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Simultaneous multiplexed detection of exosomal microRNAs and surface proteins for prostate cancer diagnosis

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A B S T R A C T

Since the tumor is extremely heterogeneous, a single biomarker cannot reflect the exact symptoms of the disease or its stage. Exosomes are biomarker reservoirs that provide disease information with a high accuracy, especially when specific markers, including microRNAs (miRNAs) and proteins, are combined. However, currently available exosomal miRNA and protein detection methods are time consuming, expensive, and laborious. Meanwhile, simultaneous detection of an exosomal miRNA and protein in a single reaction is even more challenging. Thus, development of an efficient method for detecting multiple miRNAs and proteins in a single exosomal reaction is highly needed. Herein, to increase the value of using exosomes over other circulating biomarkers for prostate cancer (PCa) liquid biopsy, a method for simultaneous multiplexed *in situ* detection of exosomal miRNAs and proteins was developed. Exosomal miRNAs and surface proteins were simultaneously detected in captured exosomes with a high specificity, using nano-sized molecular beacons and fluorescent dye-conjugated antibodies. The method allowed the quantitative analysis of various disease-specific miRNAs and surface proteins in PCa cell-derived exosomes in a single exosomal reaction. Overall, simultaneous multiplexed *in situ* detection of exosomal miRNAs and surface proteins can be developed as a simple, cost-effective, non-invasive liquid biopsy method for diagnosing PCa.

1. Introduction

Prostate cancer (PCa) is the second most commonly diagnosed cancer in males worldwide. The serum prostate-specific antigen test and digital rectal examination are commonly used methods for PCa diagnosis prior to initial tissue biopsy. However, these methods have been criticized because they cause discomfort in patients and lack the specificity and accuracy (Andriole et al., 2009; Harvey et al., 2012; Koulikov et al., 2012). Several probes and techniques for cancer diagnosis were recently developed but these are restricted to the cancer cell detection (Liu et al., 2019a; Liu et al., 2019b). Thus, there is an obvious need for a novel diagnostic tool and biomarkers that is patient friendly and can provide tumor heterogeneity information with a high accuracy.

Recently, exosomes have been a subject of great interest as sources of novel biomarkers for liquid biopsy, which can overcome the limitations of previously used biomarkers for PCa. Exosomes are nano-sized extracellular vesicles (Colombo et al., 2014) and play an important role in disease pathogenesis, including cancer progression and metastasis (Azmi et al., 2013). High concentrations of exosomes can be found in various bodily fluids, including the blood, urine, saliva, and sputum (Ramirez et al., 2018). The molecular content of exosomes reflects specific physiological conditions and functions of their parental cells (Seigneuric et al., 2016). Unlike other circulating biomarkers, exosomes, because of their endosomal origin, are enriched in virtually every type of biomolecules, such as proteins, RNAs, and lipids, which are encapsulated inside or located on the surface of exosomes (Boyiadzis and Whiteside, 2017; Yang et al., 2018). Thus, exosomes and their biomolecules can be considered ideal biomarkers for liquid biopsy.

Among exosomal biomolecules, miRNAs and surface proteins have been extensively studied for the development of novel biomarkers for liquid biopsy (Liu et al., 2018; Valentino et al., 2017). Owing to the presence of exosomes in various bodily fluids and the high stability of miRNAs in exosomes, exosomal miRNAs can serve as a new class of biomarkers for early and minimally invasive cancer diagnosis (Salehi and Sharifi, 2018). Besides, exosomal surface proteins such as epithelial cell adhesion molecule (EpCAM), epidermal growth factor receptor (EGFR), survivin, and insulin-like growth factor 1 receptor (IGF-1R) can be used as disease biomarkers, for exosome enrichment, and as

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normalization factors for exosome concentrations (DeRita et al., 2017; Khan et al., 2012; Kharmate et al., 2016; Zhou et al., 2016). Taken together, exosomal miRNAs and surface proteins provide useful information on disease progression. Moreover, it is obvious that multiplexed detection is required since a single biomarker cannot accurately reflect the stage of disease progression. Therefore, development of simultaneous detection of various types of exosome biomarkers is required to achieve disease diagnosis with a high accuracy and specificity. However, no currently available method can simultaneously detect both exosomal miRNAs and proteins because nucleic acids and proteins have entirely different properties and localizations. Thus, these biomolecules should be analyzed independently and separately, using different methods such as real-time polymerase chain reaction (PCR) and an enzyme-linked immunosorbent assay (ELISA). Since this increases the complexity of the diagnosis and requires a number of steps, all of which are time consuming and laborious, it is necessary to develop a method for simple, simultaneous, multiplexed detection of exosomal miRNAs and surface proteins.

Herein, a novel PCa diagnosis method, based on simultaneous, multiplexed *in situ* detection of exosomal miRNAs and surface proteins, was developed for the first time (Fig. 1A). To achieve this goal, exosomes from human prostate-derived tumorigenic and non-tumorigenic cells were first captured using their surface proteins followed by exosomal miRNA and surface protein detection. MiRNAs inside exosomes were detected using molecular beacons (MBs), nano-sized oligonucleotide probes (Lee et al. 2015, 2016, 2018), while exosomal surface proteins such as a general exosome marker or cancer biomarkers were detected with specific antibodies. The simultaneous multiplexed *in situ* detection of exosomal miRNAs and surface proteins can be used as a successful and beneficial platform for simple, labor- and time-saving, non-invasive liquid biopsy for the diagnosis of various diseases including PCa.

2. Materials and methods

Molecular beacon design, exosome production and characterization, real-time PCR analysis, Western blot analysis, and *in situ* detection of exosomal miRNAs and surface proteins are described in details in *Supplemental Materials*.

3. Results

3.1. Characterization of exosomes from cancerous and non-cancerous prostate cells and their disease-related miRNAs and proteins

For simultaneous detection of exosome surface proteins and miRNAs, size distributions and concentrations of exosomes from RWPE-1, DU145, and PC-3 cells were analyzed by NTA. The average sizes of exosomes from RWPE-1, DU145, and PC-3 cells were 136.4, 137.8, and 141.2 nm, respectively (Fig. 2A). It has previously been reported that different exosome sizes might affect the fluorescence signals when more than two fluorescent dyes are used (Lee et al., 2016). However, no significant differences were observed among the exosomes from different cells (Fig. 2B). To confirm the effectiveness of exosome isolation, Western blot analysis was performed against typical exosomal markers (CD63, CD81, ALIX, and TSG101) and a cell-specific marker (calnexin; Fig. 2C). The exosomal markers were clearly detected in exosome protein lysates from all cells, whereas endoplasmic reticulum-derived contaminants such as calnexin were only detected in cell lysates.

