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Research Article

Detection of Thyroid Carcinoma Antigen with Quantum Dots and Monoclonal IgM Antibody (JT-95) System

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High-intensity fluorescent nanoparticles, quantum dots (QDs), have been applied to a wide range of biological studies and medical studies by taking advantage of their fluorescent properties. On the other hand, we have reported the specificity of JT-95 monoclonal IgM antibody, which recognizes the antigen of thyroid carcinomas. Here we show that the combination of QDs and JT-95 monoclonal antibody was applicable to Western blotting analysis, ELISA-like system, and fluorescent microscopic analysis of SW1736 thyroid carcinoma cell line. We have opened up the possibility that antibodies for higher specific recognition, even IgM, are applicable to the detection system with QDs.

1. Introduction

Diagnosis of thyroid cancer involves methods such as palpation, ultrasonography, fine-needle aspiration cytology (FNAC), scintigraphy, and blood test. To achieve more precise diagnosis and alleviate burdens of both patients and doctors, however, we consider that the following 2 points need to be improved.

The first point is the blood test. Although the blood test is thought to be the simplest method without much burden on patients, blood tests currently performed are not well suited for screening tests to determine the existence or nonexistence of cancer. In the blood tests, thyroglobulin [1, 2] and calcitonin are considered to be most effective [3]. Thyroglobulin is known to exhibit high values with papillary and follicular carcinomas of the classified thyroid cancers as well as with other diseases including inflammation and benignant tumors [2]. Although calcitonin is reported to be a marker for medullary carcinoma [3], negative cases are also reported [2]. Hence, the development of a new blood test method which will replace these ones and increase the precision is required.

The second point is the judgment of the degree of malignancy of neoplasm in FNAC. The FNAC is known to be extremely precise in classifying neoplasm and judging the degree of malignancy [4, 5]. The follicular carcinoma which has the second highest rate of occurrence in Japan [6], within the classified thyroid cancers such as papillary carcinoma, follicular carcinoma, poorly differentiated carcinoma, undifferentiated carcinoma, and medullary carcinoma, however, does not exhibit any nuclear atypicality. This fact makes it difficult to judge from FNAC results whether a tumor is benignant or malignant. When such a judgment is found to be difficult before operation, surgical removal of follicular lesions is preferably carried out. Recent studies showed that in patients who had their follicular carcinoma removed only 15–20% of them were malignant, while more than 80% were benignant [7, 8]. That is, more than 80% of patients have supposedly undergone an operation to have their benignant tumors removed without such a need. Hence, we think the

availability of testing methods of high-precision is highly beneficial.

We hereby propose new detection methods of thyroid carcinoma antigen by combining the high-intensity fluorescent nanoparticles with antibodies for the specific recognition of thyroid cancer. In previous studies, we have been developing monoclonal antibodies for the specific recognition of thyroid cancer (JT-95) [9]. The JT-95 antibody belongs to IgM group. IgM antibodies are often known to have low affinity (dissociation constant (Kd) > 10^{-5} M) and therefore they are usually neglected as tools [10]. However, the result of histological stains using JT-95 and the enzyme-labeled second antibody shows the possibility of distinguishing cancer from benign lesions with precision [9]. Moreover, we have shown using enzyme-linked immunosorbent assay (ELISA) test that these antibodies react to the plasma samples from patients with thyroid carcinoma.

On the other hand, we have been conducting researches on biochemical applications of the quantum dots (QDs) [11–17], which are the high-intensity fluorescent nanoparticles [18]. We have developed a high-intensity detection method for microbial toxins [19] or myeloperoxidase from neutrophil [20] by binding QDs to antibodies, similar as the applications in the report [21].

From these studies, we can expect that the combination of the JT-95 antibody and QDs will further increase the detection sensitivity of thyroid cancer, enabling the detection with blood test and helping judge the degree of malignancy of follicular carcinoma in the future.

In the present study, we tried to detect the antigen of SW1736 thyroid cancer cell line in microscopic analysis, Western blotting analysis, and ELISA-like system with QDs. Moreover, in order to speculate the differences of reactivities between streptavidin-conjugated QDs (streptavidin-QDs) and avidin-conjugated-horseradish peroxidase (avidin-HRP) or between IgM and IgG during the immunoassays, we investigated the size distributions of immunocomplexes.

