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BRIEF COMMUNICATION

Liquid biopsy: a new source of candidate biomarkers in amyotrophic lateral sclerosis

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Abstract

Noninvasive tests to diagnose and monitor the progression of neurodegenerative disorders have been a challenge for decades. The aim of this study was to explore the feasibility of applying liquid biopsy procedures to patients with a neurodegenerative disease such as amyotrophic lateral sclerosis (ALS). We isolated plasma cell-free DNA (cfDNA) in 20 ALS patients and 20 controls and used cfDNA to identify a novel differentially methylated mark in *RHBDF2* gene in ALS patients compared to controls. Our findings support the notion that liquid biopsy may be applied to living patients as a source of potential epigenetic biomarkers for neurodegenerative disorders.

Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease causing the progressive loss of motor neurons with an ominous prognosis.[1] So far, diagnosis of ALS is only based on clinical criteria[2] after ruling out other non-degenerative disorders mimicking ALS. The discovery of reliable biomarkers is crucial to further improve ALS diagnostic certainty and predict disease progression in the clinical setting.

The etiology of ALS remains unknown though it has been suggested that interaction between a genetic susceptibility profile and certain environmental factors may contribute to the disease through epigenetic changes.[3–6] In fact, some studies have shown that ALS patients harbor specific epigenetic marks as changes in DNA methylation in the central nervous system (CNS).[7,8] These DNA methylation changes may serve as candidate epigenetic biomarkers for ALS. However, there is little chance to access this "CNS-locked" epigenetic information while patients are alive.

Recent technological advances in the field of precision medicine are rapidly changing the diagnostic and treatment paradigm of diseases. Among these advances, liquid biopsy is making progress toward true personalized medicine, particularly in clinical oncology. Liquid biopsy is a noninvasive blood test to isolate tumor-related circulating cell-free DNA (cfDNA). Both blood plasma and serum are known to contain fragments of cfDNA originating from dead cells, up to 5000 genome equivalents/mL.[9] Liquid biopsy can be used to screen early stages of cancer or disease recurrence, to select molecular targets based on the profile of tumor mutations, and to monitor response to treatment.[10–12] However, the usefulness of liquid biopsy in neurodegenerative conditions has not yet been assessed.

The aim of this proof-of-concept study was to test whether liquid biopsy is feasible in neurodegenerative

diseases such as ALS. Thus, we wanted to assess if (1) cfDNA could be readily isolated from plasma of ALS patients and (2) may serve as a source of potential epigenetic biomarkers of neurodegeneration. To this end, we measured DNA methylation levels in plasma cfDNA for a candidate gene, *RHBDF2* (Rhomboid 5 Homolog 2). Other authors have shown *RHBDF2* may be a potential epigenetic marker of neurodegeneration, as it was found to be differentially methylated in the CNS in Alzheimer's disease.[13,14] *RHBDF2* gene encodes a rhomboid-like pseudoprotease which is involved in the innate immune response[15,16] and the activation of the epidermal growth factor (EGF) receptor signaling pathway.[17] Interestingly, EGF is essential to cell survival and has been related to the pathogenesis of ALS, as low levels of this peptide were found in the cerebrospinal fluid (CSF) of ALS patients.[18] Moreover, *RHBDF2* is implicated in tissue regeneration by activating the NRF2 (nuclear factor E2-related factor 2)-antioxidant pathway.[19]

Table 1. Characteristics of participants.

Characteristics	ALS (n = 20)	Control (n = 20)	P-value
Gender, Female/Male (%)	8/12 (40%)	8/12 (40%)	1.000
Age, mean ± SD	64.5 ± 14.3	56.7 ± 11.2	0.066
cfDNA concentration	25.76 ± 11.3	29.6 ± 10.3	0.272
ALS phenotype, bulbar/spinal (%)	4/16 (20%)		
Intense physical activity[1] (%)	6/20 (30%)		
Survival[2], mean ± SD	44.05 ± 29.20		
Course of disease[3], mean ± SD	26.41 ± 27.18		

[1]Intense physical activity > 150 min/week.
[2]Time to death or end of study in months.
[3]Time between symptom onset and sample collection in months.