To assess disease-related exosomal miRNA and protein levels, relative miRNA and protein levels were compared using real-time PCR analysis and western blotting, respectively. As expected, both miR-21 and miR-574-3p were highly expressed in exosomes derived from DU145 and PC-3 cells compared with their expression in exosomes derived from RWPE-1 cells (Fig. 3A). These results were consistent with the data from previous reports (Foj et al., 2017; Hessvik et al., 2013), indicating that exosomal miR-21 and miR-574-3p can be used as potential PCa diagnostic biomarkers. Both miR-21 and miR-574–3p levels were higher in the PC-3 exosomes than in the DU145 exosomes, which could be explained by the data from a previous report, showing that PC-3 cells had a higher metastatic potential than did DU145 cells, with a moderate metastatic potential (van Leenders et al., 2001).

Western blot analysis revealed the expression levels of four disease-related proteins (EpCAM, EGFR, survivin, and IGF-1R). In general, expression of most of the proteins was higher in PCa cell-derived exosomes than in prostate normal cell-derived exosomes (Fig. 3B and C). EpCAM and EGFR showed much larger differences between PCa cell- and prostate non-cancer cell-derived exosomes than did survivin and IGF-1R (Fig. 3C). These results showed that each of the disease-related miRNA and protein biomarkers helped distinguish PCa cell-derived exosomes from normal cell-derived exosomes.

3.2. Capture temperature- and time-dependent multiplexed detection of exosomal miRNAs and surface proteins

To establish the simultaneous detection of various types of exosomal biomarkers, exosomal miR-21 and CD63 were detected after capturing PC-3 cell-derived exosomes using a CD63 antibody at 37 °C (Fig. 4A). A longer capture time was used for an exosome capture experiment performed at 4 °C to test if the method is also applicable under a different condition (Fig. S1A). The fluorescent intensities from miR-21 and CD63 gradually increased as the exosome capture time increased, irrespective of the capture temperature. The S/B ratios for both miR-21 and CD63

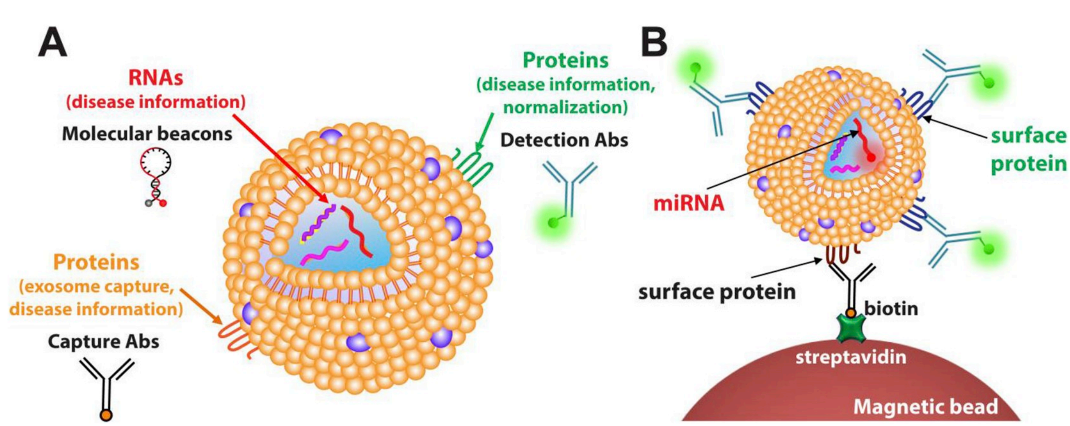

Fig. 1. Schematic representation of simultaneous multiplexed *in situ* detection of exosomal microRNA and surface protein markers. (A) Potential targets inside and on the surface of exosomes. (B) Exosomal miRNA and surface protein markers were detected using molecular beacons and fluorescent dye-conjugated antibodies in captured exosomes.

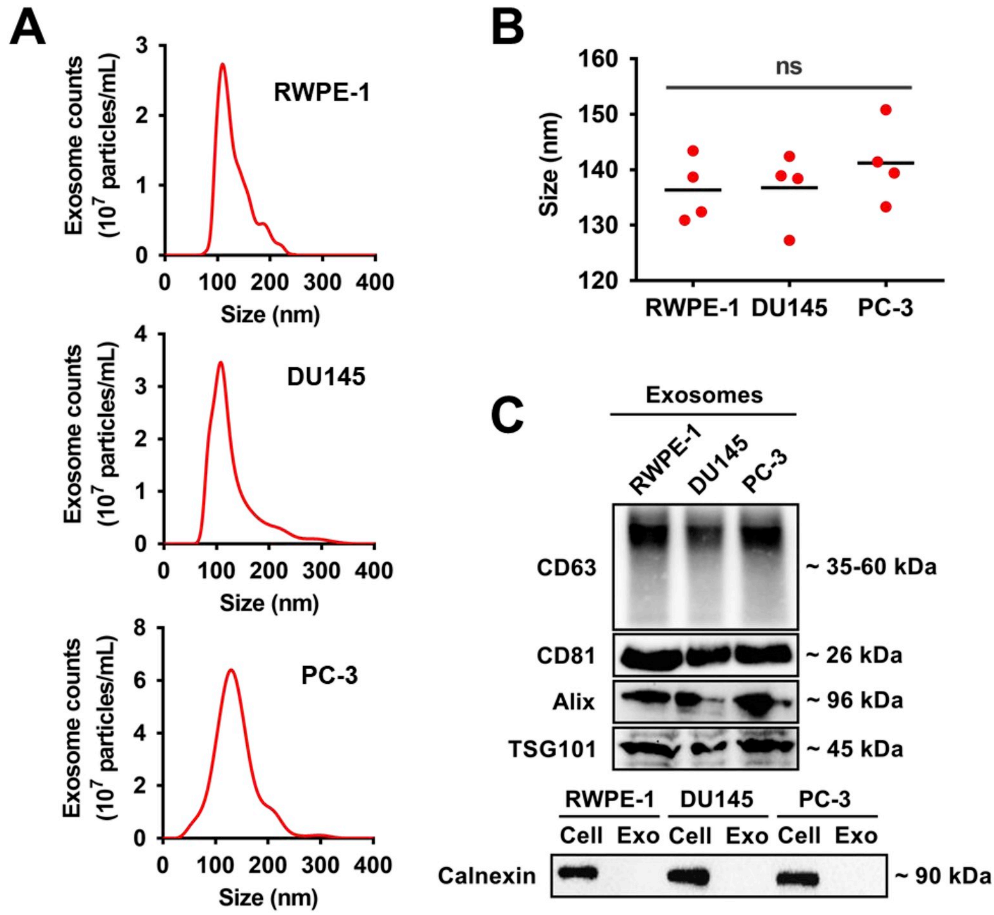

Fig. 2. Characterization of exosomes from cancerous and non-cancerous prostate cells. (A) Size distributions and concentrations of exosomes analyzed by NTA. (B) Average exosome sizes are shown (C) Western blot analysis of exosomal proteins was used for the detection of exosomal protein markers, CD63, CD81, ALIX, and TSG101, and to show negativity of the exosomal preparations for an endoplasmic reticulum protein (calnexin).