2. Materials and Methods

2.1. Cell Line. SW1736 human thyroid carcinoma cell line was used in this study. Previous study showed that the JT-95 antibody recognized the sialic-acid-modified fibronectin from the cells [22]. Pancreatic cell lines (10 cell lines: KP4, KP4-1, KP4-2, IC3, 2C6, T3M4, Panc28 Puru, Panc28 DB2M, Panc-1, and MiaPaca2) were used as negative control cells in Western blotting analysis. Green fluorescent protein- (GFP-) expressed U937 human leukemic monocyte lymphoma cell line was used as negative control cells in fluorescent microscopy analysis.

2.2. Purification of JT-95 IgM Antibody and Normal Mouse IgM Antibody. For the preparation of JT-95 antibody, antibody-producing hybridoma was cultured in serum-free medium (Cosmedium-001, CosmoBio Corporation, Tokyo, Japan). Normal mouse IgM was prepared from mouse serum (BALB/cA Jcl, Clea Japan, Tokyo, Japan). The JT-95 antibody and normal mouse IgM were purified using an affinity IgM column (HiTrap IgM purification HP column, GE Healthcare, Buckinghamshire, UK) and bound; the IgM antibody was eluted with glycine buffer (0.2 M glycine, 0.15 M NaCl, pH 2.8). Eluates were neutralized with a 1/10 volume of 1 M Tris buffer (tris hydroxymethyl aminomethane, pH 8.5), and the IgM concentrations were determined by a spectrophotometer.

2.3. QDs Imaging of Cells and Observation under Microscopy. The 4×10^4 cells of SW1736 cells were cocultured with the 4×10^4 cells of GFP-expressed U937 cells on collagen-coated chamber slides (4-well) for 2 days. The co-cultured cells were fixed with 4% (w/v) paraformaldehyde in Dulbecco's PBS (DPBS) for 20 minutes. The 9 μg of JT-95 or normal mouse IgM in 300 μL Block Ace (Snow Brand Milk Products Corporation, Tokyo, Japan) was added and incubated for 90 min. Then biotinylated anti-IgM antibody in Vectastain ABC mouse IgM kit (Vector Laboratories, Inc., CA, USA) was added as second antibody and incubated for 60 min. After the incubation, the slides were washed and 20 nM of streptavidin-Qdot 655 (Qdot 655 ITK streptavidin conjugation kit, Invitrogen Corporation, USA) or streptavidin-PE (BioLegend, CA, USA) was added and incubated for 30 min. The cells were observed with BZ-9000 fluorescent microscope (Keyence Corporation, Osaka, Japan). The slides were washed with DPBS 3 times between all procedures.

2.4. Western Blotting Analysis. In order to evaluate the immunoreactivity of the JT-95 with QDs, Western blotting analysis was conducted. SW1736 cells were dispersed in DPBS and lyed with supersonic on ice (3 seconds × 3 times). The lysates were separated by 7.5% SDS-PAGE and transferred to a membrane (Hybond-P, Amersham Biosciences, Habersham, UK) for 60 min. The membranes were blocked with the Block ace overnight at 4°C. The 7.5 μg of JT-95 or normal mouse IgM in 5 mL Block ace was added and incubated for 90 min. Then biotinylated anti-IgM antibody in the Vectastain ABC mouse IgM kit, was added as second antibody and incubated for 60 min. After the incubation, 20 nM of streptavidin-Qdot 655, or complexes of Avidin DH and Biotinylated Horseradish Peroxidase H (HRP) in the kit was added and incubated for 30 min. After washing of the membrane, fluorescence of the Qdot 655 was examined under 365 nm UV light excitation. Antigen-binding activity of JT-95 with Qdot 655 was confirmed by comparison to the HRP reaction with DAB kit (Vector Laboratories, CA, USA). The membranes were washed with PBS containing 0.05% Tween 20 (PBST) 3 times between all procedures.

2.5. ELISA-Like Assay with QDs. In order to evaluate the quantitative reactivity of the JT-95 with QDs, ELISA-like system was conducted. The supernatant of SW1736 cells culture media was plated on high-affinity plastic plates (96-Well Clear Flat Bottom Polystyrene High Bind Microplate: Corning, NY, USA) in 15–0.015 μg/100 μL PBS, and the plates were incubated at 4°C for 16 hours. The plate was blocked with the 200 μL of Block ace 1 hour at 37°C.