Patients and Methods

Patients and samples

Patients who met El Escorial revised criteria[2] of definite or probable ALS (n = 20) and controls (n = 20) were recruited in our hospital (Complejo Hospitalario de Navarra, Spain) from July 2014 to December 2015. Controls were recruited among subjects who underwent spinal anesthesia in trauma minor surgery, after ruling out the following conditions: history of atherosclerosis, neurological, tumoral, or systemic inflammatory diseases. Characteristics of participants are shown in Table 1. Sample size estimation was performed by using the Ene software version 2.0. (GlaxoSmithKline, Madrid, Spain). The minimum sample size was calculated to be 14 in each group to provide 80% power to detect a 10% difference in DNA methylation levels between ALS cases and controls assuming that independent samples t-test would be used at a two-sided significance level of $\alpha = 0.05$. The study was approved by the Navarra Ethics Research Committee and informed consent was obtained from each participant.

Liquid biopsy procedure/Isolation of cfDNA

Whole blood samples were collected from each subject by venipuncture in EDTA tubes and centrifuged within 1 h (15 min, 2400 g, 4°C). Plasma was transferred to plastic tubes and stored at −80°C until further analysis. Thereafter, cfDNA was isolated from 2 mL plasma by using QIAmp Circulating Nucleic Acid Kit (QIAGEN, Redwood City, CA, USA) following manufacturer's recommendations. Concentration, quality and size distribution of cfDNA was assessed by a Fragment Analyzer™ Automated CE System with DNF-464 High Sensitivity Large Fragment 50Kb Analysis Kit (AATI, Ankeny, IA, USA).

Pyrosequencing

From each sample, 200 ng of cfDNA was bisulfite converted using the EpiTect Bisulfite Kit (Qiagen, Redwood City, CA, USA). Primers to amplify and sequence a target region in *RHBDF2* were designed with PyroMark Assay Design version 2.0.1.15 (Qiagen) (Table 2), and PCR reactions were carried out on a Veriti™ Thermal Cycler (Applied Biosystems, Foster City, CA, USA). A quantity of 20 μL of biotinylated PCR product was immobilized using streptavidin-coated sepaharose beads (GE Healthcare Life Sciences, Piscataway, NJ, USA) and 0.3 μmol/L sequencing primer was annealed to purified DNA strands. Pyrosequencing was performed using the PyroMark Gold Q96 reagents (Qiagen) on a PyroMark™ Q96 ID System (Qiagen). For each particular *locus* (CpG1 and CpG2), methylation levels were expressed as percentage of methylated cytosines over the sum of total cytosines. Following the same procedure, we also assayed the region surrounding an intergenic CpG at chr10p15.2 as a negative control *locus*.[20] Primers for this control region are shown in Table 2.

Bisulfite cloning sequencing

Technical validations of pyrosequencing results were performed by bisulfite cloning sequencing. Bisulfite-converted cfDNA samples (n = 16) were used to amplify by PCR a 173-bp region encompassing the two CpGs assayed by pyrosequencing (Fig. 1). The negative control region was also validated using an amplicon of 265-bp length.

Table 2. Pyrosequencing and Bisulfite PCR primers.

Identification	PCR Purpose	Amplicon size	Tm	Forward primer	Tm2	Reverse primer
RHBDF2_pyro	Pyro	113 bp	61.8	TGGTGGTTAGGTAGGGATAGTT	62.1	ATTTTTCCTTACACCC ATCCTAAATCT
RHBDF2_pyro_seq	Pyro		44.2	AGGGATAGTTTTTAGGGTA		
RHBDF2_bis	Bisulfite PCR	173 bp	59.61	ATTTTAGGTTTTTTGGGAGGTAAAA	57.66	TCCAACTAAACCAAA CTAAAAAAAA
Control_pyro	Pyro	175 bp	56.1	AGTTAGGTTTTAGTGAGTTTTTGTTTAT	56.7	TCCCTAACCCTCCATTTCA TATCAATACTA
Control_pyro_seq	Pyro		44.8	ATATGTGTGTAAGTTGAATAAAAT		
Control_bis	Bisulfite PCR	265 bp	55.05	TTGGTAGTGAATATTTTTGTTGTTA	57.4	ATAAATCCTTTACAA CTCCCTAACC

Pyro, pyrosequencing; bp, base pair; Tm, Melting Temperature; seq, sequencing primer.

Primer pair sequences were designed by MethPrimer[21] (Table 2). PCR products were cloned using the TopoTA Cloning System (Invitrogen, Carlsbad, CA, USA) and a minimum of 12 independent clones were sequenced for each subject.

