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Nanopore-based detection of circulating microRNAs in lung cancer patients

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Abstract

MicroRNAs are short RNA molecules that regulate gene expression. They have been investigated as potential biomarkers because their expression levels are correlated with various diseases. However, the detection of microRNAs in the bloodstream remains difficult because current methods are not sufficiently selective or sensitive. Here, we show that a nanopore sensor based on the alpha-hemolysin protein selectively detected microRNAs at the single molecular level in plasma samples from lung cancer patients without the need for labelling or amplification. The sensor, which used a programmable oligonucleotide probe to generate a target-specific signature signal, was able to quantify sub-picomolar levels of cancer-associated microRNAs and to discriminate single nucleotide differences between microRNA family members. This approach could prove useful for quantitative microRNA detection, biomarker discovery, and the non-invasive early diagnosis of cancer.

MicroRNAs are a class of short (~18–22 nucleotides) non-coding RNAs that are important in development and cell differentiation and in the regulation of the cell cycle, apoptosis and signalling pathways^{1,2}. Since the initial discovery in *Caenorhabditis elegans* in 1993³, over 17,000 microRNAs have been identified across different species including human⁴. In the cytoplasm, mature microRNAs are associated with a RNA-induced silencing protein

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Author contributions.

Y.W. designed and performed the nanopore experiments, collected and analysed the nanopore data and co-wrote the manuscript.

D.Z. designed and performed the RT-PCR experiments, analysed the RT-PCR data and co-wrote the manuscript.

Q.T. performed molecular biology experiments including protein synthesis.

M.W. conceived the qRT-PCR experiments, provided the patients' samples and co-wrote the manuscript.

L.Q.G. conceived the principal idea, designed the nanopore experiments and wrote the manuscript.

All the authors discussed the results and commented on the manuscript.

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complex (RISC) to bind with the 3' untranslated region (3'-UTR) of target messenger RNAs^{1,5,6}. By either repressing translation or cleaving the target messenger RNA^{1,5,6}, these microRNAs regulate approximately 30% of human gene expression⁵ at the post-transcriptional level. Aberrant expression of microRNAs has been found in all types of tumours^{7,8}, and different types of cancers exhibit distinct microRNAs profiles⁹. Interestingly, microRNAs can be released from the primary tumour into the bloodstream in a stable form¹⁰. Circulating microRNAs are enveloped inside exosomal vesicles and are transferable to and functional in recipient cells^{11–13}. Therefore, the detection of tumour-specific circulating microRNAs is useful for the early diagnosis, staging and monitoring of cancer^{7,8,10–13}.

Reverse transcription real-time polymerase chain reaction (qRT-PCR) assays and microarrays have been developed for the detection of microRNA. However, these methods suffer from error-prone amplification, cross-hybridisation, and a lack of valid internal controls^{14,15} because the shortness of the microRNA sequences makes it difficult to design probes and primers. Other techniques based on colourimetry, bioluminescence, enzyme turnover and electrochemistry have been proposed; nanoparticles, molecular beacons, deep sequencing^{16,17} and single-molecule fluorescence¹⁸ have also been applied to microRNA detection with enhanced sensitivity and/or selectivity (for reviews see references 16–17), but these methods either need improvements in versatility or require expensive instruments.

The nanopore is a molecular-scale pore structure that is able to detect with great sensitivity for the position and conformation of a single molecule that is present in the pore lumen¹⁹. From the characteristic change in the nanopore conductance, one can electrically elucidate single-molecule kinetic pathways and quantify the target. Various nanopore sensors are being developed with broad biotechnological applications^{19–24} (for reviews see references 19–23), including the next generation of DNA sequencing technology^{25,26}. The development of nanopore-based microRNA detectors is a novel effort in this rapidly evolving field, and Wanunu et al first reported utilising a 3-nm synthetic pore to quantify the translocation of enriched microRNAs that were hybridised to the probe²⁷. In this report, we construct a robust protein nanopore-based sensor that enables the sensitive, selective and direct quantification of cancer-associated microRNAs in the blood by discriminating single nucleotide differences in microRNA family members.

Generation of microRNA signatures in the nanopore

We employed the α -haemolysin protein pore, a toxin from *Staphylococcus aureus* bacterium²⁸, as the sensor element. The translocation of single-stranded oligonucleotides through this 2-nm pore has been studied extensively^{29–32}. However, it is difficult to distinguish the translocation of different microRNAs because the sequences of all microRNAs are short and similar in length. One way to overcome this challenge is the use of a signature that can discriminate the target microRNA in the mixture. We identified such a microRNA signature signal in the nanopore using an oligonucleotide probe.