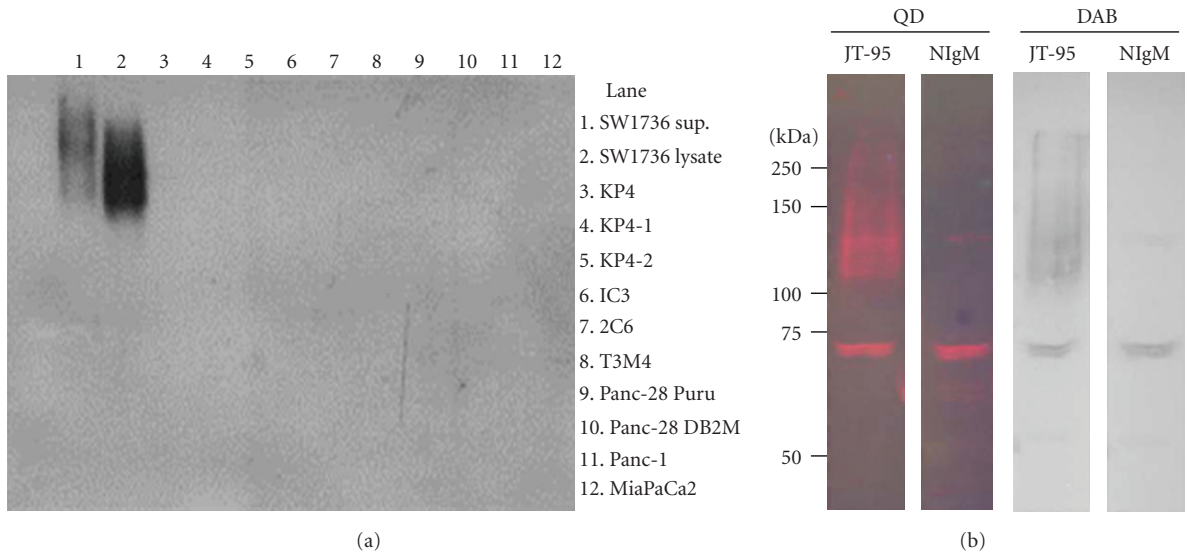

Figure 1: Western blotting analysis with JT-95 monoclonal antibody for thyroid carcinoma antigen. (a) Reactivity of JT-95 antibody with HRP-anti-IgM antibody against SW1736 thyroid carcinoma cell lines (lanes 1, 2) and lysates of pancreatic carcinoma cell lines as negative controls (lanes 3-12). This membrane image was developed with ECL Plus Western Blotting Detection System (GE Health Care). Sup.: supernatant in the culture media of SW1736 cells. (b) Reactivity of JT-95 antibody against the lysates of SW1736 cells with streptavidin-Qdot 655 staining (a) and nickel-DAB staining (b) system. The membrane stained with QDs was excited with UV (365 nm). NIgM: normal mouse IgM.

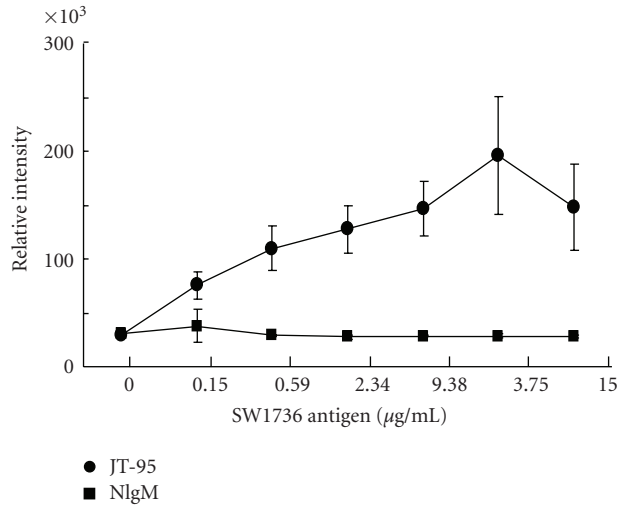

Figure 2: ELISA-like assay with QDs for the antigen of SW1736 cells. The SW1736 antigens were serially diluted and reacted with JT-95, biotinylated anti-IgM antibody, and streptavidin-Qdot 655 in immunoreaction enhancer solutions (Can Get Signal: Toyobo, Osaka, Japan).

