News Release

Title

Urinary MicroRNA-based Diagnostic Model for Central Nervous System Tumors Using Nanowire Scaffolds

Key Points

- **We have developed a mass-producible and sterilizable nanowire-based device which can extract urinary microRNAs efficiently in order to perform an accurate mass screening for early detection of central nervous system tumors.**
- **Analyses of glioblastoma organoid-derived micro RNAs suggested that many CNS tumor-derived microRNAs could be identified in urine directly.**
- **Diagnostic model based on expression of the selected microRNAs was able to differentiate patients and non-cancer individuals at a sensitivity and specificity of 100% and 97%, respectively.**

Summary

There are no accurate mass screening methods for early detection of central nervous system (CNS) tumors. Recently, liquid biopsy has received a lot of attention for less-invasive cancer screening. Unlike other cancers, CNS tumors require efforts to find biomarkers due to the blood-brain barrier, which restricts molecular exchange between parenchyma and blood. Additionally, because a satisfactory way to collect urinary biomarkers is lacking, urine-based liquid biopsy has not been fully investigated despite the fact that it has some advantages compared to blood or cerebrospinal fluid-based biopsy. Here, we have developed a mass-producible and sterilizable nanowire-based device which can extract urinary microRNAs efficiently. Urinary microRNAs from patients with CNS tumors (n = 119) and non-cancer individuals (n = 100) were analyzed using a microarray to yield comprehensive microRNA expression profiles. To clarify the origin of urinary microRNAs of patients with CNS tumors, glioblastoma organoids were generated. Glioblastoma organoid-derived differentially expressed microRNAs (DEMs) included 73.4% of the DEMs in urine of patients with parental tumors, but included only 3.9% of those in urine of non-cancer individuals, which suggested that many CNS tumor-derived microRNAs could be identified in urine directly. We constructed the diagnostic model based on expression of the selected microRNAs and found it was able to differentiate patients and non-cancer individuals at a sensitivity and specificity of 100% and 97%, respectively, in an independent dataset. Our findings demonstrate that urinary microRNAs extracted with the nanowire device offer a well-fitted strategy for mass screening of CNS tumors.

Research Background

Liquid biopsy using microRNAs (miRNAs) in biofluids (*e.g.* blood, cerebrospinal fluid, and urine) has received a lot of attention for early cancer detection and screening. miRNA expression profiles of cancer samples are different from those of non-cancer samples. miRNAs are secreted from various cells and exist stably in biofluids within extracellular vesicles (EVs). Several research groups have reported that miRNAs in blood could be used in clinical applications of cancer screening. Urine-based liquid biopsy has some advantages compared to blood-based liquid biopsy, such as being noninvasive, and providing easy handling and sampling. Since urine sampling can be self-performed and repeated at any location and with a minimal effort, urinary miRNAs seem to be the best biomarker candidate for cancer screening. However, urine-based liquid biopsy has not been fully investigated for patients with non-urological tumors because none of the conventional methodologies (*e.g.* ultracentrifugation and polymeric precipitation methods like ExoQuick-TC) have a satisfactory way to collect urinary miRNAs.
Here, first we have developed a sterilizable and mass-producible zinc oxide (ZnO) nanowire-based device for extracting urinary miRNAs efficiently with the long-term goal of achieving liquid biopsy using urinary miRNAs. Next, we extracted urinary miRNAs in urine samples from patients with CNS tumors and non-cancer individuals. Microarray analysis of urinary miRNAs revealed a characteristic miRNA expression pattern, and based on it, we constructed a diagnostic model of patients with CNS tumors.

Research Results

Assembly-type Microfluidic Nanowire Device.

We designed an assembly-type microfluidic nanowire device for extracting urinary miRNAs and acquiring miRNA expression profiles. We fabricated the assembly-type microfluidic nanowire device by two processes: first, we grew ZnO nanowire scaffolds from a thermally oxidized chromium layer on silicon (Si) substrate; and second, we assembled the ZnO nanowire scaffolds, cyclo-olefin polymer (COP) resin microfluidic substrate, COP resin substrate, two stainless steel holders, and polyether ether ketone (PEEK) tubes into the device (Figure 1A). The device was connected to PEEK tubes for introduction of urine and lysis buffer and collection of flow-through urine and miRNA-containing solution. Since no bonding process was required, each component of the assembly-type microfluidic nanowire device could be sterilized to prevent contamination by miRNAs in saliva and sweat of persons handling the device. Furthermore, by simplifying the fabricating processes, fabrication time was shortened and the device could be mass produced.