Data analysis

Statistical analysis was performed with SPSS 21.0 (IBM, Inc., USA). Normality was assessed by Kolmogorov–Smirnov test. Data represents the mean ± SD. Statistical significance for pyrosequencing intergroup differences was evaluated by *T*-test with a significance level of 0.05. Pearson correlation coefficient was calculated to search for correlation between methylation levels and age. The diagnostic performance of *RHBDF2* methylation levels was determined by receiver operating characteristic (ROC) curves. The area under the curve (AUC) was calculated and optimum cut-of points were selected based on their sensitivity and specificity. GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA) was used to draw graphs except for methylation figures that were obtained by QUMA software.[22]

Results

There were no significant differences in age or gender between ALS patients ($n = 20$) and controls ($n = 20$) (Table 1). We succeeded to isolate plasma cfDNA from each participant. Concentration of cfDNA ranged between 8.9 and 58.9 ng/μL and was similar in both groups (Table 1). Fragment separation of cfDNA usually follows a pattern that mirrors cfDNA cleavage by the nucleosome positioning, showing peaks at around 165 bp, 350 bp, and 565 bp. In our experiment, capillary electrophoresis performed on a Fragment Analyzer system showed the typical pattern of fragmented cfDNA (Fig. 1) with no genomic DNA contamination.

Next, we measured methylation levels of two distinct CpGs (CpG1 and CpG2) located at the promoter-enhancer region of *RHBDF2* gene. Pyrosequencing showed that averaged methylation levels at CpG1 were significantly increased in ALS patients compared to controls [73 ± 11% vs. 65 ± 12%; *P*-value < 0.05]. Similarly, CpG2 showed a significant increase in methylation levels in ALS patients compared to controls [88 ± 6% vs. 81 ± 10%; *P*-value < 0.05] (Fig. 1). As expected due to their genomic proximity, methylation levels of CpG1 and CpG2 were positively correlated [$r = 0.807$; *P*-value < 5E-07). In contrast, no correlation was observed between age and methylation levels at CpG1 ($r = 0.231$; $P = 0.157$) or CpG2 ($r = 0.195$; $P = 0.233$). Regarding gender, no differences in methylation levels at CpG1 [female vs male, 71 ± 12% vs. 68 ± 13%; *P*-value = 0.334] or CpG2 [87 ± 9% vs. 83 ± 9%; *P*-value = 0.125] were found.

We wanted to validate the pyrosequencing results and explore methylation levels within the genomic region where the two CpGs were located. To that end, we performed bisulfite cloning sequencing to assess methylation levels for a 173 bp-amplicon harboring CpG1 and CpG2. In line with the previous pyrosequencing results, we found that average methylation levels for the whole amplicon were significantly increased in ALS patients compared to controls [85 ± 7% vs. 68 ± 14%; *P*-value < 0.01](Fig. 1).

In order to avoid false positives, we selected a region to be explored as a negative control locus for changes in cfDNA methylation. An intergenic CpG (cg09787504) located at chr10p15.2 was chosen as other authors had previously used it as a brain-derived methylation marker in cfDNA[20] and no changes have been reported for this locus in neurodegenerative conditions. In our study, no changes in methylation were observed for this CpG by pyrosequencing analysis between ALS patients and controls [98.97 ± 2.34% vs. 98.13 ± 3.47%; *P*-value = 0.434]. Moreover, these results were validated and

