Increased circulating microRNAs miR-342-3p and miR-21-5p in natural sheep prion disease

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Abstract

Scrapie is a transmissible spongiform encephalopathy (TSE), or prion disease, of sheep and goats. As no simple diagnostic tests are yet available to detect TSEs *in vivo*, easily accessible biomarkers could facilitate the eradication of scrapie agents from the food chain. To this end, we analysed by quantitative reverse transcription PCR a selected set of candidate microRNAs (miRNAs) from circulating blood plasma of naturally infected, classical scrapie sheep that demonstrated clear scrapie symptoms and pathology. Significant scrapie-associated increase was repeatedly found for miR-342-3p and miR-21-5p. This is the first demonstration, to our knowledge, of circulating miRNA alterations in any animal suffering from TSE. Genome-wide expression studies are warranted to investigate the true depth of miRNA alterations in naturally occurring TSEs, especially in presymptomatic animals, as the presented study demonstrates the potential feasibility of miRNAs as circulating TSE biomarkers.

Transmissible spongiform encephalopathies (TSEs), also known as prion diseases, are fatal neurodegenerative disorders that include Creutzfeldt–Jacob disease and Gerstmann–Sträussler–Scheinker syndrome in humans, bovine spongiform encephalopathy in cattle and scrapie in sheep and goats [1]. According to the prion hypothesis, TSEs result from misfolding of a native cellular prion protein, PrP, encoded by the gene *PRNP*, to an infectious, pathological conformation, PrPSc (named after PrP scrapie). This process is template driven and perpetual: conformationally altered PrPSc molecules can repeatedly convert their correctly folded, soluble counterparts into infectious prion deposits. The *PRNP* gene is highly, although not exclusively, expressed in the central nervous system (CNS) where its pathologically transformed products may spread from cell to cell. The accumulation of PrPSc in the CNS generally corresponds with the progression of neurodegeneration as evidenced by correlation with severity of common histopathological lesions observed in TSEs, such as neuropil spongiosis, vacuolation and activation of astrocytes and microglia.

Recent work in experimental TSE models warrants further investigation on the yet ill-defined zoonotic potential of scrapie [2, 3]. To date, there are no epidemiological studies that indicate that scrapie could be transmitted to humans. However, scrapie has both social and economic impact due to its effect on animal health and the resulting practical complications impinge on animal production and surveillance. The current measures for scrapie eradication are selective breeding (to replace susceptible *PRNP* genotypes with resistant ones) and culling of symptomatic, PrPSc-positive livestock. However, like in human TSE patients and in primate models of prion disease, long incubation periods occur in sheep during which asymptomatic but infected animals are a potential source of disease spread. Scientific opinion by the European Food Safety Authority [4] suggests that post-mortem testing of infected sheep stocks is not likely to be a sufficient measure to eradicate the disease.

At present, the only biochemical diagnostic marker for TSEs is considered to be PrPSc accumulation in post-mortem brain tissue or lymphoid tissue. The use of PrPSc as a preclinical diagnostic biomarker is difficult because the levels of

PrPSc in easily accessible tissues or body fluids are minimal [5]. Accumulation of PrPSc may be detected in the lymphoreticular system of preclinical animals [6]. However, this requires genetic predisposition to peripheral PrPSc accumulation [7, 8] and, hence, may be prone to false negatives as a diagnostic method. Equally, assays based on PrPSc detection may perform worse for identifying atypical scrapie than classical scrapie cases [9]. An ideal biomarker would be one capable of diagnosing all types of TSEs at the clinical and preclinical phases from easily obtainable tissue source.