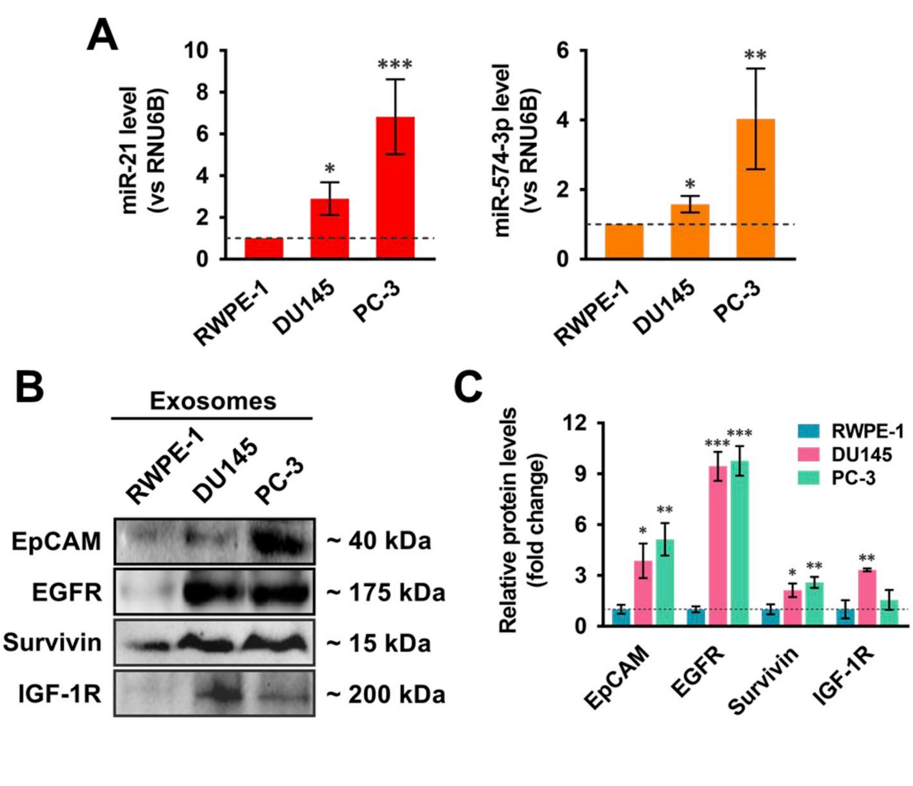

Fig. 3. Quantification of disease-related exosomal microRNAs and proteins. (A) Real-time PCR analysis was performed to quantify exosomal miR-21 and miR-574-3p. The miRNA levels were normalized to those of U6 snRNA. (B) Western blot analysis was performed to assess exosomal EpCAM, EGFR, survivin, and IGF-1R levels. (C) Graphs show quantitative integrated densities measured using ImageJ. All values are the mean ± standard deviation (SD; *p<0.05, **p<0.01, ***p<0.001; n = 3–6).

from the exosomes captured at 37 °C were higher than those from the exosomes captured at 4 °C after 2 h of capture. Considering the detection sensitivity and efficiency, two different exosome capture conditions were chosen, including 37 °C for 4 h and 4 °C for 12 h, for further experiments. Additionally, these findings indicate that exosome capture at 37 °C was more efficient than at 4 °C, which could reduce the overall diagnosis time for cancer. Moreover, the use of one temperature for all the detection processes (including exosome capture and biomarker detection) is more effective than using different temperatures for each process. Thus, the capture of exosomes at 37 °C is more beneficial and efficient than that at 4 °C.

3.3. Exosome capture specificity and efficiency using CD63 antibody-coated magnetic beads

The specificity of simultaneous detection of exosomal miRNA and surface protein biomarkers was determined *in situ*. There were negligible amounts of fluorescence when exosome capture antibody-coated magnetic beads were incubated without exosomes, followed by incubation with MB-21 and the anti-CD63 detection antibody (Fig. 4B), indicating negligible non-specific binding of MB-21 or the anti-CD63 detection antibody to CD63 antibody-coated magnetic beads, regardless of the capture temperature (Fig. S1B). When exosomes were incubated with magnetic beads without capture antibody or with isotype control antibody coating, once again, there were no significant increases in the S/B ratios of MB-21 and CD63. Meanwhile, high fluorescence signals were observed when PC-3 cell-derived exosomes were added to the reactions. These findings indicated that the anti-CD63 exosome capture antibody specifically captured exosomes.

Concentrations of total RNA extracted from captured exosomes were measured to assess the capture efficiency of exosomes derived from each prostate cell line. The concentrations of total RNA extracted from exosomes derived from each prostate cell line before capture were used as controls. The exosome recovery yields after exosome capture at 37 °C were 32, 19, and 28% for RWPE-1, DU145, and PC-3 exosomes, respectively (Fig. 4C). Similar patterns were obtained for exosome recovery yields of exosome capture at 4 °C (Fig. S1C). Notably, regardless of the exosome capture temperature, the exosome recovery yield for DU145 cell-derived exosomes was the lowest. No significant difference was observed between the exosome recovery yields for RWPE-1 and PC-3 cell-derived exosomes. These results were also consistent with the Western blot results (Fig. 2C), which showed that exosomes from DU145 cells had the lowest amount of CD63.