The 1.13 μg of JT-95 or normal mouse IgM in 50 μL immunoreaction enhancer solution 1 (Can Get Signal: Toyobo, Osaka, Japan) was added and incubated for 2 hours. Then the 1.25 μg of biotinylated anti-IgM antibody in the 50 μL enhancer solution 2 was added and incubated for 60 min. After the incubation, 5 nM of streptavidin-Qdot 655 in the 50 μL enhancer solution 2 was added and incubated for 60 min. After 100 μL of PBS was added, the reacted QDs were detected with a filter set of excitation 360 nm/emission 650 nm in DTX880 plate reader (Beckman Coulter, CA, USA). The plates were washed with PBST 3 times between all procedures.

2.6. Dynamic Light-Scattering Analysis. To investigate the conformation changes of immunoglobulin complexes during the process of immunoassays, we attempted to measure the particle diameter of the complexes by dynamic light scattering (DLS) with Zetasizer Nano (Malvern Instruments, Worcestershire, UK). The 0.5 mg/mL of JT-95 antibody or Mouse IgG_1 antibody (Mouse IgG_1 Isotype Control, R&D Systems, MN, USA) in DPBS was incubated with 20 μL of biotinylated anti-IgM antibody or biotinylated anti-IgG antibody in the Vectastain ABC kit for 60 min, respectively. After the incubation, the streptavidin-Qdot 655 was added to the concentration of 200 nM, or 20 μL of the avidin-HRP