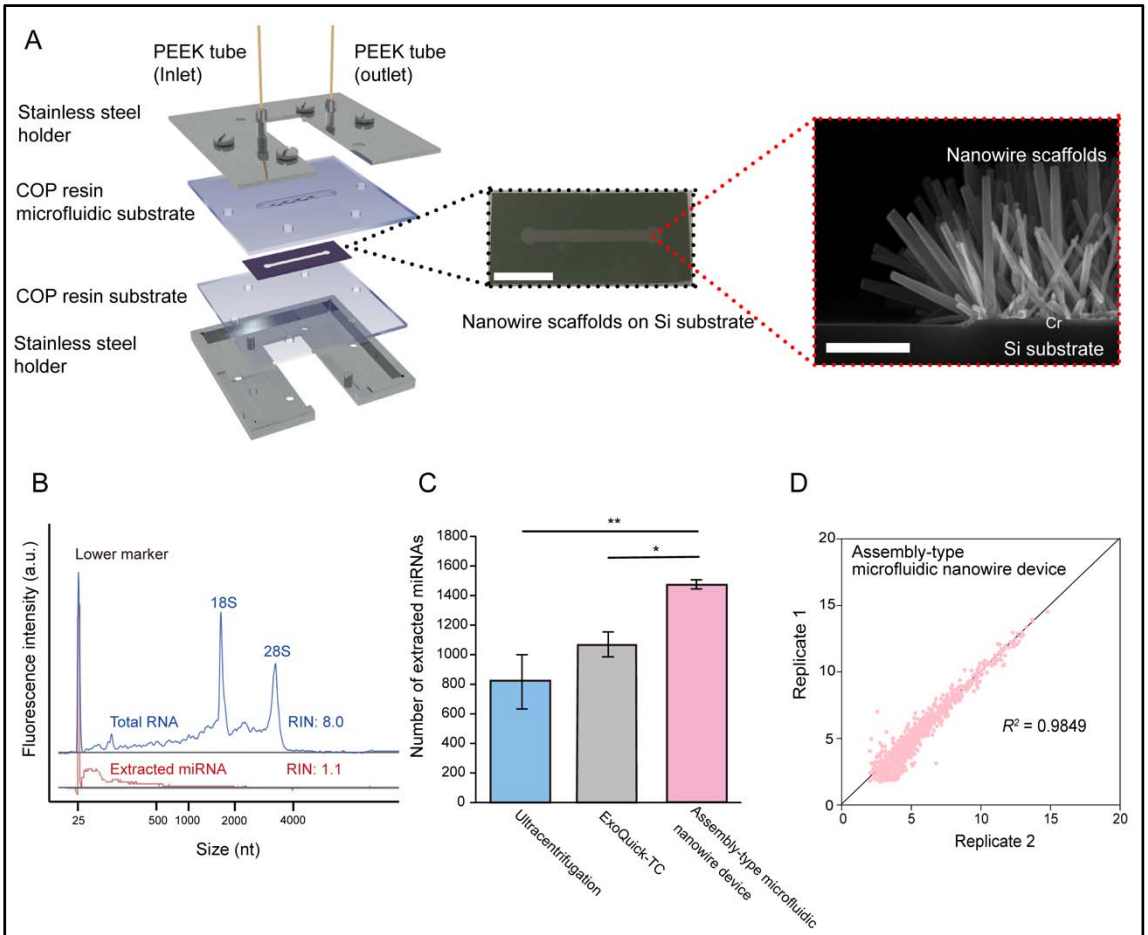

Figure 1. The assembly-type microfluidic nanowire device. (A) Schematic illustration of the assembly-type microfluidic nanowire device (left panel); photograph of the ZnO nanowire scaffolds on Si substrate (middle panel; scale bar, 1cm), and vertical cross-sectional field-emission scanning electron microscopy image of nanowire scaffolds grown on Si substrate (right panel; scale bar, 1μm). (B) Electropherograms of the extracted miRNAs (red) and total RNA (blue). For the extracted miRNAs, a high peak was observed in the miRNA (25 nt) region, but not in the 18S (1,9000 nt) or 28S (4,700 nt) ribosomal RNA region, indicating high-purity miRNAs. (C) A bar graph illustrating the number of captured miRNAs in non-cancer individuals as obtained by ultracentrifugation (cyan; n = 3), ExoQuick-TC (gray; n = 3), and the assembly-type microfluidic nanowire device (pink; n = 100). Data are represented as mean ± standard error; *P < 0.05; **P < 0.01 (Wilcoxon rank sum test). (D) The scatterplot of miRNA expression levels from the microarray data indicated reproducibility of technical replicates in the assembly-type microfluidic nanowire device. The coefficient of determination (R^2) was 0.9849.

Performance of the Assembly-type Microfluidic Nanowire Device.

To investigate whether our assembly-type microfluidic nanowire device could extract urinary miRNAs efficiently, we extracted urinary miRNAs from non-cancer individuals (Figure 1B). All urinary miRNAs were analyzed using a miRNA microarray, and that yielded comprehensive miRNA expression profiles which included 2565 species of miRNAs. Compared to the ultracentrifugation method (n = 3) or ExoQuick-TC (n = 3), the assembly-type nanowire device (n = 100) showed a significantly higher number of extracted miRNAs (P = 0.01 or 0.03, respectively) in miRNA microarray analysis of non-cancer individuals (Figure 1C). In addition, to assess reproducibility of the assembly-type nanowire device, we extracted miRNAs from duplicate urinary samples and performed microarray analyses, and showed that the nanowire