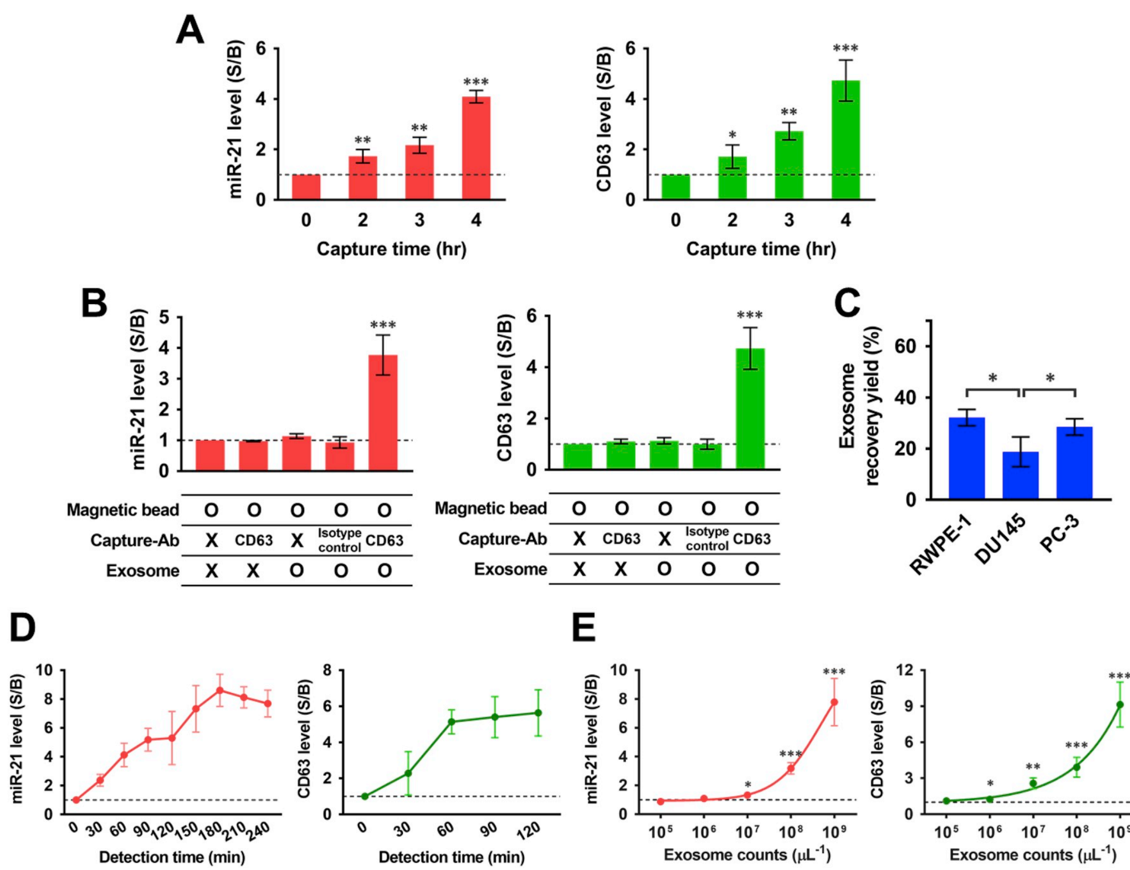

Fig. 4. Detection of exosomal miRNA and surface protein markers in captured exosomes. (A) Effects of the exosome capture time on exosome miRNA and surface protein detection was assessed. PC-3 exosomes (10^8 particles/μL) were incubated with CD63 antibody-coated magnetic beads at 37 °C. The captured exosomes were separated and further incubated with MB-21 (left) and CD63 antibody (right), respectively. The fluorescence intensities obtained from MB and the CD63 antibody in the absence of exosomes were used as background signals, and S/B ratios of each reaction were normalized to the control (without exosomes). (B) Specificity of exosomal miRNA and surface protein marker detection was assessed. The control experiments were performed in the absence of exosomes using magnetic beads functionalized (i) without antibodies and (ii) with the anti-CD63 capture antibody, as well as in the presence of exosomes using magnetic beads functionalized (iii) without antibodies, (iv) with an isotype control antibody, and (v) with the anti-CD63 capture antibody. The captured exosomes were incubated with MB-21 and CD63 antibody to detect exosomal miR-21 (left) and CD63 (right). (C) Exosome recovery yields during exosome capture using CD63 antibody-coated magnetic beads. (D) Effects of the detection time on multiplexed detection of exosomal miRNA and surface protein markers. The captured exosomes were incubated with MB-21 (left) and the fluorescent dye-conjugated CD63 antibody (right), and fluorescent intensities were measured. (E) Dynamic detection ranges of simultaneous *in situ* detection are shown for exosomal miR-21 (left) and CD63 (right), respectively. All values are the mean ± SD (*$p<0.05$, **$p<0.01$, ***$p<0.001$; n = 3–5).

3.4. Detection time and dynamic detection range of simultaneous in situ detection of exosomal miRNA and surface protein biomarkers

Time-dependent changes in S/B ratios were analyzed for MB or the antibody to find the optimal detection time for multiplexed detection of exosome biomarkers. In general, the S/B ratios of exosomal miR-21 and CD63 gradually increased as the incubation time increased (Fig. 4D). There was no further increase in the S/B ratio of exosomal surface CD63 after 60 min of incubation, whereas that of exosomal miR-21 continuously increased up to 180 min. We have previously demonstrated that the MB penetrates the exosomes and hybridizes with the target miRNA inside the exosomes (Lee et al. 2015, 2016, 2018). Thus, these results