The probe structure is shown in Fig. 1a. The capture domain of the probe was used to bind the target microRNA by Watson-Crick base pairing in the solution. Each end (3' and 5') of

the capture domain was extended with a poly(dC)₃₀ signal tag. Our first target was *miR-155*, a lung cancer associated microRNA^{12,13,33}. Fig. 1b illustrates a sequence of nanopore current events in the presence of *miR-155* and its probe, *P₁₅₅*, on the *cis* side of the pore. The boxed events represent a characteristic type of multi-level block that was generated by the *miR-155*•*P₁₅₅* hybrid. This block type was not observed in the presence of *miR-155* or *P₁₅₅* alone in the *cis* solution. In a multi-level block (Fig. 1c), the Level 1 state lasted for 250±58 ms, which is almost equal to the entire event duration, and greatly reduced the nanopore current, with a relative residual conductance (g/g_0) of 0.15. The Level 1 state was followed by a discrete current increase to the Level 2 state, which persisted for 410±20 μ s, with a g/g_0 of 0.42. Finally, the current discretely dropped to the Level 3 state and remained there briefly for 270±30 μ s before returning to the full open base level. Similar to Level 1, Level 3 almost fully reduced the pore current, with a g/g_0 of 0.08. The right panel in Fig. 1c depicts the molecular configurations that corresponded to the multi-level block. The amplitude and duration of Level 1 were consistent with a configuration in which the *miR-155*•*P₁₅₅* complex was trapped in the nanopore at the 2.6-nm *cis* opening, with either the 3' or 5' signal tag of *P₁₅₅* occupying the 1.6- to 2.0-nm β -barrel. Driven by the transmembrane voltage, the signal tag in the β -barrel induced the dissociation of *miR-155*•*P₁₅₅*. The dissociation time (the duration of Level 1) was comparable to previously reported time scales for DNA unzipping in the pore^{34,35}. After unzipping, *P₁₅₅* left the pore through the narrower (*trans*) opening, and the Level 1 state was terminated. The Level 2 state featured a large residue conductance, which should correspond to a configuration in which *miR-155* unzipped from *miR-155*•*P₁₅₅* temporarily resided in the nanocavity. This result is consistent with earlier reports that an oligonucleotide trapped in the nanocavity can generate partial blocks³⁶. The *miR-155* in the nanocavity finally passed through the β -barrel to yield the short-lived Level 3 state. The duration of Level 3 (270 μ s) was consistent with the translocation duration of *miR-155* alone (220 μ s) and the time scale for DNA or RNA translocations in previous studies^{29–32}. The molecular mechanism described above was further evidenced by the voltage-dependent durations of Levels 1 and 3. Level 1 was shortened by 23-fold to 11 ms and Level 3 by 2-fold to 150 μ s as the voltage increased from +100 mV to +180 mV (Fig. 1c, lower panel), which indicated that the voltage both enhanced the unzipping of *miR-155*•*P₁₅₅* and accelerated the translocation of *miR-155*³⁰. After the electrical recording, we quantified *miR-155* in the *trans* solution using qRT-PCR (Fig. 1d and Supplementary Information S3). For 0.5, 1 and 10 nM of *cis* *miR-155* (1 μ M of *P₁₅₅*), the corresponding *trans* *miR-155* was 14, 34 and 63 aM (10⁻¹⁸ M) respectively. The *trans* *miR-155* identification verifies that the microRNA unzipped from a microRNA•probe hybrid indeed translocated to the *trans* side of the pore.

The *miR-155*•*P₁₅₅* hybrid also produced single-level long blocks that had similar conductances ($g/g_0=0.15$) to Level 1 of the multi-level block (Fig. 1e). The single-level block was formed by the trapped *miR-155*•*P₁₅₅* complex that returned to the *cis* solution without unzipping and translocation. The frequency ratio of multi-level to single-level events monotonically increased with the voltage, from 0.42 at +100 mV to 1.4 at +180 mV (Table S2). This voltage-dependent frequency variation was consistent with the expectation that higher voltage increases the unzipping probability of *miR-155*•*P₁₅₅* and decreases its escaping probability. In addition to the characteristic long events, we also observed spike-

like short blocks in the same recordings (Fig. 1f). Both their duration ($220 \pm 21 \mu\text{s}$) and conductance ($g/g_0 = 0.16$) were similar to the translocation of unhybridised *miR-155* or *P₁₅₅* present in the *cis* solution (Fig. S1).