device had high reproducibility of extracted miRNA species (R^2 = 0.9849) (Figure 1D). The assembly-type microfluidic nanowire device was seen to be sterilizable, to be mass-producible, to offer time savings for miRNA extraction, and to be efficient and reproducible for miRNA extraction.

Origin of Urinary miRNAs Characteristic of Patients with CNS Tumors.

To assess whether urinary miRNAs which showed significantly higher or lower expression in patients with CNS tumors were derived from the tumor itself, we established two glioblastoma (GBM) organoids (named GBOs) from patients with GBM. Since organoids are *in-vitro* 3D cell aggregates derived from primary tissue, organoid-secreted EV-encapsulated miRNAs and EV-free miRNAs could be detected in organoid culture supernatants as well as tumor cells. We extracted miRNAs from culture supernatants of two GBOs and immortalized human astrocytes (NHAs) with the nanowire device and performed miRNA microarray analyses. We also extracted urinary miRNAs from two patients for whom organoids were established and 117 patients with CNS tumors, and performed miRNA microarray analyses (Figure 2A). By investigating whether differentially expressed miRNAs (DEMs) of each GBO corresponded to urinary DEMs of patients with parental tumors, diffuse gliomas, and other CNS tumors and non-cancer individuals, DEMs of the organoid culture supernatant were much more frequently identified as DEMs in urine of patients with parental tumors (73.4%), diffuse gliomas (30.6%), and other CNS tumors (25.0%) than the urine of non-cancer individuals (3.9%) (Figure 2B). These results suggested that many tumor-derived DEMs could be detected in urine as well as serum of patients with parental tumors directly.