Alterations in RNA metabolism, including that of microRNAs (miRNAs), are frequently found as contributors in neurodegenerative diseases [10]. miRNAs are small non-protein-coding RNAs that, as a part of the miRNA-induced silencing complex, regulate gene expression posttranscriptionally and play a critical role in neuronal survival and function [11]. miRNAs have also gained attention as minimally invasive disease biomarkers as they are often secreted by pathologically affected tissues into plasma and other body fluids where they show remarkable stability due to proteic chaperones or vesicular (such as exosomal) protection from ribonucleases [12]. Interestingly, human PRNP binds essential components of the miRNA-induced silencing complex and promotes its stability or formation [13]. However, it is not known if this interaction is perturbed in TSEs. Only a few studies have investigated how miRNA expression is affected in prion diseases. Brains of mice intracerebrally inoculated with scrapie displayed alterations in 15 miRNAs, including miR-342-3p, let-7b, let-7d, miR-128a and miR-146a [14, 15]. MicroRNA miR-342-3p was also increased in post-mortem brain samples from bovine-spongiform-encephalopathy-infected macaques and in sporadic Creutzfeldt–Jacob disease (sCJD) patients [16], and miR-146a was upregulated in sCJD and Gerstmann–Sträussler–Scheinker patients [17]. Studies on hippocampal neurons and synaptoneurosomes imply that dynamic modulation of miRNAs occurs between the preclinical and terminal stages of the prion disease [18, 19]. Finally, exosomes released *in vitro* by prion-infected neuronal cells have altered miRNA profiles (including miR-342-3p, let-7b, miR-128a, miR-21 and miR-146a) compared with non-infected exosomes [20]. If neuronal cells modulate their miRNA release (or secretion) in prion disease also *in vivo*, these could be possibly detected directly from the circulation of animals and humans suffering from TSEs.

Here, the aim was to carry out the first set of experiments in order to investigate circulating miRNA alterations in symptomatic, naturally infected cases of classical scrapie. Selected candidate miRNAs for the study (Table S1, available in the online Supplementary Material) were based on published data on miRNA alterations in the CNS of mouse models of prion disease [14, 15, 17–19] and prion-infected neuronal cells [20] as well as on very limited data on human sCJD patients [16, 17]. Breed Rasa Aragonesa of genotype ARQ/ARQ (homozygous for polymorphisms at codons 136, 154 and 171 of the *PRNP* gene) was used as it is the most

frequently found genotype in the scrapie-affected animals of this breed [21]. All classical scrapie animals were at clearly clinical stage and were derived from several regional flocks in Aragón, Spain, and the samples from healthy controls were harvested from the same region but from a flock negative for scrapie history. The mean age ($\pm$SD) of the scrapie sheep was 37.1$\pm$13.4 months and that of control sheep 30.9$\pm$4.0 months. None of the sheep was pregnant as this could possibly alter the abundance of circulating miRNAs [22]. The whole PRNP coding region was sequenced from the experimental sheep to confirm the ARQ genotype and to reveal any other polymorphisms present (Table S2). Earlier described protective polymorphisms in PRNP amino acid residues 112 and 141 [23] were not found in any of the sheep. However, dimorphic variations in codons 101, 143 and 175 were found, each in one individual sheep. Additionally, two silent polymorphisms at codons 231 and 237 were present in approximately half of the sheep.

Using methods previously described [24], we first analysed histopathological lesions [spongiosis and vacuolation, haematoxylin and eosin (HE) staining], astrogliosis [glial fibrillary acidic protein (GFAP), rabbit polyclonal antibody Z0334 by Dako] and pathological PrPSc levels (mAb L42, R-Biopharm) in the medulla oblongata (MO) and cervical spinal cord (SC) of classical scrapie and control groups (Fig. 1a–e). The PrPSc levels were measured from proteinase-K-digested sections to remove any non-pathological PrPC. The pathology in each case was quantified as an average score from mild to severe (1–5) in five nuclei of MO or three regions of SC. Both MO and SC in classical scrapie sheep were found to be affected to a similar extent for spongiosis (Fig. 1a), astrogliosis (Fig. 1c) and PrPSc levels (Fig. 1d), whereas vacuolation in MO was significantly higher than in SC (Fig. 1b, P=0.001). Vacuolation or PrPSc expression was not observed in any of the control sheep. However, spongiosis and GFAP expression were detected at low levels in MO and SC of controls, and in each case, the levels were significantly lower than those in MO or SC from classical scrapie sheep (P<0.0001). These results demonstrate that scrapie pathology was detected consistently in the two CNS regions of symptomatic classical scrapie sheep but not in control animals.