The above analysis indicated two important functions performed by the signal tag of the probe: the guidance of the microRNA•probe complex entrapment in the pore and the inducement of the dissociation of the microRNA•probe complex. The configuration change during the unzipping process gave rise to the signature current patterns, which enabled the recognition of single target microRNA molecules. Because of the probe's specificity, the frequency of the signature signal (f_{sig}) was independent of the presence of multiple nucleic acid components (Fig. S3) and could, therefore, be used to quantify the target microRNA in the mixture. Overall, the signature signal ensured the high selectivity that is required for microRNA detection in plasma RNA extract.

Quantification of microRNA using probes with optimised sequences

The frequency of signature events can be used to quantify microRNA by the equation $f_{sig} = k_{on} [miR]_0$, where $[miR]_0$ is the microRNA concentration and k_{on} is the occurrence rate constant of signature events (Supplementary Information S2). k_{on} is a key determinant of quantification sensitivity and can be vastly improved by the optimisation of the probe structure. Fig. 2a shows that the probe without the signal tag (*P_{nt}*) gave the lowest k_{on} at $2.8 \pm 0.6 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$. k_{on} tripled to $6.8 \pm 1.3 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ when the probe was attached to a poly(dC)₃₀ tag at the 5' end (*P_{5'-C30}*). However, k_{on} was vastly increased by 50-fold to $1.4 \pm 0.3 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ when the poly(dC)₃₀ tag was attached to the 3' end (*P_{3'-C30}*). This orientation discrimination of single-stranded oligonucleotides in the nanopore is consistent with previous studies^{37,38}. As expected, the *P₁₅₅* probe that contained both 3' and 5' poly(dC)₃₀ achieved the highest k_{on} at $2.0 \pm 0.2 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$. In addition to the tag directionality, k_{on} is also dependent on the tag length. For example, the poly(dC)₃₀ tag showed much higher efficiency in the generation of signature events than a shorter tag, such as poly(dC)₈, and was more efficient than poly(dA)₃₀ and poly(dT)₃₀ tags (unpublished data).

Using *P₁₅₅* as the probe, we verified that the frequency of the signature event was proportional to the *miR-155* concentration range from 10 to 100 nM (Fig. 2b, left panel). This correlation was measured at +100 mV in 1 M KCl. The frequencies in any two *miR-155* concentrations, such as 10 and 25 nM, were significantly separated ($p < 0.005$). Wanunu et al. have reported that a gradient of salt concentration across a synthetic nanopore vastly increases the capture rate of dsDNA³⁹. Similarly, the use of asymmetrical KCl solutions (0.2 M/3 M, *cis/trans*) allowed for the measurement of the frequency of microRNA events at far lower concentrations, from 0.1 to 100 pM (Fig. 2b, right panel). In addition to the use of asymmetric solutions, the application of high voltage (Fig. S2) and the use of an engineered nanopore⁴⁰ also proved effective in increasing the event frequency for high sensitivity.

Discrimination of single nucleotide differences between microRNAs

Among the over 1,400 human microRNAs that have been identified, many members of the same microRNA family possess similar sequences or single nucleotide polymorphisms

(SNPs). The SNPs are associated with significant biological properties of cancers, such as susceptibility, prognosis, and the response to therapeutic agents⁴¹. However, sequence-similar microRNAs or SNPs are difficult to distinguish using current PCR or hybridisation-based methods^{14–16}. Because dsDNAs that contain a single nucleotide mismatch are identifiable in the nanopore based on their unzipping time^{35,42–44}, we decided to study whether the nanopore could discriminate single nucleotide differences in microRNA family members.