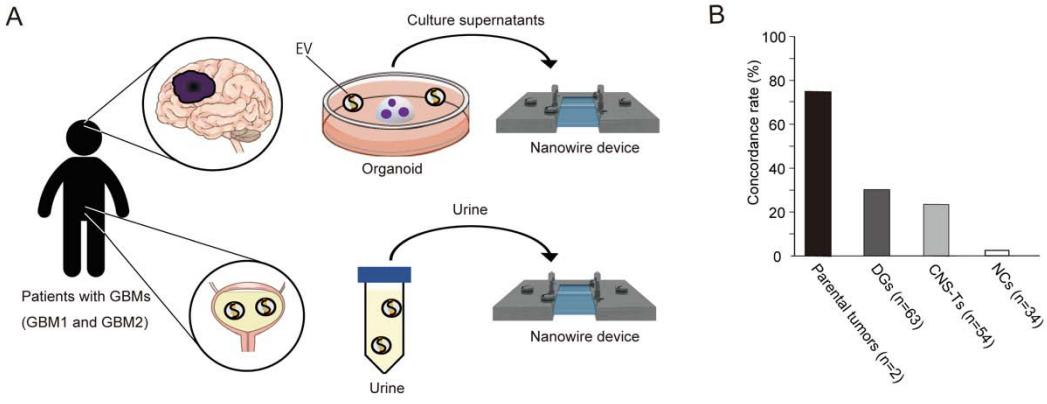

Figure 2. GBM organoid-derived miRNAs. (A) A schematic diagram of the analyses of miRNAs extracted from organoids and urine of the patients with parental tumors. (B) The percentages of organoid-derived DEMs in urinary DEMs of each group. Data are represented as mean. DGs, diffuse gliomas; CNS-Ts, central nervous system tumors (not including diffuse gliomas); NCs, non-cancer individuals.

Identification of the Best Combination of miRNAs for Detection of CNS Tumors.

In order to evaluate the usefulness of the assembly-type microfluidic nanowire device for CNS tumor screening, we developed a diagnostic model for CNS tumors using the miRNA expression

profiles. To construct a discriminant function as a diagnostic model between patients with CNS tumors and non-cancer individuals, we randomly divided 100 non-cancer individuals and 102 patients with CNS tumors (*i.e.* diffuse gliomas, meningiomas, schwannomas, and metastatic brain tumors) into two groups designated as: the training set and validation set (Figure 3). The remaining CNS tumor patients (n = 15) were allocated to an exploratory set which was intended to evaluate whether the model could also detect other kinds of CNS tumors. A 23-miRNA classifier was developed with data from the training set. A diagnostic index was calculated based on the individualized values of 23 miRNAs to differentiate between patients with CNS tumors and non-cancer individuals; a diagnostic index ≥ 0.4 indicated a CNS tumor, and a diagnostic index < 0.4 indicated its absence. This diagnostic model provided the best discrimination in the training set; and the following values were obtained: AUC, 1.00 (95% confidence interval (CI), 1.00-1.00); sensitivity, 1.00; specificity, 1.00 (Figure 4A, B). The performance of the diagnostic model was confirmed using the validation set, and the model was very accurate: AUC, 1.00 (95% CI, 1.00-1.00); sensitivity, 1.00; specificity, 0.97 (Figure 4A).
Our diagnostic model was found to be able to accurately discriminate CNS tumors from non-cancer samples irrespective of tumor grade (Figure 4B, left and middle panels). In addition, the model also successfully classified all CNS tumors (n = 15) in the exploratory set as positive: sensitivity, 1.00 (Figure 4B, right panel).