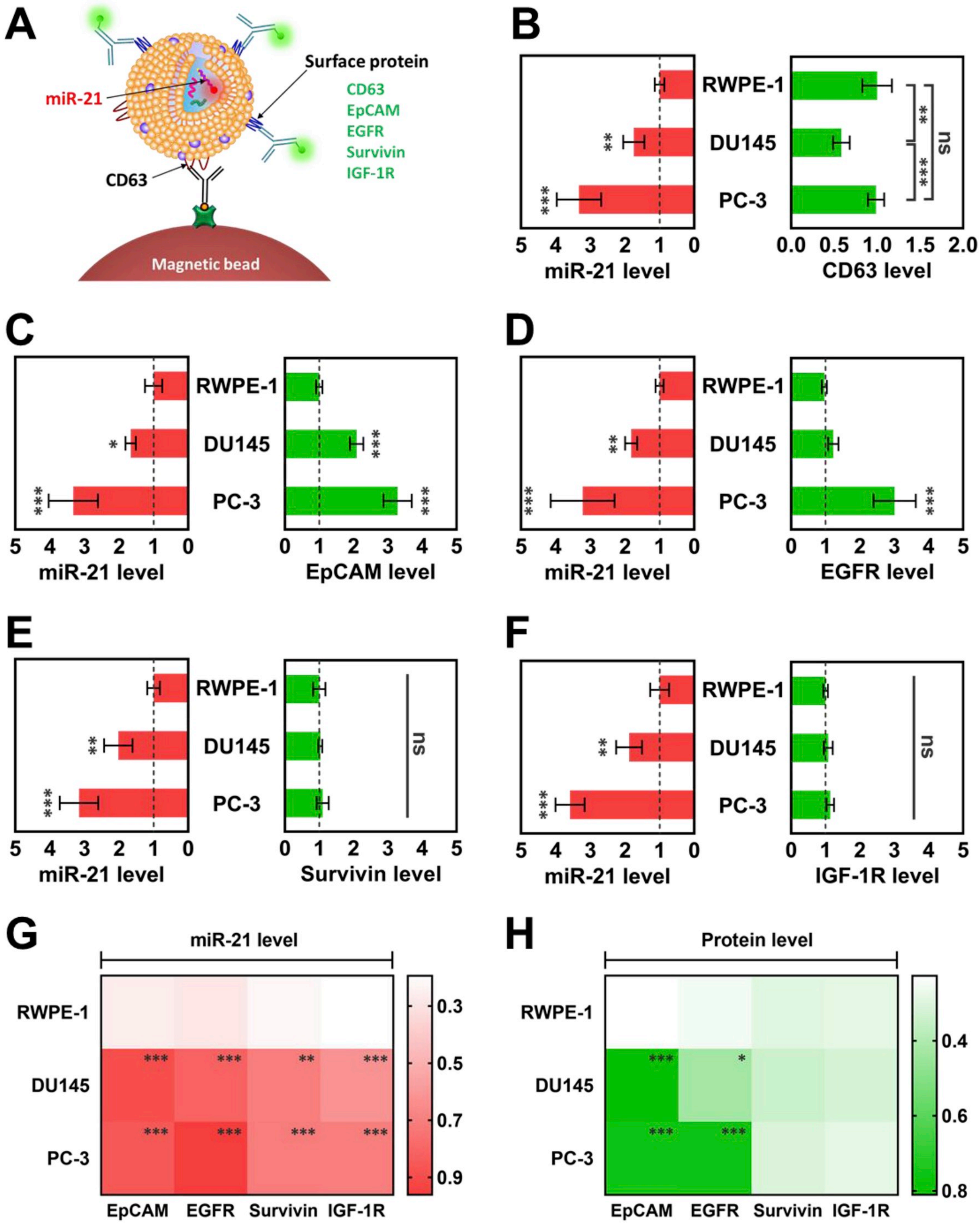

Fig. 5. Simultaneous *in situ* detection of miR-21 and surface protein biomarkers in captured exosomes. (A) Schematic representation of *in situ* detection of exosomal miRNA and protein combinations. (B–F) miR-21 was detected in combination with CD63 (B), EpCAM (C), EGFR (D), survivin (E), and IGF-1R (F). Results are displayed after normalization to the fluorescent intensity from RWPE-1 exosomes. (G, H) Post-normalization heatmaps of simultaneous *in situ* PCa-related biomarker detection, showing normalized levels of (G) miRNA and (H) surface protein, detected in combination in exosomes derived from prostate cell lines. The biomarker levels were determined by normalizing each marker signal to that of anti-CD63 to account for the variation in exosomal counts across samples. Red and green intensities indicate fold changes of miRNA and surface protein levels, respectively. All values are the mean ± SD (*$p<0.05$, **$p<0.01$, ***$p<0.001$; n = 3–6). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

were probably due to the target localization so that MB-21 required more time to penetrate into exosomes and hybridize with miR-21, while the CD63 antibody did not need as much time since the target CD63 resides on the exosome surface. Both miR-21 and CD63 S/B ratios showed statistical significance after 30 min of detection. Based on these findings, the exosomal miRNA and surface protein detection times were fixed at 60 min for further experiments.

To assess the dynamic detection range of simultaneous *in situ* exosomal miRNA and surface protein detection, various concentrations of PC-3 cell-derived exosomes were used for exosome capture. The corresponding fluorescence intensities from MB-21 and the CD63 antibody gradually and significantly increased in exosome concentration-dependent manners (Fig. 4E). The lower limit of detection (LOD) was estimated to be 10^7 particles/μL for exosomes captured at 37 °C for 4 h. Considering high exosome concentrations in human bodily fluids, including blood and urine ($1–3 \times 10^9$ exosomes/μL in the blood and $3–8 \times 10^6$ exosomes/μL urine), the simultaneous *in situ* detection method described here provides sufficiently low LODs for exosome PCa biomarkers (Li et al., 2014).

3.5. Simultaneous in situ detection of miR-21 and different surface proteins in CD63-specific captured exosomes

Based on the results obtained, simultaneous *in situ* detection of miR-21 and different exosomal surface proteins was carried out. The same amounts of exosomes from non-cancer (RWPE-1) and cancer (DU145 and PC-3) cells were captured using CD63 antibody-conjugated magnetic beads. CD63 was used as a marker for exosome quantification, and therefore, its detection signal was used for normalization of detection signals from exosomal miRNA and other disease-related proteins. EpCAM, EGFR, survivin, and IGF-1R were used as disease-related protein markers (Fig. 5A). After each exosomes were incubated with CD63 antibody-coated magnetic beads, the captured exosomes were further incubated with MB-21 and the CD63 antibody. The exosomal miR-21 levels were significantly higher, by 1.74- and 3.82-fold, in DU145 and PC-3 exosomes than in RWPE-1 exosomes, respectively (Fig. 5B, left), and this expression pattern was consistent with that obtained by real-time PCR (Fig. 3A). Similar patterns were observed for exosomal miR-21 levels in combination with the other surface proteins, indicating consistent *in situ* miRNA detection in captured exosomes, regardless of the protein combination (Fig. 5C–F). At the same time, the fluorescence signals of CD63 from captured DU145 and PC-3 exosomes were 58% and 98%, respectively, of that from captured RWPE-1 exosomes (Fig. 5B, right). The fluorescent intensity of CD63 from DU145 exosomes was the lowest since DU145 exosomes had the lowest amount of the CD63 protein, as demonstrated by western blotting (Fig. 2C). Thus, this resulted in both the lowest amount of captured exosomes (Fig. 4C), and the lowest CD63 fluorescent signal when using the CD63 detection antibody.

Among the disease-related protein markers, the EpCAM levels in DU145 and PC-3 exosomes were 2.09- and 3.28-fold higher than that in RWPE-1 exosomes, respectively (Fig. 5C, right). On the contrary, the EGFR level was only significantly higher in PC-3 exosomes (Fig. 5D, right). The EGFR levels in DU145 and PC-3 exosomes were 1.22- and 3.12-fold of that in RWPE-1 exosomes, respectively. There were no significant differences in exosomal survivin and IGF-1R levels among the prostate cell-derived exosomes (Fig. 5E and F, right). Since both survivin and IGF-1R levels in DU145 and PC-3 exosomes were higher than those in RWPE-1 exosomes (Fig. 2C), we speculated that the absolute amounts of survivin and IGF-1R were relatively low to obtain sufficient fluorescence signals using their respective detection antibodies and below the detection limits. Overall, these results demonstrated that simultaneous *in situ* detection of exosomal miRNA and surface proteins was successfully developed and could be further used for PCa diagnosis with a high accuracy, once suitable biomarkers are chosen.