We selected the *Let-7* tumour-suppressing microRNA family^{7–9} as the target. *Let-7* is down-regulated in lung cancer and, therefore, is useful as a biomarker and potential therapeutic agent⁴⁵. *Let-7a* and *-7b* have two nucleotide differences (see sequences in Table S1), and their probes were P_a and P_b , respectively. The hybrids *let-7a*• P_a and *let-7b*• P_b were full-matched, but *let-7b*• P_a and *let-7a*• P_b contained 2 mismatches. Fig. 3a shows that the unzipping time, τ_{sig} , at +120 mV decreased 3.2-fold from *let-7a*• P_a (155 ± 28 ms) to *let-7b*• P_a (48 ± 11 ms) ($p < 0.005$) and 6.9-fold from *let-7b*• P_b (165 ± 47 ms) to *let-7a*• P_b (24 ± 2 ms) ($p < 0.005$) (Fig. 3a). Similarly, *Let-7a* and *-7c* only have one nucleotide difference. Fig. 3b shows that when using P_a and P_c to target *Let-7a* and *-7c*, respectively, at +100 mV, τ_{sig} decreased 2.4-fold from *let-7a*• P_a (303 ± 45 ms) to *let-7c*• P_a (124 ± 39 ms) ($p < 0.005$) and 2.0-fold from *let-7c*• P_c (342 ± 49 ms) to *let-7a*• P_c (179 ± 38 ms) ($p < 0.005$).

We constructed receiver operating characteristic (ROC) curves to compare durations of events for fully-matched microRNA•probe hybrids and the microRNA•probe hybrids that containing mismatches (Fig. 3c). Based on ROC curves, we measured the area under the ROC curve (AUC, Fig. 3d), which is an indicator of the ability to discriminate nucleotide differences between microRNAs. The AUCs varied between 0.72 and 0.83, and increased with the ratio of event duration between fully-matched hybrids and mismatches. This duration ratio-dependent AUC was further verified through a simulation method (Fig. 3d, Fig. S5 and Table S6). The ROC analysis strongly suggested a power of the nanopore in discrimination of microRNA SNP based on the duration of signature events. This discriminatory ability was tested in a mixture of 100 nM *Let-7a* and *Let-7b* and probed with P_a . Based on the AUCs, the optimal cut-point (OCP) duration was calculated to be ~190 ms, which was the threshold duration that provided the best discriminatory ability (Table S6). Block events that were longer than the OCP occurred for *Let-7a*• P_a , and events that were shorter than the OCP occurred for *Let-7c*• P_a . The numbers of events were transformed into the concentrations of *Let-7a* and *Let-7c*, which were 85 nM and 120 nM, respectively. Both concentrations varied 15–20% from the real concentration (100 nM).

Detection of microRNA levels in lung cancer patients

Lung cancer is the leading cause of cancer mortality in men and women worldwide and is responsible for approximately 1.2 million deaths each year⁴⁶. Because there is no effective screening procedure available, more than 70% of patients are diagnosed at advanced stages, with a 5-year survival rate of less than 15%⁴⁶. Currently over one hundred microRNAs have been identified to be dysregulated in lung cancer^{7–13,17,33,41}. Notably, high levels of *miR-155* and low levels of *let-7a-2* correlate with significantly poor prognoses and shorter survival times in lung cancer patients^{47,48}.

We detected plasma *miR-155* in lung cancer patients using the nanopore sensor. Peripheral blood samples were obtained from six lung cancer patients and six healthy volunteers after local IRB approval. Total plasma RNAs, which contained microRNAs, were extracted from 350 μ l of each plasma sample using a mirVana PARIS Kit (Ambion, Austin, TX), with a final elution volume of 100 μ l. The elution volumes were then divided into two 50- μ l aliquots for the nanopore and RT-PCR assays, respectively. One aliquot was mixed in the recording solution with or without the P_{155} probe. The nanopore current retained a low level of noise even in the presence of plasma extracts. In the absence of P_{155} , only short blocks were observed as a translocation of single-stranded oligonucleotides, such as free microRNAs (Figs. 4a and b). In the presence of the probe, we identified distinct short and long blocks in both the control (Fig. 4c) and lung cancer groups (Fig. 4d). The characteristic long blocks shared the same current profile and properties with the synthetic *miR-155* RNA (Fig. 1). More frequent short events were also observed in the presence of P_{155} (Figs. 4c and d) than that without P_{155} (Figs. 4a and b). The majority of these short events may be contributed by the P_{155} probe, since its translocation rate was 8.4 times higher than that of the microRNA (Fig. S6). Overall, the characteristic long blocks could be attributed to *miR-155*• P_{155} hybrids and served as signatures for the identification of single molecules of *miR-155*.