Next, blood plasma samples from healthy (n=10) and classical scrapie (n=11) sheep were analysed for the expression of eight candidate miRNAs (let-7b-5p, let-7d-5p, miR-128-3p, miR-132-3p, miR-146a-5p, miR-21-5p, miR-342-3p and miR-342-5p) (Table S1) that were shown earlier to be altered in the brains of intracraneally inoculated mice and other models of prion disease [14–19]. One plasma sample from each individual sheep was used, i.e. the number of samples reflects the number of experimental sheep. Briefly, blood was collected to EDTA tubes, plasma was separated by centrifugation at 1300 *g* for 10 min at 4 °C and samples were frozen at −80 °C within 1 h of blood collection. Total RNA was extracted from 0.2 ml plasma using Norgen Total RNA Purification kit (Norgen Biotek) modified for plasma
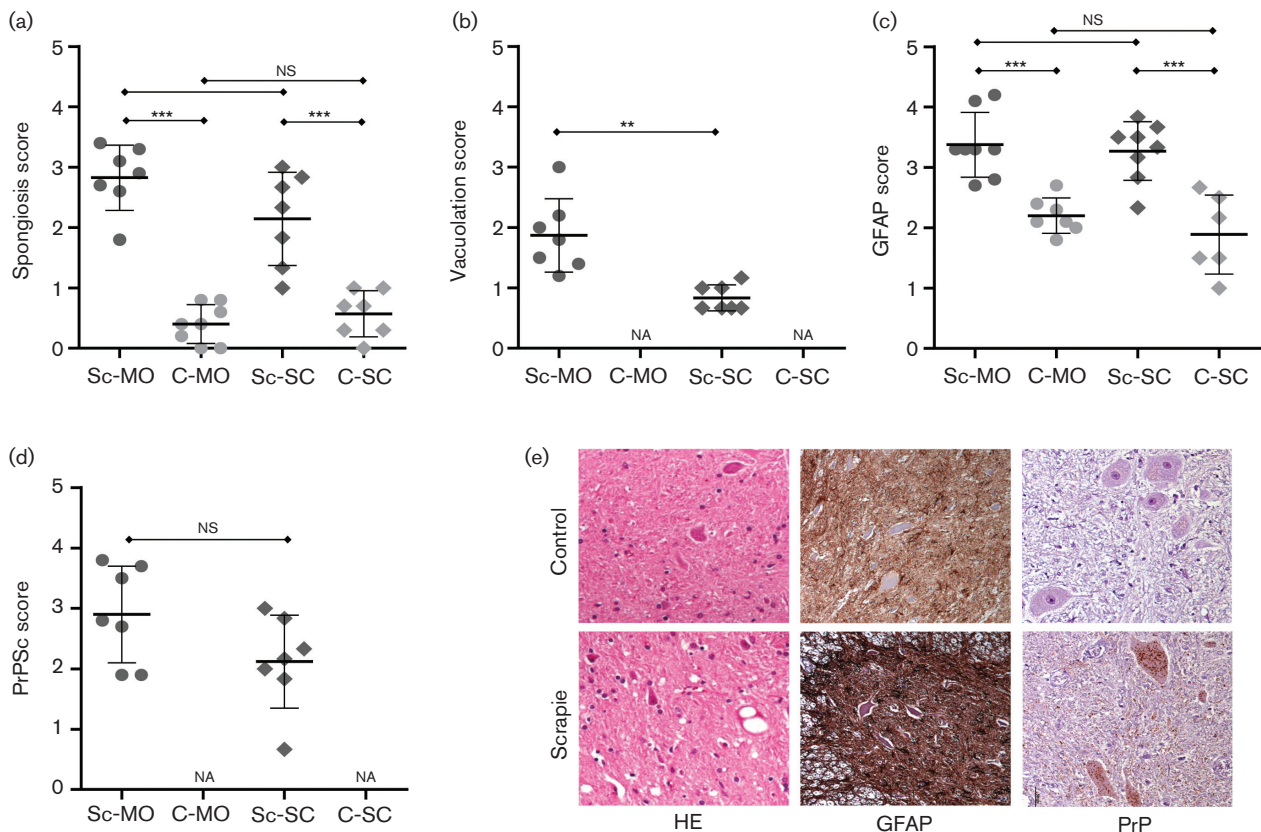
Fig. 1. Scrapie-associated histopathology in the CNS. (a–d) Histopathological scores (1–5) in MO (circles) and SC (diamonds) of scrapie (Sc, dark grey) and control (C, light grey) sheep. Statistical significance was assessed by one-way ANOVA (*$P<0.05$, **$P<0.01$, ***$P<0.001$). (a) Spongiosis, detected by HE staining, was increased in MO and SC of scrapie sheep with no significant differences between the two CNS regions within scrapie or control groups. (b) Vacuolation (HE staining) was observed in all scrapie animals but not in controls and was more pronounced in MO compared with SC in scrapie sheep. (c) Astrogliosis, as measured by immunohisto-chemical quantification of GFAP, was increased in MO and SC of scrapie sheep, but there were no significant differences between the two CNS regions within scrapie or control groups. (d) PrPSc, as measured by immunohistochemical quantification of pathological, pro-teinase-K-resistant form of PrP, was observed in all scrapie animals but not in controls, and there were no significant differences between MO and SC. (e) Representative images demonstrating the presence of scrapie-associated pathology. HE staining for spongio-sis and vacuolation and immunohistochemistry for astrogliosis marker (GFAP) and proteinase-K-resistant PrP (PrP) are shown for one control (upper row) and one scrapie animal (lower row).