3.6. Normalized heatmap results of simultaneous in situ PCa-related exosomal biomarker detection

Many biomolecules are located inside and on the surface of exosomes. Some are disease-related molecules, which are aberrantly expressed in diseased cells and therefore encapsulated in exosomes. On the contrary, some molecules show no correlation with specific diseases but are highly encapsulated in exosomes during biogenesis. Different individuals may have different concentrations of exosomes in bodily fluids, including blood and urine. As a consequence, fluorescent signals from disease-related biomarkers located in exosomes can be over- or underestimated, depending on the amount of exosomes captured. Thus, it is necessary to compensate for individual differences in exosome concentrations in bodily fluids (Im et al., 2014; Shao et al., 2012). For this reason, the fluorescent intensities of the PCa-related exosomal biomarkers (miR-21, EpCAM, EGFR, survivin, and IGF-1R) were normalized to that of PCa-unrelated exosomal biomarker, CD63, to obtain relative molecular levels of disease biomarkers per single exosome. Thus, the fluorescence intensity of CD63 from each type of exosomes was used as a normalization factor, and all the fluorescence intensities for PCa-related biomarkers, including miR-21, EpCAM, EGFR, survivin, and IGF-1R, were normalized to obtain normalized heatmaps (Fig. 5G and H). As a result, all the normalized miR-21 levels significantly increased in DU145 exosomes and further increased in PC-3 exosomes compared with those in RWPE-1 exosomes, regardless of the surface protein biomarkers (Fig. 5G). The results were similar for the exosome surface protein biomarkers EpCAM and EGFR (Fig. 5H). Notably, the fold differences of miR-21, EpCAM, and EGFR fluorescence intensities between RWPE-1 and DU145 exosomes, shown in Fig. 5C–F, further increased. For instance, the fold difference in miR-21 between RWPE-1 and DU145 exosomes was 1.48 in Fig. 5C but increased to 3.43 in Fig. 5G after normalization to each CD63 level. These data are due to the fact that DU145 exosomes had less CD63 than other exosomes had, and these differences contributed to the increases in miR-21, EpCAM, and EGFR levels in DU145 exosomes after normalization. Since normalization compensated for the low capture efficiency of DU145 exosomes, due to a lower CD63 level on their surface, the normalized heatmaps provide more accurate detection profiles by showing the levels of disease-related biomarkers per exosome. In particular, the EGFR level in DU145 exosomes became significantly higher than that in RWPE-1 exosomes after normalization to the CD63 levels (Fig. 5H), suggesting that normalization may compensate for the low EGFR level caused by a lower exosome capture efficiency of DU145 exosomes, due to a low amount of CD63 on their surface. Similar results were observed for exosomes captured at 4 °C (Fig. S2), indicating that the method is applicable, regardless of the capture condition. Consequently, by using this approach, quantitative and disease-related exosome information could be obtained using various exosomal markers, and integration of this information will provide enhanced accuracy of disease diagnosis.

3.7. Multiplexed detection of miR-21, miR-574-3p, and EpCAM in prostate cell-derived exosomes

One of the advantages of our simultaneous *in situ* detection of exosomal miRNA and surface proteins is biomarker scalability and variability, allowing the selection of additional biomarkers to increase the accuracy and efficiency of diagnosis in a single reaction. Herein, two different exosomal miRNAs were detected together with EpCAM as a surface protein biomarker in CD63-specific captured exosomes (Fig. 6A). miR-574-3p was chosen as an additional target miRNA, and a Cy5-labeled miR-574-3p-targeting MB (MB-574-3p) was synthesized. From real-time PCR results, the miR-574-3p level was higher in DU145 exosomes than in RWPE-1 exosomes, and the highest S/B ratio was observed in PC-3 exosomes (Fig. 3A). To investigate the possibility of triplex detection of exosomal miRNAs (miR-21 and miR-574-3p) and a surface protein (EpCAM) for discrimination between cancerous and non-