To normalize the assay, the frequencies of *miR-155* signature events (f_{155}) for all the samples of the lung cancer and control groups were measured in the presence of a spiked-in synthetic *C. elegans* microRNA *miR-39*. Fig. 4e shows that all of the f_{155} values in the lung cancer group were higher than those in the control group ($p < 0.001$). However, Fig. 4f indicates that the frequencies of spiked-in *miR-39* events (f_{39}) were independent of the samples ($p > 0.05$). In order to evaluate the variability during sample preparation, the spiked-in *miR-39* was used as an internal control and the ratio f_{155}/f_{39} was used to normalise the assays. Fig. 4g shows that the mean f_{155}/f_{39} in the lung cancer group (0.62 ± 0.06) was significantly higher than the f_{155}/f_{39} in the control group (0.24 ± 0.05) ($p < 0.001$). The relative levels of *miR-155* that were measured with the nanopore were also compared using the qRT-PCR method (Fig. 4h). Collectively, the mean level of *miR-155* was increased 2.6-fold in the lung cancer group compared to the control group as measured by the nanopore sensor ($p < 0.001$), but a 4.3-fold increase was obtained using the qRT-PCR method ($p < 0.02$). In addition, a greater variability was observed in the qRT-PCR assay. Therefore, although both the nanopore and qRT-PCR assays indicated a significant elevation of *miR-155* in lung cancer patient samples, the nanopore method demonstrated higher accuracy with no requirement for labelling or amplification (Fig. 4h).

In conclusion, we have designed a nanopore-based microRNA sensor that utilizes an oligonucleotide probe to generate a signature electrical signal for the direct and label-free detection of the target microRNA in a fluctuating background, such as a plasma RNA extract. The key component of the nanopore sensor is the probe, whose sequence is programmable and can be optimized to achieve high sensitivity and selectivity. We expect that the probe composition is not limited to the four nucleotides. The incorporation of unnatural compounds such as locked nucleic acids (LNA) and peptide nucleotide acids (PNA) into the probe sequences may enhance the selectivity due to the strengthened

hybridisation between the probe and the target. The probe can also be engineered with a specific barcode through chemical modification^{31,49}, such that multiple microRNAs can be simultaneously detected using distinct probes. Regarding the target, this versatile sensor can not only detect microRNAs, but various nucleic acids fragments including genetic alternations and pathogenic DNAs/RNAs for fighting infectious diseases. Compared with the synthetic nanopore-based microRNA detection reported recently²⁷, the protein pore sensor needs improvement in the membrane stability and high throughput capability. We think new lipid bilayer platforms (exemplified in reference⁵⁰) might be useful in constructing stable and manipulable nanopore arrays for microRNA profile detection. Overall, the nanopore method could prove a useful tool for quantitative studies of microRNAs and the discovery of disease markers. Once the microRNA markers are established, this approach will have the potential for the noninvasive screening and early diagnosis of diseases, such as cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Capturing single microRNA molecules in the nanopore. **a**, Molecular diagram of a microRNA (red) bound to a probe (green) bearing signal tags on each end. **b**, A sequence of nanopore current blocks in the presence of 100 nM *miR-155* and 100 nM *P₁₅₅* in the *cis* solution. Traces were recorded at +100 mV in solutions containing 1 M KCl buffered with 10 mM Tris (pH 8.0). Red boxes represent the multi-level current pattern. **c**, Multi-level long block (from b) at +100 mV generated by the *miR-155*•*P₁₅₅* hybrid. Right panel: diagram showing the mechanism of translocation. Level 1 indicates the trapping of the microRNA•probe hybrid in the pore, the unzipping of the microRNA from the probe and the translocation of the probe through the pore; Level 2, the unzipped microRNA residing in the nanocavity of the pore; Level 3, the translocation of the unzipped microRNA through the pore. Lower panel: multi-level blocks at +150 mV and +180 mV. Increasing the voltage reduced the duration of Level 1 and Level 3, which supports the above mechanistic model. **d**, *miR-155* levels detected by qRT-PCR in *trans* solutions. Before PCR detection, the pore current has been monitored in the presence of 1 μ M *P₁₅₅* and 0.5, 1 or 10 nM of *miR-155* in the *cis* solution, respectively. The asymmetric 0.5 M/3M (*cis/trans*) KCl solutions and high

voltage at +180 mV increased the frequency of signature events. The much higher probe concentration than microRNA was to enhance their hybridisation in the *cis* solution. The method is detailed in Supplementary Information S3 and S4. **e**, A single-level block (from **b**) generated by a trapped *mir-155*•*P₁₅₅* hybrid that exited the pore from the *cis* entrance without unzipping. **f**, A spike-like short block generated by the translocation of unhybridised *miR-155* or *P₁₅₅* from the *cis* solution.