samples, as instructed in the kit. After inactivation of nucle-ases, samples were spiked with 25 fmol synthetic RNA nor-malizer cel-miR-39 (Qiagen), and 0.7 µl MS2 RNA (Roche) was added as a carrier. Samples were eluted in 100 µl nucle-ase-free water, from which 9 µl was used as a template to prepare cDNA for each miRNA under study using stem–loop primers from TaqMan MicroRNA Assays (Life Tech-nologies) combined with TaqMan MicroRNA Reverse Transcription kit (Life Technologies). cDNAs were sub-jected to quantitative PCR in three technical replicate reac-tions consisting of 2.5 µl 2× TaqMan Fast Universal PCR Master Mix, no AmpErase UNG (Life Technologies), 0.25 µl TaqMan miRNA probe (Life Technologies) and 2.25 µl of cDNA diluted 1:7 in nuclease-free water. Cycling condi-tions were 95 °C for 20 s followed by 40 cycles of 95 °C for 1 s and 60 °C for 20 s. Sample/probe combinations where C_t values exceeded 32 cycles were omitted. For each sample,

the mean C_t of technical replicates for each miRNA was normalized using the mean C_t of spiked-in cel-miR-39. Rel-ative miRNA expression was determined with the $2^{-\Delta\Delta C_t}$ method [25] using healthy control sheep as calibrators. The statistics were analysed using the Student's (equal variances) or Welch's (unequal variances) *t*-test for normally distrib-uted data and the Mann–Whitney *U* test when the test for normality failed. See detailed protocols in Supplementary Methods.