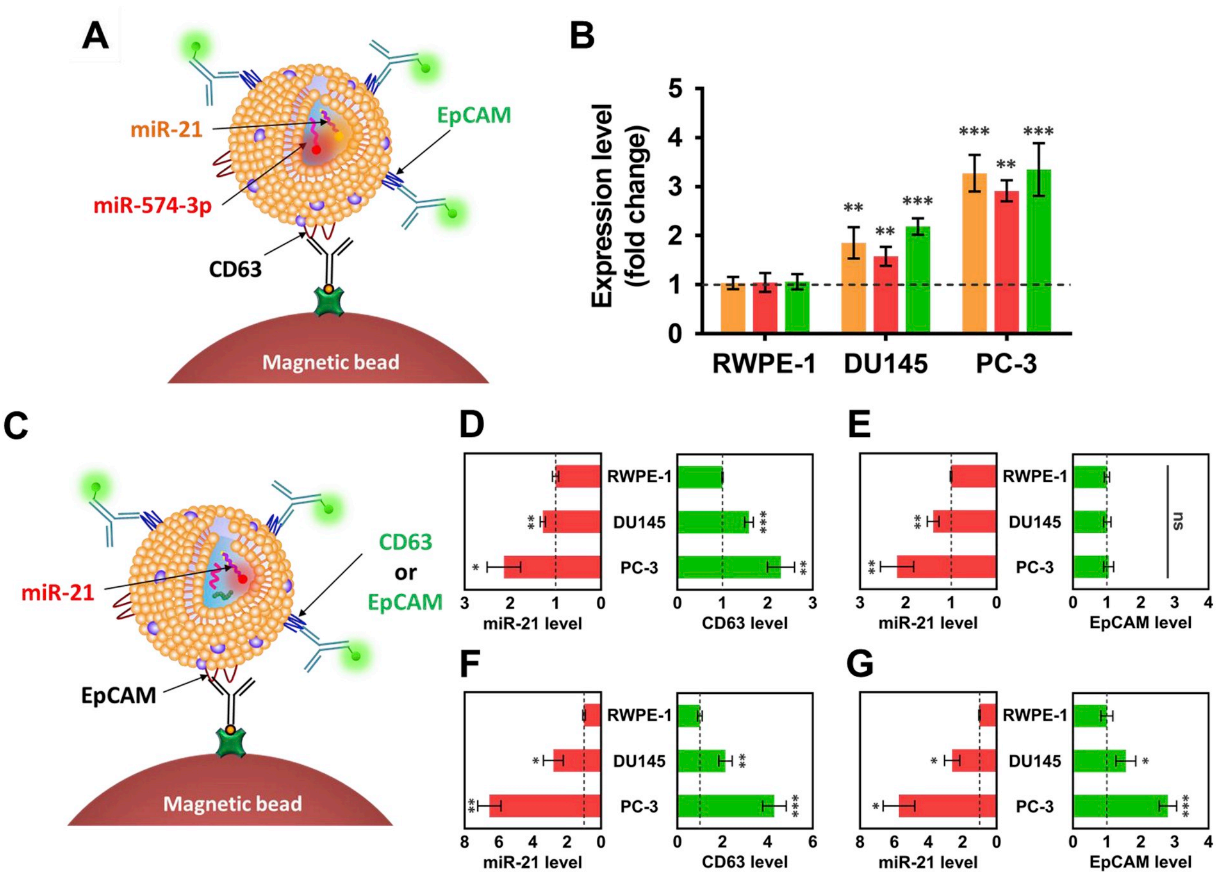

Fig. 6. Multiplexed simultaneous *in situ* detection of miRNAs and surface protein biomarkers. (A, B) Multiplexed detection of miR-21, miR-574-3p, and EpCAM in prostate cell-derived exosomes. (A) Schematic representation of simultaneous *in situ* detection of the two miRNAs and the protein in captured exosomes. (B) Disease-related exosomal miRNA and protein expression levels in prostate cell-derived exosomes, obtained using the multiplexed *in situ* detection technique. (C–G) Simultaneous *in situ* detection of miRNA and surface protein biomarkers in EpCAM-positive exosomes. (C) Schematic representation of *in situ* detection of miRNA and proteins in EpCAM-specific exosomes. (D–G) Prostate cell-derived exosomes [10^8 particles/μL (D, E) or 10^9 particles/μL (F, G)] were incubated with EpCAM antibody-coated magnetic beads. The captured EpCAM-positive exosomes were then incubated with MB-21 and the anti-CD63 (D, E) or anti-EpCAM (F, G) antibody to detect exosomal miR-21 (left) and CD63 or EpCAM (right) in combination. Results are displayed after normalization to the S/B ratio of RWPE-1 exosomes. All values are the mean ± SD (*$p<0.05$, **$p<0.01$, ***$p<0.001$; n = 3–5).

cancerous prostate cell-derived exosomes, exosomes were incubated with CD63 antibody-coated magnetic beads and further incubated with MB-21, MB-574-3p, and the EpCAM antibody. As a result, increases in the S/B ratios of the three different biomarkers were observed, indicating that the multiplexed detection of the exosomal miRNA and protein biomarkers was successfully accomplished (Fig. 6B). High detection signals were observed in the order of PC-3, DU145, and RWPE-1 exosomes for all the biomarkers, which was consistent with the results shown in Figs. 3 and 5. Thus, these results strongly confirmed that simultaneous detection of multiple exosomal biomarkers using this method could be efficiently performed for the diagnosis of PCa with a high accuracy. There could be signal interference among the fluorescent dye labeled probes, including between the MBs and antibody, though the distance between the MBs and antibody may be too large. To minimize the fluorescence interference among the probes, relevant fluorescent dyes should be used to avoid any fluorescence overlap among the dyes.

3.8. Simultaneous in situ detection of exosomal miRNA and protein biomarkers in disease-specific captured exosomes

Since exosomes are released from cells in different tissues, a heterogeneous population of exosomes from different sources circulates in bodily fluids. If exosomes originated from normal cells outnumber those from cancer cells, the detection signals from disease-related biomarkers can be underestimated because exosomes from healthy cells contain low levels of these markers. Thus, it is crucial to specifically isolate exosomes from diseased cells before biomarker detection. Besides, unlike other circulating biomarkers such as cell-free DNA, proteins, or miRNAs, exosomes are vesicles with multiple components; therefore, disease-specific exosome capture, followed by *in situ* exosomal biomarker detection, is expected to contribute to the enhanced efficiency and accuracy of PCa diagnosis.

Accordingly, simultaneous *in situ* miRNA and surface protein detection was carried out in exosomes captured using a PCa-specific protein with the EpCAM-specific antibody, instead of the CD63-specific antibody. As mentioned, EpCAM is an epithelial surface antigen (glycoprotein) found in epithelial intercellular junctions (Schnell et al., 2013), which is overexpressed in PCa and metastatic PCa (Massoner et al., 2014). EpCAM-positive exosomes were captured from prostate cell-derived exosomes (10^8 particles/μL), followed by incubation with MB-21 and the CD63 or EpCAM antibody (Fig. 6C). The S/B ratios were measured and normalized to those from RWPE-1 exosomes for comparison. As shown in Fig. 6D, higher fluorescent intensities of both miR-21 and CD63 were observed in EpCAM-positive exosomes from PCa cells than in those from non-cancer cells. The CD63 level could be interpreted in this case as reflecting the concentration of EpCAM-positive exosomes, and these results corroborated the data from a previous report, showing a higher EpCAM expression level on tumor-derived cells versus normal cells (Liu et al., 2014). Interestingly, the levels of EpCAM in EpCAM-positive exosomes from PCa exosomes were not higher than that from non-cancer exosomes (Fig. 6E), although significantly higher miR-21 levels were observed in the former, indicating that the exosome capture efficiency using EpCAM antibody-coated magnetic beads was far lower than that of CD63 antibody-coated magnetic beads. This problem could be solved by simply increasing the initial exosome concentration. As shown in Fig. 6F and G, the fluorescence levels from miR-21 and EpCAM increased compared with those shown in Fig. 6D and E. Moreover, there were significant differences in the EpCAM levels in DU145 and PC-3 exosomes compared with that in RWPE-1 exosomes. Thus, the described method of *in situ* detection of exosomal biomarkers offers a successful and beneficial platform for simultaneous detection of miRNA and surface protein biomarkers in tumor-derived exosomes. The method can be used as a more reliable and effective diagnostic tool than currently available methods by maximizing the difference in biomarker levels between cancer patients and normal individuals in clinical practice.