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Figure 2.

Enhancing the detection sensitivity by optimising the probe sequence. **a.** Left panel, current traces showing the frequency of signature events for *miR-155* hybridised to the probes $P_{5'-C30}$ (top), $P_{3'-C30}$ (middle) and P_{155} (bottom), monitored at +100 mV in 1 M KCl. Right panel, the occurrence rate constant of the signature events for *miR-155* detection with different probes (Table S3). The results using any two probes were statistically significant ($p < 0.005$); **b.** Left panel, the [*miR-155*] - f_{155} correlation for target concentration ranges between 10 and 100 nM in 1 M KCl. Right panel, the [*miR-155*] - f_{155} correlation measured in 0.2 M/3 M (*cis/trans*) KCl with much lower target concentrations (between 0.1 and 100

pM). Data in both left and right curves was measured at +100 mV. The detection results between any two *miR-155* concentrations were statistically significant ($p<0.01$).

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Figure 3.

The differentiation of *let-7* microRNAs containing one or two different nucleotides. The sequences of *let-7a*, *-7b*, and *-7c* are provided in Table S1. **a**, The detection of *let-7a* and *let-7b* using the probe P_a or P_b at +120 mV. Left, current traces. Right, the signature event duration. **b**, The detection of *let-7a* and *-7c* using the probe P_a or P_c at +100 mV. Left, current traces. Right, the signature event duration. A representative histogram is shown in Fig. S4, and the data are listed in Table S4. **c**, Receiver operating characteristic (ROC) curves for the discrimination of events for fully-matched microRNA•probe hybrids (defined

as positive events) and microRNA•probe hybrids containing mismatches (defined as negative events). □: $let-7a \bullet P_a / let-7b \bullet P_a$, ○: $let-7b \bullet P_b / let-7a \bullet P_b$, ■: $let-7a \bullet P_a / let-7c \bullet P_a$, and ●: $let-7c \bullet P_c / let-7a \bullet P_c$. **d**, Correlations between the areas under the ROC curves (AUCs) and the ratio of event duration for fully-matched hybrids versus mismatches. ●: AUCs measured from the ROC curves in panel **c** (Table S5), ○: AUCs calculated from ROC analyses based on simulated datasets (Fig. S5 and Table S6). The computer-generated event duration followed an exponential distribution. The ratios of event duration for the ROC analysis were 1, 2, 3, 4, 5 and 10.

Figure 4.

The detection of *miR-155* in the plasma of lung cancer patients. **a** through **d**, Current traces for total plasma RNAs from healthy volunteers and lung cancer patients without and with the P_{155} probe. The traces were recorded in 1 M KCl at +100 mV. Signature events were marked with red arrows. No signature events were observed in the absence of the probe (**a** and **b**), whereas the signature events were found in the presence of 100 nM probe for both healthy volunteers (**c**) and lung cancer patients (**d**). **e**, The frequencies of *miR-155* signature events (f_{155}) from six healthy individuals (#1 to #6) and six patients with lung cancer (#7 to #12) in the presence of spiked-in synthetic *miR-39*. **f**, The frequencies of *miR-39* signature events detected using P_{39} (sequence in Table S1) from all the samples that were used in **e**.

Each sample was measured n times ($n = 4$) using independent nanopores. The data are displayed as means $\pm$ SD. The patient conditions were the following: #7, metastatic squamous lung carcinoma; #8, recurrent small-cell cancer; #9, early-stage small-cell carcinoma, status post-chemotherapy and -radiation; #10, early-stage small-cell cancer, status post-chemotherapy; #11, late-stage non-small cell carcinoma, status post-resection and -chemotherapy; #12, late-stage adenocarcinoma, status post-chemotherapy. **g**, f_{155}/f_{39} calculated from panels e and f. **h**, Box and whiskers plots of the relative *miR-155* levels in the healthy and lung cancer groups measured with the nanopore sensor and qRT-PCR. The boxes mark the intervals between the 25th and 75th percentiles. The black lines inside the boxes denote the medians. The whiskers denote the intervals between the 5th and 95th percentiles. The filled circles indicate data points outside of the 5th and 95th percentiles. The data are provided in Table S7.