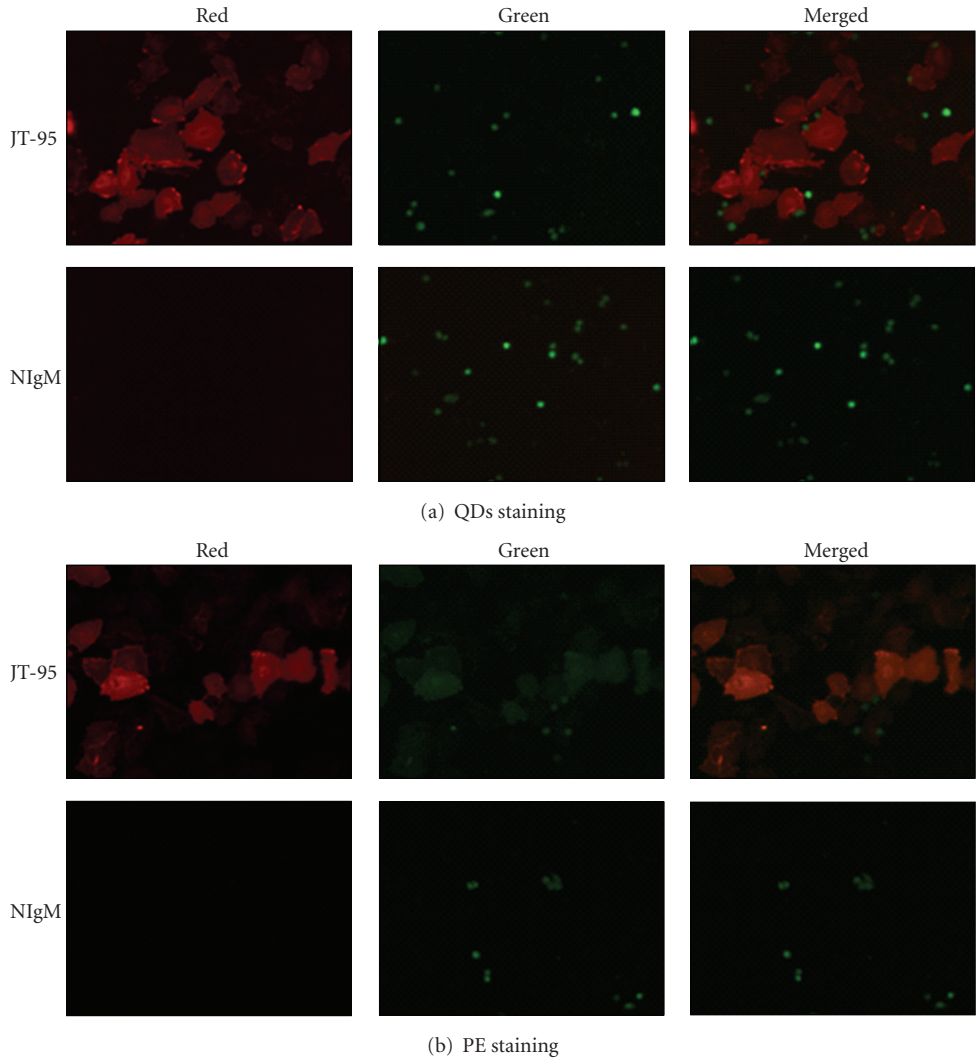

Figure 3: Fluorescent microscopic analysis of SW1736 cells with JT-95 monoclonal antibody. (a) Streptavidin-Qdot 655 staining. (b) Streptavidin-PE staining. NIgM: normal mouse IgM (Magnification: ×80).

complexes (Avidin DH and Biotinylated Horseradish Peroxidase) was added.

3. Results and Discussion

In previous study, we showed that the JT-95 antibody recognized antigen from SW1736 thyroid carcinomacell lines as well as malignant lesions and serum from patients [9]. In order to reaffirm the specificity, we investigated the affinities of JT-95 antibody against several carcinoma cell lines (Figure 1(a)). Western blotting analysis with HRP-enhanced chemiluminescent (ECL) system showed that the JT-95 antibody recognized the supernatant in cultured medium and whole lysate of SW1736, while the JT-95 antibody did not recognize10 kinds of pancreatic carcinoma cell lines as negative controls.

Although the ECL system has high sensitivity, the system needs development procedure in a dark room. For the usability, we attempted Western blotting analysis using streptavidin-QDs or nickel-diaminobenzidine (DAB) system as concise methods. The results showed a band in the range of 100–250 kDa was detected in both the detection system using QDs and the system using DAB (Figure 1(b)). In comparison to DAB system, the visibility of the band is higher with QDs. With normal mouse IgM as a negative control, bands were found in 2 places. These can possibly be attributed to nonspecific binds of IgM.

Quantitative reactivity was measured with ELISA-like assay. The JT-95 bound to the SW1736 antigens in a dose-dependent manner in the range of 0.15–3.75 μg/mL (Figure 2). Previous report using peroxidase ELISA system showed that the JT-95 bound to the membrane fraction of papillary carcinoma in the range of 1–100 μg/mL [9]. Since the range of QDs assay are sufficiently-lower than that of previous ELISA system, our QDs system will be applicable to antigen screening.

In order to investigate the possibility of usage in pathology assessment, we applied QDs and JT-95 antibody to stain