Tree of the studied miRNAs were significantly increased in the circulation of classical scrapie sheep (Fig. 2a). miRNA miR-342-3p was 2.3-fold increased ($P=0.022$, Welch's test), miR-21-5p was 3.3-fold increased ($P=0.007$, Mann–Whit-ney test) and let-7b showed 4.0-fold increase in classical scrapie sheep ($P=0.008$, Mann–Whitney test). In the latter, however, the let-7b levels were sufficiently high for reliable quantification ($C_t <32$) only in 4 out of 12 controls and 11
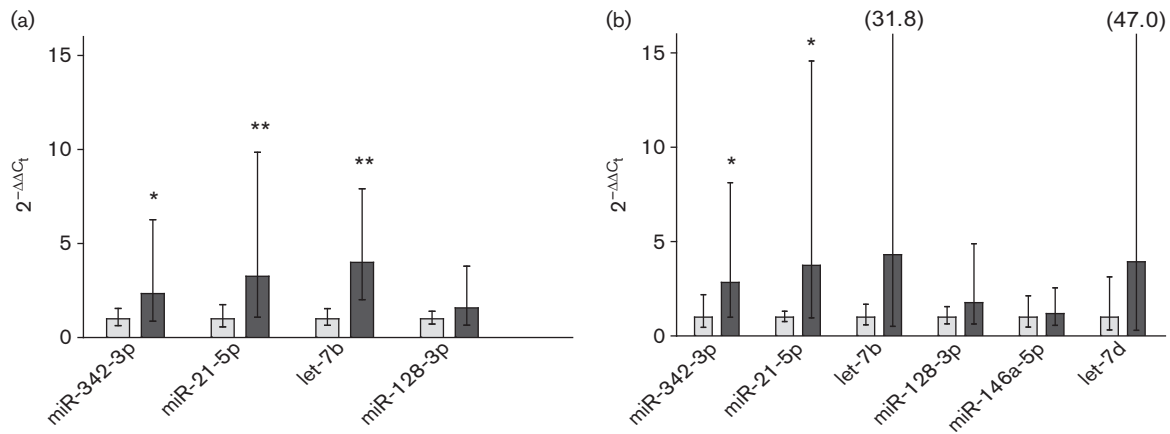
Fig. 2. Circulating plasma miRNA alterations in scrapie sheep. Relative expression of miRNAs in blood plasma of scrapie sheep (dark-grey bars) compared with control sheep (light-grey bars) as measured by quantitative RT-PCR. Each plasma sample was normalized (ΔC_t) using synthetic, spiked-in cel-miR-39, and the mean ΔC_t of the controls was used as a calibrator ($\Delta\Delta C_t$). (a) First experiment carried out from 0.2 ml plasma. Four miRNAs were found at detectable levels. Significantly increased levels were found for miR-342-3p (P=0.022), miR-21-5p (P=0.007) and let-7b (P=0.008). (b) Repeat experiment carried out from 1.5 ml plasma. Six miRNAs were found at detectable levels. Significantly increased levels were found for miR-342-3p (P=0.041) and miR-21-5p (P=0.031). Additionally, miRNAs of the let-7 family showed a tendency towards increase, but their levels were extremely variable in the scrapie group. Note: for clarity, the value of positive error for let-7b and let-7d is shown in brackets above the bar. The data in both graphs are expressed as a relative expression value after $2^{-\Delta\Delta C_t}$ conversion. The error bars represent ±SD of ΔC_t values after $2^{-\Delta\Delta C_t}$ conversion, expressed as positive and negative errors. Statistical significance was assessed by Student's *t*-test (equal variances) or Welch's *t*-test (unequal variances) for normally distributed data and the Mann–Whitney *U* test when the test for normality failed (*P<0.05, **P<0.01).

out of 12 scrapie samples. Additionally, let-7d was detected only in 1 out of 12 control samples (preventing relative quantification), whereas it was detected in 7 out of 12 scrapie samples (data not shown). miRNA miR-128a-3p was not affected and three miRNAs (miR-132-3p, miR-146a and miR-342-5p) were not found at quantifiable levels in any of the plasma samples. In an attempt to increase the number of reliably detected circulating miRNAs, the experiment was repeated (*n*=8 for each group) with increased volume (1.5 ml) of plasma as a starting material using Norgen Plasma/Serum RNA Purification Midi kit (Norgen Biotek). In this experiment (Fig. 2b), increased circulating levels of miR-342-3p (2.8-fold, *P*=0.041, Student's *t*-test) and miR-21-5p (3.8-fold, *P*=0.031, Welch's test) in scrapie sheep were confirmed. Additionally, let-7b was now detectable in all RNA samples studied. Although there was a tendency towards let-7b upregulation in scrapie sheep (3.9-fold increase, similar to the previous experiment), this was not significant (*P*=0.205, Welch's test). miRNAs miR-128a-3p, miR-146a and let-7d were all found at quantifiable levels but were not significantly altered in scrapie sheep. The descriptive statistics for the two quantitative reverse transcription PCR (RT-PCR) experiments in plasma are shown in Tables S3 and S4.

