD [9]. Likewise, there is no consistent evidence for an association between AD and genetic variation of mitochondrial DNA (mtDNA) [10].

There is increasing effort to develop molecular diagnostic markers that meet requirements like easy accessibility, for example, from blood, sufficiently high specificity and sensitivity, low costs and applicability by laboratories with standard equipment. Several blood, plasma, or serum born AD biomarkers have been proposed to meet these criteria. Doecke et al. recently presented a panel of protein biomarkers to reliably detect AD with an accuracy of 85% [11]. Moreover, Tan et al. provided evidence that the proteins p53 and p21 can be used to detect AD using blood samples. A receiver operating characteristic curve analysis revealed a specificity of 76% and a sensitivity of 84% for p53, 88% and 82% for p53(ser15), 80% and 75% for p21, and 84% and 68% for p21(thr145) [12].

Besides proteins microRNAs (miRNAs) have also demonstrated their potential as non-invasive biomarkers from blood and serum for a wide variety of human pathologies [13]. A deregulation of miRNA expression might be involved in neurological dysfunction or neurodegenerative processes. Interestingly, Liang et al. [14] showed that the expression pattern of brain and blood PBMC cluster together which might be an indication that a specific blood based expression signature might prove to be useful as biomarker for AD and other neurological diseases. MiRNA expression analyses can be readily applied for *in vitro* diagnostic testing by molecular diagnostics and CLIA (Clinical Laboratory Improvement Amendments) laboratories.

While altered miRNA patterns have been exhaustively investigated in AD patients' tissue samples or cell cultures [15-18], less information on circulating miRNAs in AD is known. A recent serum profiling of AD patients provided first evidence that expression changes of circulatin NAs may be valuable biomarkers for AD [19].

We describe our results obtained by applying the generation sequencing (NGS) approach to scre expression of all human miRNAs in blood from sively characterized AD patients and healthy co Patient blood was obtained from the SAMPLE (Alzheimer diseaseand MCI Prospective Longitudin luation) Registry of PrecisionMed (San Diego, CA, and blood from age-matched healthy donors fro ACE (Aging Cognition Evaluation) Registry, a sionMed- UBC (The University of British Columbi laboration. We identified 140 unique differer expressed miRNAs between AD patients and co Validation of a 12-miRNA signature was carried RT-qPCR in a cohort of 202 samples encomp patients suffering from other neurological disc including mild cognitive impairment as a potentia minary stage of AD, and other neurodegenerati eases like Parkinson disease and multiple sclerosis as mental diseases like schizophrenia (SCHIZ), depression (DEP), and bipolar disorder (BD).

A combination of AD-specific miRNA expression tures with the rapidly developing and expanding ar load imaging techniques may be useful as non-in diagnostic tools in AD diagnosis in the future [20].

Results

Initial biomarker screening using next-generation sequencing

To detect potential AD biomarkers we examined from well-characterized patients and control obtained blood from the SAMPLE (Serial Alzheimer seand MCI Prospective Longitudinal Evaluation) R of PrecisionMed (San Diego, CA, USA). SAMPI sample depository resulting from a longitudinal stu evaluates cognition in women and men, who are rec evaluated, cognitively studied, and sampled from 1 experienced investigative sites in USA. All partic underwent several tests (that is, Alzheimer Disease A ment Scale-cognitive subscale (ADAS-Cog), C Dementia Rating (CDR), Wechsler Memory Scal Mini-Mental State Exam (MMSE)) to evaluate cog Blood from age-matched healthy donors was ob from the Ace Registry, which is a biological sampl of serial patient samples with linked serial cognitio based on a cognition battery selected from UBC's pr tary computerized testing platform.

We carried out high-throughput NGS of 22 h control samples (C) and 48 AD patient samples usir minaHiSeq 2000 sequencing with eight multiplexe ples on each sequencing lane. We detected no known human miRNAs, but also novel miRNA cand that have previously not been included in the mi

v18 [21,22]. These miRNA candidates are, however, much less abundant compared to the known human miRNAs. After removing the least abundant miRNAs (that is, all miRNAs with <50 read counts summed up across all samples of each group) we detected a total of 383 different miRNA precursors resulting in 416 unique mature miRNA forms.