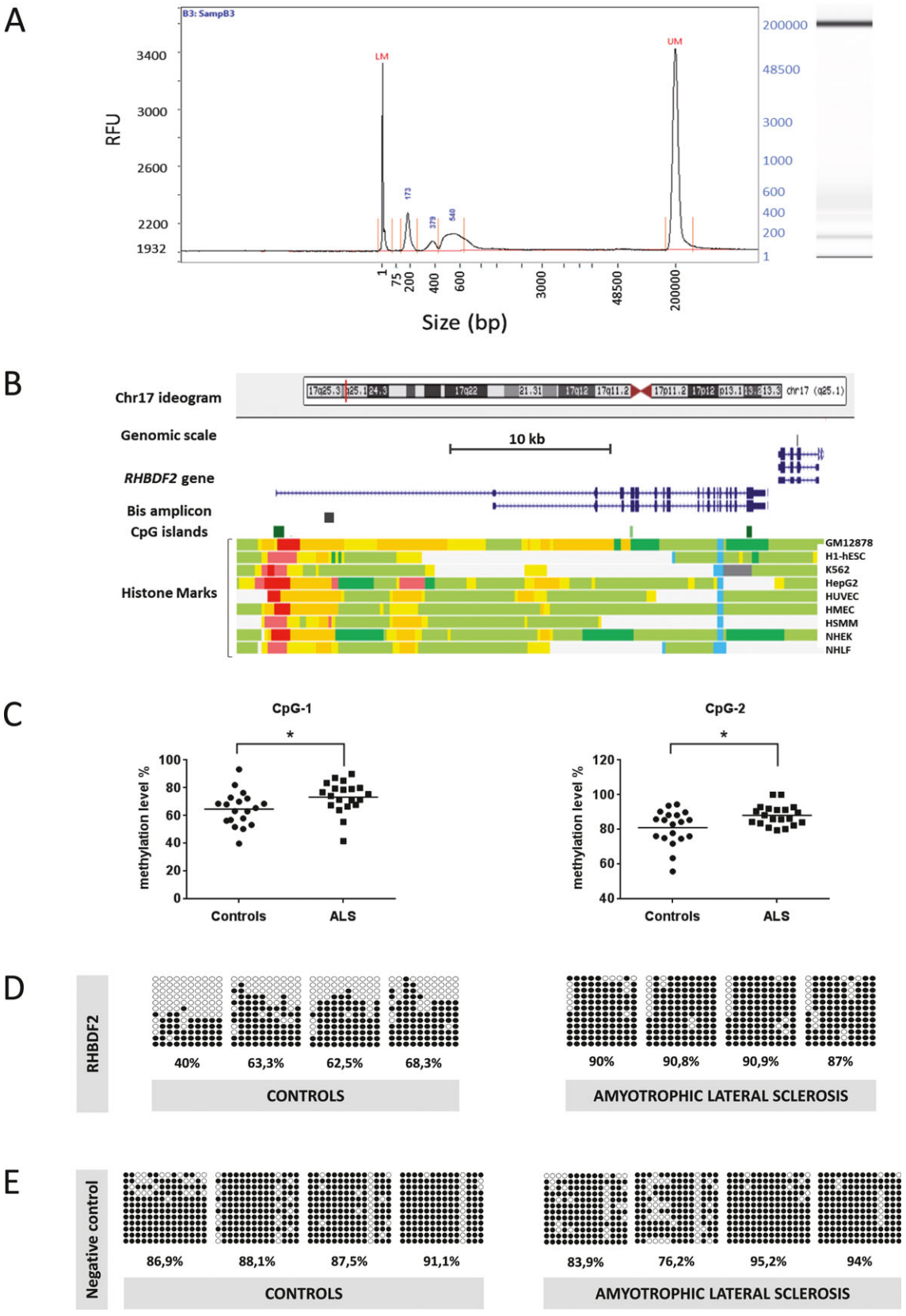

Figure 1. *RHBDF2* DNA methylation levels in cfDNA in ALS. (A) The diagram represents the typical pattern of cfDNA with peaks at around 165 bp, 350 bp and 565 bp after performing capillary electrophoresis on a Fragment Analyzer Automated CE System. (B) The graph shows genomic position of the amplicon (black box) validated by bisulfite cloning sequencing which contains the cytosines assayed by pyrosequencing (CpG1 and CpG2) within a promoter-enhancer region of the *RHBDF2* gene. *RHBDF2* is located on the long arm of chromosome 17 (chr17:74,466,975-74,497,509-GRchr17/hg19 coordinates). CpG islands are represented by isolated green boxes. At the bottom of the graph, predicted functional elements are shown for each of nine human cell lines explored by Chromatine imunoprecipitation (ChIP) combined with massively parallel DNA sequencing. Boxes represent promoter regions (red), enhancers (yellow), transcriptional transition & elongation (dark green), weak transcribed regions (light green) and insulators (blue). The track was obtained from Chromatin State Segmentation by HMM from ENCODE/Broad track shown at the UCSC Genome Browser. (C) Dot-plot charts representing pyrosequencing methylation levels for CpG1 and CpG2 (C) are shown. Horizontal lines represent median methylation values for each group. (D) Representative examples of bisulfite cloning sequencing validation for the amplicon containing both CpGs are shown**.** (E) In the bottom line, representative examples of bisulfite cloning sequencing validation for the negative control locus surrounding cg09787504 are depicted. Boxes represent individual controls (left side) and ALS patients (right side). Black and white circles denote methylated and unmethylated cytosines, respectively. Each column symbolizes a unique CpG site in the examined amplicon and each line represents an individual DNA clone. **P*-value < 0.05.

extended by bisulfite cloning sequencing for a 265-bp amplicon which included cg09787504. No difference in methylation levels was found for the whole amplicon between ALS patients and controls (Fig. 1).