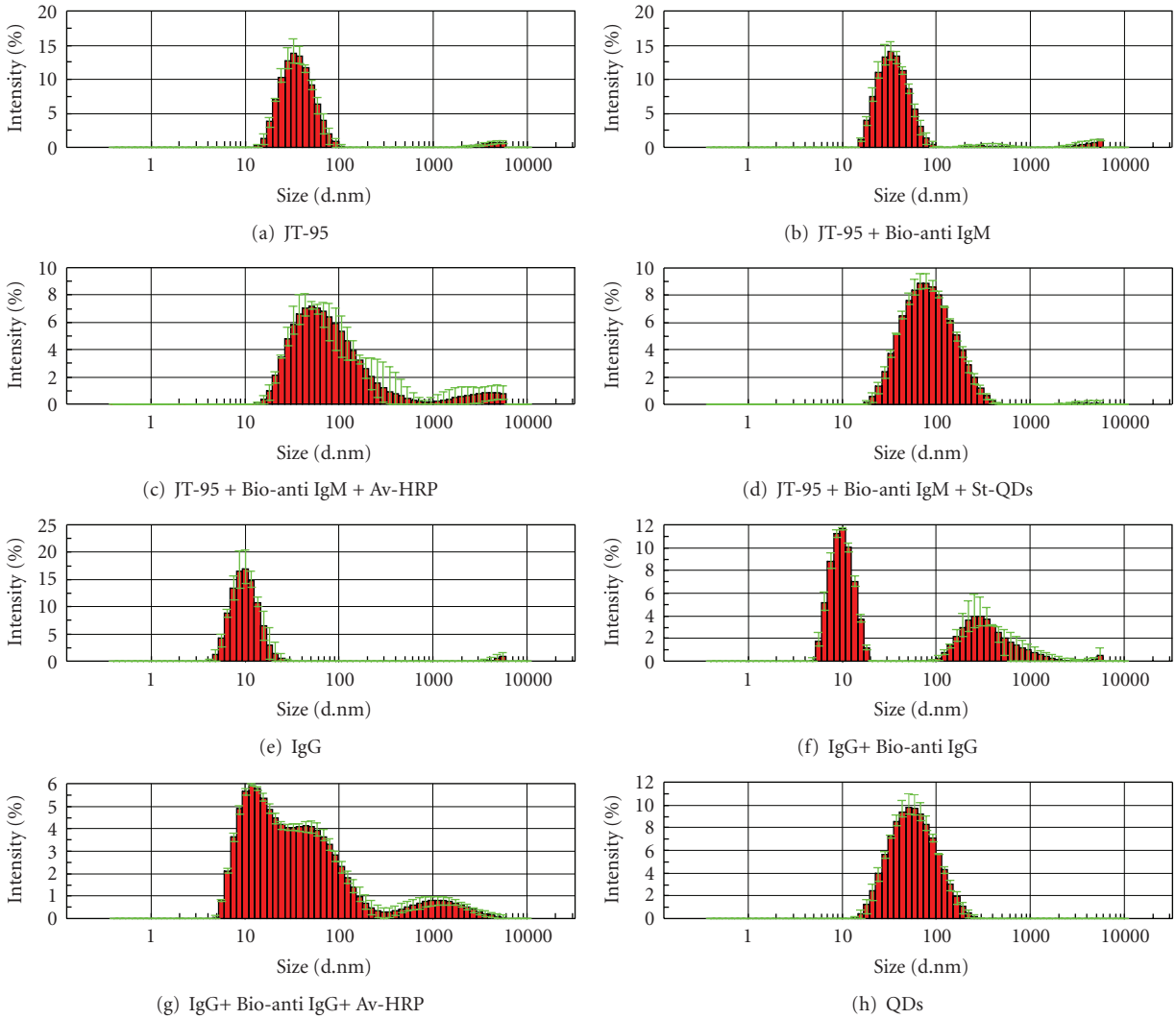

Figure 4: Size distributions of immunoglobulin and immunocomplexes in immunoassay procedures. The size distributions during reactions of JT-95 antibody were shown in (a)-(d). JT-95 antibody in DPBS (a) was reacted with biotinylated anti-IgM antibody for 60 min (b). After the reaction, the solution was incubated with avidin-HRP (c) or streptavidin-Qdot 655 (d) for 30 min. As a comparison, the size distributions during reactions of normal mouse IgG_1 were shown in (e)-(g). The IgG_1 (e) was reacted with biotinylated anti-IgG antibody for 60 min (f). After the reaction, the solution was incubated with avidin-HRP (g). On the other hand, aggregation occurred, in the solution which incubated with streptavidin-Qdot 655 (data not shown). The size distribution of only streptavidin-Qdot 655 in DPBS (h).

SW1736 cells. Figure 3(a) shows the images where thyroid cancer cell lines were stained with the combination. The JT-95 antibody is known to recognize the mutant fibronectin on cell membranes, which are also found to be strongly stained in this study. The co-cultured U937 cells expressing GFP are not stained with this system, denoting its specificity of antigen staining. No QDs were detected using normal mouse IgM. As a comparison with a conventional method, we conducted phycoerythrin (PE) staining (Figure 3(b)). The combination of PE and JT-95 antibody also stained the membrane of SW1736 cells. However, the emission from PE was detected not only in the Red channel but also in the Green channel, since the fluorescence of PE has broad emission band. When double staining was conducted in pathological assessment, narrow-emission QDs may be better suited.

There are problems to solve, however. It needed 3 seconds to identify the fluorescence from QDs by a microscopic observation, which is low in sensitivity when compared to conventional detection systems which require less than 1 second. This may be possibly attributed to the high molecular weights or large size of the combination of QDs and IgM.