To compare the NGS results of the AD patient samples with the samples from healthy donors we first computed Wilcoxon-Mann-Whitney (WMW) test and adjusted the significance values for multiple testing using Benjamini-Hochberg adjustment. All miRNAs with adjusted significance values <0.05 were considered statistically significant. We also computed the area under the receiver operator characteristics curve (AUC). In total, we detected 180 significantly dys-regulated miRNAs (140 unique mature miRNAs) including 90 miRNAs (58 unique mature miRNAs) that were downregulated and 90 miRNAs (82 unique mature miRNAs) that were upregulated in AD samples compared to healthy control samples (see Additional file 1-Table S1). Additional file 2-Figure S1 shows a heatmap for 180 significantly dys-regulated miRNAs. The most upregulated miRNA was hsa-miR-30d-5p (AUC of 0.0819) with a *P* value of $8.35*10^{-6}$and the most downregulated miRNA was hsa-miR-144-5p (AUC of 0.9138) with *P* value of $8.35*10^{-6}$. While the high AUC value indicates that each of these miRNAs has sufficient power to differentiate between AD and healthy controls, they are not specific for AD since both miRNAs have already been described for many other human pathologies, including different neoplasms [13]. Among the significantly dys-regulated miRNAs are also 15 novel miRNA candidates (called brain-miR) that were all upregulated in AD compared to controls. A list of all novel mature miRNAs is provided in Additional file 3-Table S2. To gain first insight into the biological function of the mature miRNAs that were dys-regulated between AD patients and healthy control individuals, we applied a miRNA over-representation analysis for these miRNAs using the TAM (tool for annotations of human miRNAs) database [23,24]. The TAM database classifies over- or under-represented miRNAs according to the categories miRNA family, miRNA cluster, miRNA function, miRNA associated diseases, and tissue specificity. We detected for all dys-regulated miRNAs 56 significant categories (*P* value <0.05 after adjustment for multiple testing), with the

To determine whether the 140 unique differe expressed miRNAs between AD patients and h controls cluster together within a same genomic r which would suggest presence of common regu mechanisms for their expression, we sorted all mi according to their position on each chromosome. we assigned the miRNAs to one of the following classes: not dys-regulated; upregulated in AI downregulated in AD. Finally, we searched for r that contain at least three different dys-regulated r miRNAs by applying window sizes varying be 1,000 and 100,000 base pairs. Within regions e passing <1,000 base pairs we detected two cl including one on chromosome 19 with the upreg miRNAs hsa-miR-99b-5p and hsa-miR-125a-5p a downregulated miRNA hsa-let-7e-5p and a secon ter on chromosome 22 with the downregulated m hsa-let-7a-5p and hsa-let-7b-5p and the upreg miRNA hsa-let-7b-3p. Analyzing regions of up to base pairs, we found on chromosome 9 a dense c with a total of five dys-regulated miRNAs includi downregulated miRNAs hsa-let-7a-5p, hsa-let-7f-5 hsa-let-7d-5p and the upregulated miRNAs hsa-le 3p and hsa-let-7d-3p. For regions up to 30,00 pairs, we discovered one region on chromosome three co-located miRNAs including hsa-miR-3 hsa-miR-30a-3p, and hsa-miR-30a-5p, all of whic upregulated. To understand whether the miRN regulated by specific transcription factors (T extracted potential TF binding sites from the UCS ome browser but found no evidence for a signi enrichment for specific TF binding sites.

In the next step, we performed classification of A control samples using a standard machine lea approach. In a cross-validation loop, we stepwise the miRNAs with lowest significance values and edly carried out radial basis function support machines (SVM). As shown in Figure 1, a signat 250 miRNAs yields an accuracy, specificity, and se ity of 90%. Since this set of miRNAs contains a sign amount of redundant miRNAs with largely ide information and high correlation among many mi a significantly smaller set of miRNAs is likely t comparably accurate distinction between AD sa and samples from healthy controls. We selected 1 NAs with limited cross-correlation, including st dys-regulated miRNAs that show a potential to se

with nine downregulated miRNAs (P value $5.65 \cdot 10^{-7}$), and the disease category Alzheimer disease for which six dys-regulated miRNAs were relevant, including hsa-miR-21, hsa-miR-17, hsa-miR-29a, hsa-miR-29b, hsa-miR-106b, and hsa-miR-107 (P value 0.0139).

and non-cancer diseases [13] in order to ensure th selected miRNAs are not dys-regulated in several diseases. Besides known miRNAs we also include unknown miRNAs, namely brain-miR-112 and miR-161. Finally, the selected 12-miRNA sign