Finally, to ascertain the performance of *RHBDF2* methylation levels for ALS diagnosis, ROC analysis was performed. The AUC was 0.724 (CI = 0.559-0.888; *P*-value = 0.017) for CpG1 and AUC=0.695 (CI = 0.527–0.863; *P*-value = 0.038) for CpG2. The optimum cutoff points to differentiate between ALS patients and controls were 65.97% (sensitivity = 0.850, specificity = 0.526) for CpG1 and 83.26% (sensitivity = 0.800, specificity = 0.474) for CpG2.

Discussion

In this study, we have demonstrated that plasma cfDNA can be readily isolated from ALS patients and controls and may serve as a source of potential epigenetic biomarkers in neurodegenerative diseases.

Our approach sought to apply liquid biopsy beyond the oncology field to examine its usefulness in a different discipline, the neurodegenerative disorders. Some late evidence supports the innovative idea of using liquid biopsy in neurological disorders. Most of the liquid biopsy applications so far are based on detecting DNA sequence variability, for example, tumor-specific somatic mutations. That makes it challenging to use liquid biopsy in other medical fields, including neurodegenerative diseases, where DNA of affected cells does not undergo any change in DNA sequence. However, a remarkable work established proof-of-concept for using liquid biopsy in other conditions nonrelated to DNA sequence variation, including brain diseases. Notably, authors were capable of identifying tissue-specific cell death based on profiling tissue-specific methylation patterns of cfDNA.[20] For instance, they found increased brain-derived cfDNA in the serum of patients after traumatic brain injury.[20] Therefore, it is tempting to speculate that liquid biopsy may be able to detect some DNA fragments originated in dying neurons or other CNS cells that may cross the disrupted blood–brain barrier (BBB) in neurodegenerative disorders.

In contrast to the previous work, we performed our experiment to assess not tissue-specific cell death but ALS-related differential methylation marks. Thus, we revealed a novel differentially methylated region located in the promoter-enhancer region of *RHBDF2* gene in ALS patients. *RHBDF2* gene encodes a rhomboid protease-like protein expressed in spinal cord and involved in the EGF factor receptor signaling pathway and inflammation.[23,24] Notably, EGF is crucial to cell survival and proliferation and was found to be decreased in the CSF of ALS patients.[18] As an upstream regulator of EGF signaling pathway and due to its potential to activate the NRF2-antioxidant pathway,[19] *RHBDF2* may be considered an interesting candidate biomarker and therapeutic target in ALS and other neurodegenerative conditions. While future research is required to assess the performance of *RHBDF2* methylation marks as an actual biomarker of ALS, our results prove that studying methylation changes in cfDNA is feasible in a neurodegenerative condition.

Although the origin of the cfDNA in our study cannot be properly demonstrated, we hypothesize that cfDNA derived from dying CNS cells might cross the altered blood-brain-barrier and be isolated from peripheral blood in ALS patients. Indeed, this fact has been proved for multiple sclerosis and brain trauma in previous work performed by Lehmann-Werman et al.[20] While these methylation marks may be a biomarker of disease regardless their origin, the use of brain-derived cfDNA to identify new epigenetic biomarkers is relevant because these marks may reflect more accurately the molecular and epigenetic changes that are occurring in the damaged brain tissue.

Altogether, previous work[20] and the results of this study open up the door for using liquid biopsy in living patients, by isolating plasma cfDNA and measuring gene-

specific methylation levels, as a source of novel epigenetic biomarkers for neurodegenerative disorders.

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Authors Contributions

MM and IJ were responsible for the conception and design of the study and drafted the manuscript. LM was responsible for subject recruitment and sampling. MM, LM, IBL, AU, and MR participated in data acquisition, experimental work, and analysis. All authors revised the final version of the manuscript.

Conflicts of Interest

The authors declare no competing interests.