4. Discussion

Exosomal biomolecules, including proteins and miRNAs, are attractive biomarkers, enabling minimally invasive or non-invasive disease diagnosis. The lipid bilayer provides a shield against the environment, and thus exosomal biomarkers are protected during exosome circulation in bodily fluids. Moreover, various biomolecules that are encapsulated inside or reside on exosomes provide disease information as well as information on their tissue origin. Exosomal surface proteins can be used 1) as disease biomarkers; 2) for exosome capturing and enrichment; and 3) as normalization factors, which afford quantitative information on exosomes. The use of circulating biomarkers for liquid biopsy has been hindered by a lack of standardization factor that can reflect differences among individuals. The amount of a cancer-related biomarker per volume of blood or urine can be variable even in the same individual. Due to the absence of a normalization factor for circulating or exosomal miRNA detection, the detection of miRNAs was criticized because of its concentration variation in human urine or blood and due to a lack of reference molecules (Bissels et al., 2009; Ramirez et al., 2018). However, the simultaneous detection of the exosome surface proteins offers a normalization factor for PCa-related miRNA quantification by dividing the fluorescence intensity from the miRNAs with that from exosome surface proteins such as CD63. Thus, a method developed should not only be highly accurate and specific but also reproducible and robust (Wang et al., 2015). Taken together, it can be concluded that multiplexed detection of exosome biomarkers offers great opportunities for developing highly accurate diagnostic tools. The key is how these diverse biomarkers, present inside and on the surface of exosomes, are utilized to receive all benefits from exosomes.

To increase the value of using exosomes over other circulating biomarkers in liquid biopsy, we developed a method of simultaneous multiplexed *in situ* detection of exosomal miRNA and surface proteins in exosomes in a single reaction. Exosomes were captured by targeting their surface proteins, and then miRNAs located inside and proteins located on the surface were detected using MBs and detection antibodies, respectively, in a concentration-dependent manner and with a high specificity. The first type of surface proteins (e.g., CD63) provides quantitative exosome information and thus can be used to normalize the exosome concentration. The second type of surface proteins (e.g., EpCAM and/or EGFR) can be used to obtain disease-specific information, if the proteins are aberrantly overexpressed in diseased exosomes.

In addition, circulating tumor-derived exosomes are rich in tumor antigens, providing a unique opportunity for cancer diagnosis (Fang et al., 2017). However, tumor-derived exosomes may be diluted in the circulatory system, resulting in biomarkers of tumor-derived exosomes being mixed with those of healthy cells and diluted. Exosomes from healthy cells also contain low levels of PCa-related biomarkers, including miRNAs and proteins, which may interfere with the detection signals from tumor-derived exosomes. Therefore, it is necessary to remove exosomes derived from healthy cells and specifically isolate exosomes originated from cancer cells before attempting to detect exosomal miRNAs and proteins. Thus, development of a method for capturing and enriching tumor-derived exosomes from bodily fluids, followed by simultaneous *in situ* detection of miRNAs and surface proteins, will accelerate the development of an efficient and accurate method for cancer diagnosis. As shown in Fig. 6, our method provided a successful platform for simultaneous detection of miRNA and surface protein biomarkers in tumor-derived exosomes after capturing EpCAM-specific exosomes. A more reliable and effective diagnostic tool can be developed by maximizing the signal differences in biomarker levels between exosomes derived from cancer patients and normal individuals in clinical practice.

Various prominent techniques have been recently developed for multiplexed biomarker detection, which can be applied to PCa diagnosis. For example, Frei et al. (2016) reported the simultaneous detection of RNAs and proteins in a single cell named PLAYR (Proximity Ligation Assay for RNA). This technique enabled the detection of more than 40 RNAs and proteins using flow and mass cytometry. Thus, this method is capable of providing more information on the biomarkers contained in the samples. However, this method requires fixation and permeabilization, and multiple washing steps. Thus, this may not be an ideal technique for exosome biomarker detection due to the nano-sized nature of the exosomes. Moreover, the number of biomarker miRNAs and proteins can be simply increased using our *in situ* simultaneous multiplexed method by preparing samples treated with different MBs and antibodies for additional biomarker detection. Tang et al. (2016) reported microchip-based microfluidic electrochemical arrays for the multiplexed detection of cancer proteins in solution. Although this method enabled the sensitive and efficient analysis of multiple proteins, it can only be applied for exosomal protein detection and not for simultaneous exosomal miRNA and protein detection. Thus, the simultaneous multiplexed detection of miRNAs and proteins from exosomes developed in this research could offer a representative method for PCa diagnosis using exosomes.

Furthermore, the total cost of the materials used for simultaneous multiplexed detection in this research might be lower than that in typical exosomal miRNA and protein biomarker detection. Besides considering the time required for miRNA analysis using typical qPCR analysis, which requires multiple steps such as exosome capture, miRNA isolation, cDNA synthesis, and PCR, our *in situ* detection method is expected to enable faster analysis of the PCa biomarkers. In this context, our proposed method offers various advantages (Supplemental Table 2) and it could be used for a high-throughput diagnosis of PCa.

Additional important processes should be followed for the clinical application of our method to human urine or plasma samples for prostate cancer diagnosis. For example, exosomes from clinical samples (e.g. plasma, serum, urine) should be collected with high purity and yield before applying our method. High levels of impurities from the samples would result in false positive signals. Exosomes can be directly captured from the sample without an isolation step. In this case, simple sample pretreatment could be added and optimized to achieve the best performance. For example, ultrafiltration could be applied to concentrate the exosomes and remove small impurities before exosome capture, which would increase the signal-to-background level. Additionally, relevant surface proteins and miRNA biomarkers for prostate cancer should be determined from the clinical samples. Our proposed method offers the advantage of easily adopting and replacing different biomarkers. Thus, combined with the above-mentioned technical and molecular developments, the *in situ* simultaneous multiplexed exosome biomarker detection technique could be successfully applied for clinical prostate cancer diagnosis.

5. Conclusion

To increase the value of using exosomes over other circulating biomarkers for PCa liquid biopsy, we present a method for simultaneous multiplexed *in situ* detection of exosomal miRNAs and proteins. Exosomal miRNAs and surface proteins were simultaneously detected in captured exosomes with a high specificity, using nano-sized molecular beacons and fluorescent dye-conjugated antibodies. The method allowed the quantitative analysis of various disease-specific miRNAs and surface proteins in PCa cell-derived exosomes in a single exosomal reaction. Simultaneous multiplexed *in situ* detection of exosomal miRNAs and surface proteins can be used for simple, labor- and time-saving, non-invasive liquid biopsy for the diagnosis of various diseases, including PCa, and can provide a much-needed platform to predict the prognosis and monitor treatment responses in a clinical stage.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Seongcheol Cho: Conceptualization, Data curation, Methodology, Software, Writing - original draft. **Hee Cheol Yang:** Methodology, Writing - review & editing. **Won Jong Rhee:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing - original draft, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bios.2019.111749.

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