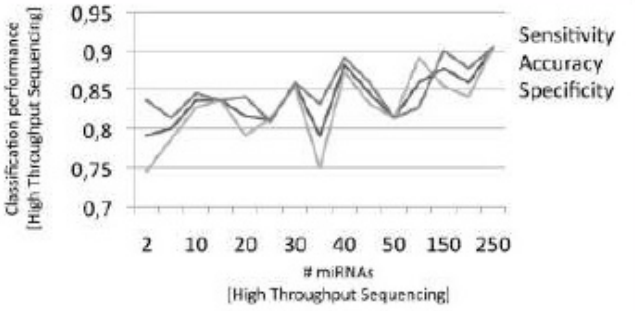

Figure 1 Classification performance dependent on miRNA combinations. With increasing number of miRNAs the accuracy, specificity, and sensitivity increases towards convergence at 90%.

contains the miRNAs brain-miR-112, brain-miR-161, hsa-let-7d-3p, hsa-miR-5010-3p, hsa-miR-26a-5p, hsa-miR-1285-5p, and hsa-miR-151a-3p, all of which are upregulated in AD and the downregulated miRNAs hsa-miR-103a-3p, hsa-miR-107, hsa-miR-532-5p, hsa-miR-26b-5p, and hsa-let-7f-5p.

Validation of a 12-miRNA signature by RT-qPCR

To validate the 12-miRNA signature we employed RT-qPCR and included not only additional patients with AD, but also patients with other diseases including neurological disorders. In total, we analyzed 12 miRNAs in 202 samples as detailed in Table 1.

We first considered the miRNA fold quotients that were obtained for AD samples and controls. We compared the fold quotients of each of the 12 miRNAs between initial NGS screening cohort and the RT-qPCR validation cohort. All but two of the 12 miRNAs, namely hsa-miR-1285-5p and hsa-miR-26a-5p, have been dys-regulated in the same direction in both approaches, indicating a high degree of concordance between screening and validation study. Both hsa-miR-1285-5p and hsa-miR-26a-5p have been significantly upregulated in AD in the NGS screening experiment while downregulated in

might be due to the duplication of the AD sample c However, SVM classification on the RT-qPCR d separate AD and controls using linear kernels in 1 cross-validations with 100 repetitions reached on a an accuracy of 93.3%, a specificity of 95.1%, and a tivity of 91.5%. The computed means, standard tions, and confidence intervals for the repe concerning specificity, sensitivity, and accuracy ar sented in Table 2, as well as the results for the c classifications with the randomly permuted class lal

To evaluate whether the selected miRNAs are dependent we further grouped the AD patients a ing to their MMSE score into mild AD (MMS n = 39) and moderate AD (MMSE 12-19, n = 46 MMSE is a short test of 30 questions used to scre cognitive impairment. Each question to be answe scored with points, with a maximum possible sc 30 points. This questionnaire can be used to est the severity of cognitive impairment and to follo course of cognitive changes in an individual over Normally, patients reaching 27 to 30 points do no fer from dementia, 10 to 26 points are indicati mild-to-moderate dementia, and less than 9 point cates severe dementia. We found no significant e sion differences of the 12-miRNA signature betwe mild AD group and the moderate AD group.

As patients with other neurological disorders can similar symptoms as AD patients, we decided to v our AD NGS results also with samples from pa with several neurological diseases. Specifically, we whether other neurological disorders show signi deviations in the expression of the 12 miRNAs results of this validation help to determine wheth investigated miRNAs have the potential for clinical cations. We analyzed patients with neurodegene diseases (MCI, Parkinson disease (PD), multiple sc (clinically isolated syndrome, CIS)) and patients wit chiatric disorders (SCHIZ, BD, and DEP) for the

Table 1 Overview of the blood samples analyzed using NGS and RT-qPCR

Sample group	*N*	Age (mean ± SD)	Sex (female/male)	MMSE (mean ± SD)	Cohort size NGS	Cohort RT-qPC
AD	106	72.7(10.4)	53/53	18.9 (3.4)	48	94
Healthy control	22	67.1 (7.5)	11/11	29.3 (1.2)	22	21
Mild cognitive impairment	18	73.9 (6.2)	9/9	25.3 (1.4)	-	18
Multiple sclerosis	16	32.3 (10.7)	12/4	NA	-	16
PD	9	69.7 (9.0)	1/8	25.2 (4.2)	-	9
DEP	15	45.2 (9.1)	0/15	NA	-	15
BD	15	41.9 (13.7)	14/1	29.5 (1.6)	-	15
SCHIZ	14	41.7 (7.9)	1/13	26.1 (4.3)	-	14

AD: Alzheimer disease; BD: bipolar disorder; DEP: major depression; MMSE: Mini-Mental State Exam; NA: not available; NGS: next-generation sequencing; Parkinson's disease; RT-qPCR: quantitative real-time PCR; SCHIZ: schizophrenia.