References

1. Zarei S, Carr K, Reiley L, et al. A comprehensive review of amyotrophic lateral sclerosis. Surg Neurol Int 2015;6:171.
2. Brooks BR, Miller RG, Swash M, Munsat TL, World Federation of Neurology Research Group on Motor Neuron D. El Escorial revisited: revised criteria for the diagnosis of amyotrophic lateral sclerosis. Amyotroph Lateral Scler Other Motor Neuron Disord 2000;1:293–299.
3. Belzil VV, Katzman RB, Petrucelli L. ALS and FTD: an epigenetic perspective. Acta Neuropathol 2016;132:487–502.
4. Kerry JA, Priddy MA, Jervey TY, et al. Multiple regulatory events influence human cytomegalovirus DNA polymerase (UL54) expression during viral infection. J Virol 1996;70:373–382.
5. Jimenez-Pacheco A, Franco JM, Lopez S, et al. Epigenetic mechanisms of gene regulation in amyotrophic lateral sclerosis. Adv Exp Med Biol 2017;978:255–275.
6. Chestnut BA, Chang Q, Price A, et al. Epigenetic regulation of motor neuron cell death through DNA methylation. J Neurosci 2011;31:16619–16636.
7. Figueroa-Romero C, Hur J, Bender DE, et al. Identification of epigenetically altered genes in sporadic amyotrophic lateral sclerosis. PLoS ONE 2012;7:e52672.
8. Morahan JM, Yu B, Trent RJ, Pamphlett R. A genome-wide analysis of brain DNA methylation identifies new candidate genes for sporadic amyotrophic lateral sclerosis. Amyotroph Lateral Scler 2009;10:418–429.
9. Schwarzenbach H, Hoon DS, Pantel K. Cell-free nucleic acids as biomarkers in cancer patients. Nat Rev Cancer 2011;11:426–437.
10. Crowley E, Di Nicolantonio F, Loupakis F, Bardelli A. Liquid biopsy: monitoring cancer-genetics in the blood. Nat Rev Clin Oncol 2013;10:472–484.
11. Karachaliou N, Mayo-de-Las-Casas C, Molina-Vila MA, Rosell R. Real-time liquid biopsies become a reality in cancer treatment. Ann Transl Med 2015;3:36.
12. Murtaza M, Dawson SJ, Tsui DW, et al. Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. Nature 2013;497:108–112.
13. De Jager PL, Srivastava G, Lunnon K, et al. Alzheimer's disease: early alterations in brain DNA methylation at ANK1, BIN1, RHBDF2 and other loci. Nat Neurosci 2014;17:1156–1163.
14. Lord J, Cruchaga C. The epigenetic landscape of Alzheimer's disease. Nat Neurosci 2014;17:1138–1140.
15. Adrain C, Zettl M, Christova Y, et al. Tumor necrosis factor signaling requires iRhom2 to promote trafficking and activation of TACE. Science 2012;335:225–228.
16. Luo WW, Shu HB. Emerging roles of rhomboid-like pseudoproteases in inflammatory and innate immune responses. FEBS Lett 2017;591:3182–3189.
17. Zettl M, Adrain C, Strisovsky K, et al. Rhomboid family pseudoproteases use the ER quality control machinery to regulate intercellular signaling. Cell 2011;145:79–91.
18. Cieslak D, Szulc-Kuberska J, Stepien H, Klimek A. Epidermal growth factor in human cerebrospinal fluid: reduced levels in amyotrophic lateral sclerosis. J Neurol 1986;233:376–377.
19. Hosur V, Burzenski LM, Stearns TM, et al. Early induction of NRF2 antioxidant pathway by RHBDF2 mediates rapid cutaneous wound healing. Exp Mol Pathol 2017;102:337–346.
20. Lehmann-Werman R, Neiman D, Zemmour H, et al. Identification of tissue-specific cell death using methylation patterns of circulating DNA. Proc Natl Acad Sci U S A 2016;113:E1826–E1834.
21. Li LC, Dahiya R. MethPrimer: designing primers for methylation PCRs. Bioinformatics 2002;18:1427–1431.
22. Kumaki Y, Oda M, Okano M. QUMA: quantification tool for methylation analysis. Nucleic Acids Res 2008;36(Web Server issue):W170–W175.
23. Li X, Maretzky T, Weskamp G, et al. iRhoms 1 and 2 are essential upstream regulators of ADAM17-dependent EGFR signaling. Proc Natl Acad Sci U S A 2015;112:6080–6085.
24. Christova Y, Adrain C, Bambrough P, et al. Mammalian iRhoms have distinct physiological functions including an essential role in TACE regulation. EMBO Rep 2013;14:884–890.