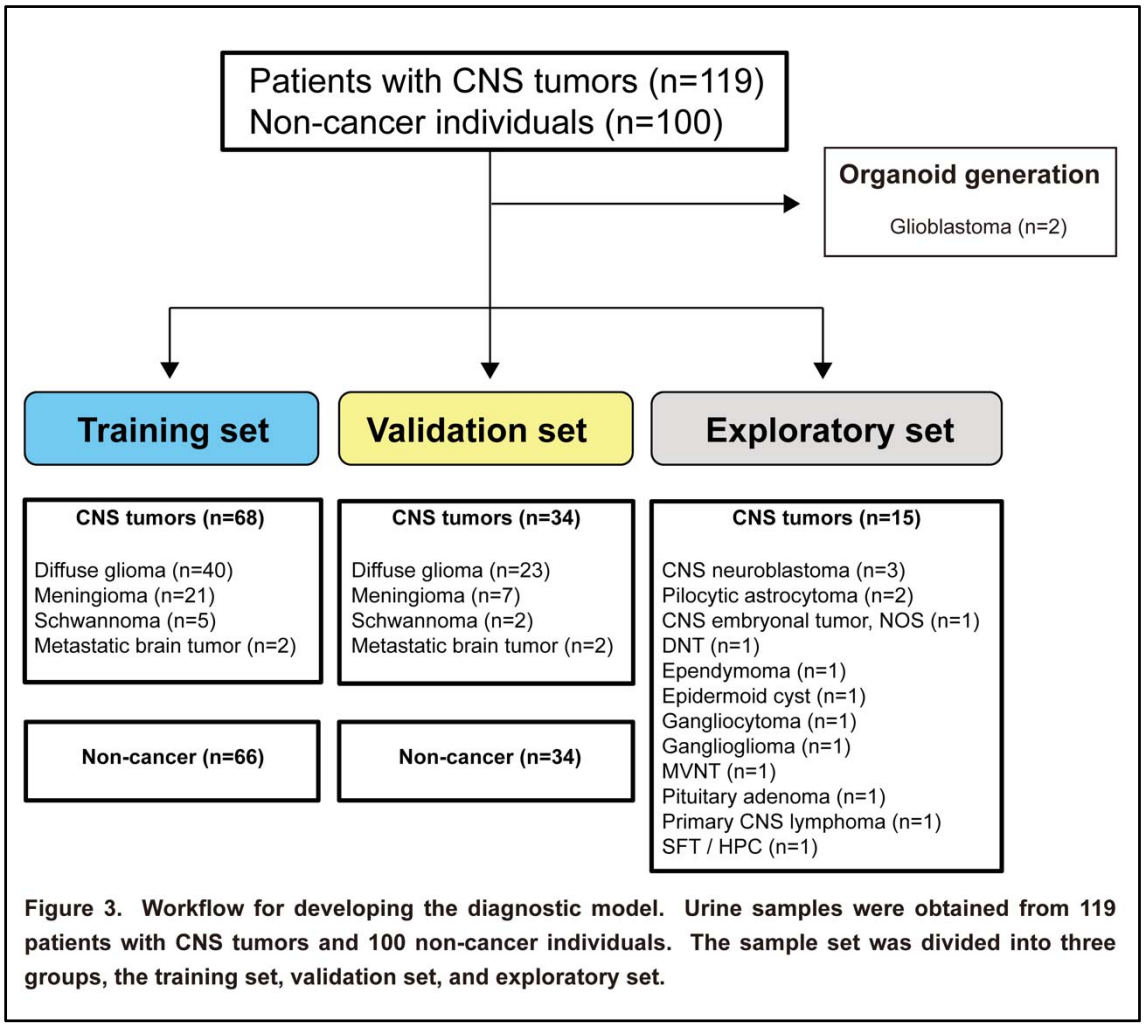

Figure 3. Workflow for developing the diagnostic model. Urine samples were obtained from 119 patients with CNS tumors and 100 non-cancer individuals. The sample set was divided into three groups, the training set, validation set, and exploratory set.