The two CNS regions studied here displayed clear scrapie pathology, including the presence of PrPSc. Interestingly, however, we did not find significant alterations in any of the studied candidate miRNAs in the CNS of clinical scrapie sheep (Fig. S1), although miR-21-5p displayed a close-to-significant increase in the scrapie sheep compared with healthy controls of the same PRNP genotype (*P*=0.056,

Student's *t*-test). This may indicate true differences with naturally infected sheep versus experimentally infected mice and *in vitro* models. However, it is equally possible that the discrepancy between earlier studies reflects larger variability in naturally infected (non-inoculated) animals. Under experimental inoculation, most animals reach symptomatic stage within a narrow interval depending on the prion strain and dose used. In natural scrapie sheep, however, the incubation period may vary substantially. Additionally, increase or decrease in a specific set of miRNAs may be observed depending on the disease stage in mouse CNS [18, 19]. Due to this dynamic nature of miRNA levels, greater variability in time between infection and clinical disease in natural scrapie cases may mask some differences observed in laboratory models. Lastly, natural scrapie in the affected flocks spreads most likely by oral transmission, and the route of infection (oral, peripheral or intracerebral) is known to influence neuropathological phenotype [26, 27]. In this respect, it would be important to compare the miRNA profiles of natural scrapie cases (such as those used in here) with those of experimentally infected scrapie sheep in the future.

Experiments on neuronal cells suggest that release of miRNAs is altered upon prion infection [20]. If this were to occur *in vivo*, miRNAs could serve as easily accessible biofluid-derived markers of TSEs. However, earlier studies have not explored this possibility. Here, miR-342-3p and miR-21-5p were found to be repeatedly increased in plasma of the natural scrapie sheep, providing the first demonstration of circulating miRNA alterations in any TSE. This

suggests that altered circulating miRNAs may be feasible candidates for TSE biomarkers. Genomic-wide expression studies are required to systematically investigate which miRNAs, if any, show required specificity and sensitivity to serve as tools for TSE detection, especially in the preclinical phase. Interestingly, miR-342-3p and miR-21-5p, as well as let-7b, which were found here to be elevated only in one of the repeat experiments, are also increased in exosomes released *in vitro* by prion-infected neuronal cells [20]. Future studies should address the possible location of these miRNAs in plasma exosomes *in vivo*. In this line of work, close-to-significant increase for miR-21-5p found in the CNS of classical scrapie sheep (Fig. S1) may suggest that elevated plasma levels of this miRNA are a consequence of increased expression and release from the CNS. miRNA-21 has not been previously associated with neurodegenerative disorders but is known to be upregulated and protective in traumatic brain injury [28], as well as a key mediator of the anti-inflammatory response [29].

The elucidation of true depth of miRNA alterations in TSEs, like in many other neurodegenerative diseases, is complicated by yet ill-defined temporal changes in different disease stages. In this light, it remains possible that miRNA alterations found in clinical sheep are not identical to those observed in preclinical stage. To provide useful alternatives for current diagnostic methodology for early scrapie eradication, the next essential step is to conduct similar experiments using preclinical sheep. In addition to classical scrapie, potential miRNA alterations should also be addressed in atypical scrapie sheep. Investigation of circulating miRNAs as TSE biomarkers should preferably also employ complementary models. Parallel experiments with scrapie-inoculated sheep and mouse TSE models should reveal common and model-specific miRNA networks, which could give further indications for their use in translational medicine.

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