On the other hand, although we succeeded in constructing an ELISA system with avidin-HRP and JT-95 antibody in PBS, we failed to construct an ELISA-like assay with streptavidin-QDs and JT-95 antibody in PBS. Therefore we conducted the latter assay in immunoreaction enhancer solution (see Materials and Methods section). In order to speculate the difference of reactivity between avidin-HRP and streptavidin-QDs, we measured the size distributions of immunocomplexes in the immunoassay procedures by

dynamic light scattering (Figure 4). The JT-95 antibody showed the peak size of 32.7 nm in DPBS (Figure 4(a)). After adding biotinylated anti-IgM antibody, the peak size was 32.7 nm as same (Figure 4(b)). Additionally, the new slight peak appeared within 100–1,000 nm. When avidin-HRP was added, the peak size moved to 50.7 nm (Figure 4(c)). The complexes within 100–1,000 nm increased drastically and the complexes over 1,000 nm increased slightly. While adding streptavidin-QDs, the peak size moved to 78.7 nm (Figure 4(d)). The complexes in 100–1,000 nm increased similarly. Different from the avidin-HRP complexes, QDs complexes within 2,300–4,800 nm were detected slightly. These results suggested that the dispersed larger size complexes over 1,000 nm, which was supposed to be constructed from several biotin-avidin or -streptavidin complexes, determine the detection abilities.

As a comparison with another immunoglobulin reactivity, we conducted the similar experiment with normal mouse IgG_1 antibody. The IgG_1 antibody showed the peak size of 10.1 nm (Figure 4(e)). After adding biotinylated anti-IgG antibody, the peak size was 10.1 nm as same (Figure 4(f)). Drastically, the new peak of complexes appeared within 100–2,000 nm. When avidin-HRP was added, the peak size moved to 13.4 nm (Figure 4(g)). Different from the final JT-95 antibody complexes with avidin-HRP (Figure 4(c)), the second highest peak of complexes appeared in 43.8 nm. Additionally, complexes at the peak size of 1,110 nm increased. When we added streptavidin-QDs with the IgG antibody and biotinylated-anti-IgG antibody dispersion, the complexes aggregated and precipitated (data not shown).

From these results, we revealed the ratio of IgG and the biotinylated antibody was higher than that of IgM case (Figure 4(b), (f)). For further detection ability, it may require the conformation change from IgM to IgG, or stronger binding ability of biotinylated anti-IgM antibody. Additionally, as a reason for fail in the ELISA-like assay with streptavidin-QDs, high molecular weight or aggregations of QDs may affect an antigen-ELISA plate interaction. The streptavidin-QDs have 5–10 streptavidins per one QD label (manufacturer's protocol). The complexes of JT-95 antibody and the biotinylated antibody were cross-linked with streptavidin-QDs and may become higher-molecular weight complexes or aggregation in near antigen area, since the QDs, which were added with the IgG_1 antibody and biotinylated-anti-IgG antibody dispersion, aggregated and precipitated. For the amelioration in the ELISA-like assay with QDs, the JT-95 antibody may need direct labeling or fragmentation for saving molecular weight.

In this study, we have succeeded in the detection of thyroid carcinoma antigen using the Western blotting analysis, ELISA-like assay, and staining thyroid cancer cell lines with QDs using a microscopy. Thus, we have opened up the possibility that antibodies for higher specific recognition, even IgM, will be applicable to the detection system with QDs.

In our previous study, the JT-95 antibodies in combination with the enzyme-labeled second antibody showed a diagnostic accuracy of 95% in papillary carcinoma and 75% in follicular carcinoma [9], when stained with sections of thyroid cancer tissues. In the future it will be necessary to compare the accuracies between the enzyme system and the QDs system. Especially in the Western blotting system, the visibility was higher and a stronger band was detected than when stained with DAB in this study. As some cancer cells express different sugar chains in differentiation or organs [23], the higher sensitive detection of bands may be helpful for judging the degree of malignancy.

4. Conclusion

We have succeeded in building new detection systems by the combination QDs and JT-95 antibody for recognition of thyroid carcinoma. These methods involved the use of IgM antibody with QDs and that is academically meaningful in the antibody detection methods where IgG is the mainstream. Although these methods need amelioration for clinical use, we are of the opinion that in the future this can become a complementary technique to the existing methods by applying to pathological assessment and blood test.

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