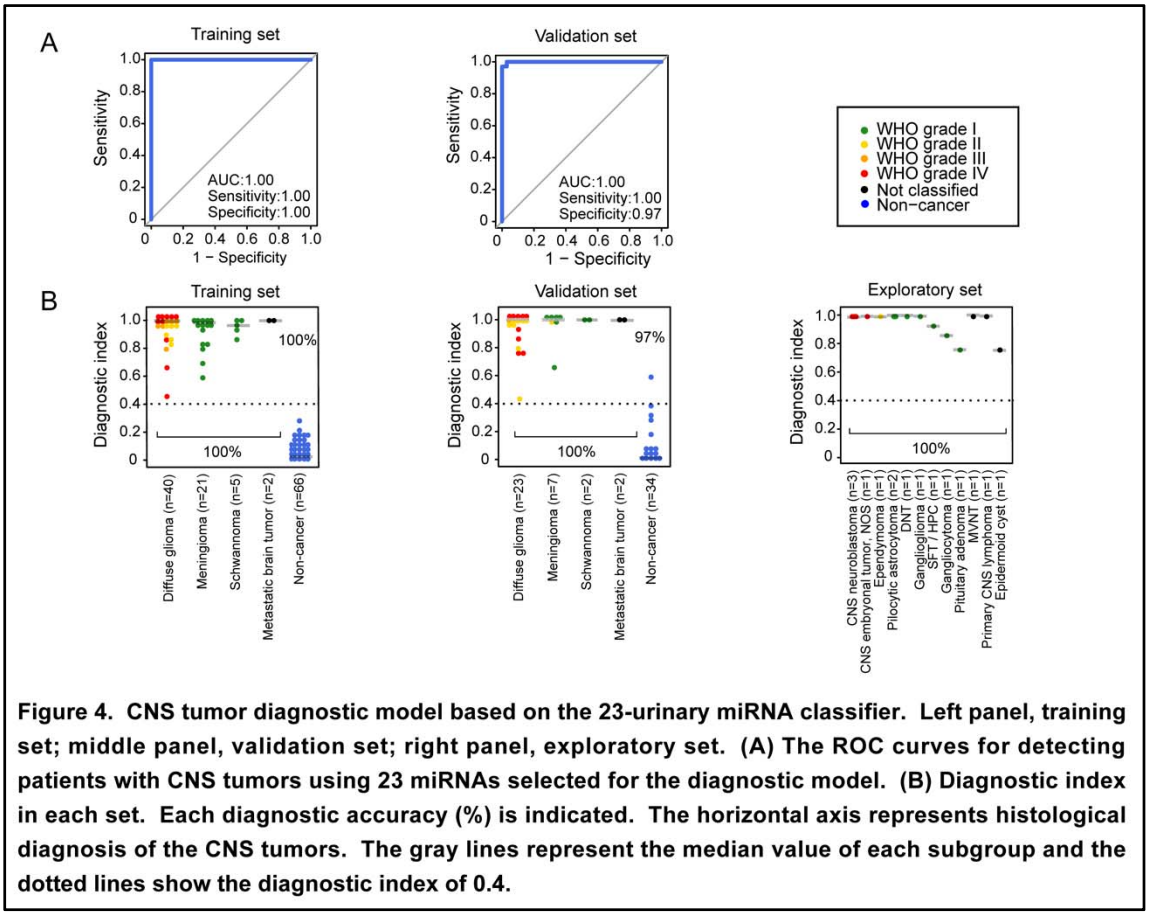

Figure 4. CNS tumor diagnostic model based on the 23-urinary miRNA classifier. Left panel, training set; middle panel, validation set; right panel, exploratory set. (A) The ROC curves for detecting patients with CNS tumors using 23 miRNAs selected for the diagnostic model. (B) Diagnostic index in each set. Each diagnostic accuracy (%) is indicated. The horizontal axis represents histological diagnosis of the CNS tumors. The gray lines represent the median value of each subgroup and the dotted lines show the diagnostic index of 0.4.

Research Summary and Future Perspective

The nanowire device enabled us to extract many more urinary miRNAs than conventional methods, analyze them comprehensively using the microarray, and develop the accurate diagnostic model for CNS tumors. In the future, the methodology of our study can be applied to different types of cancer, which benefits the development of diagnostic models for pan-cancer using only 1 mL of urine.

Publication

Yotaro Kitano, Kosuke Aoki, Fumiharu Ohka, Shintaro Yamazaki, Kazuya Motomura, Kuniaki Tanahashi, Masaki Hirano, Tsuyoshi Naganawa, Mikiko Iida, Yukihiro Shiraki, Tomohide Nishikawa, Hiroyuki Shimizu, Junya Yamaguchi, Sachi Maeda, Hidenori Suzuki, Toshihiko Wakabayashi, Yoshinobu Baba, Takao Yasui, and Atsushi Natsume.

Urinary MicroRNA-based Diagnostic Model for Central Nervous System Tumors Using Nanowire Scaffolds.

ACS applied Materials & Interfaces, published online on April 1, 2021.

DOI: 10.1021/acsami.1c01754.

Japanese ver.

https://www.med.nagoya-u.ac.jp/medical_J/research/pdf/ACS_App_Mat_210402.pdf