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ARTICLE TYPE

A rapid and convenient method for detecting a broad spectrum of malignant cells from malignant pleuroperitoneal effusion of patients using a multifunctional NIR heptamethine dye

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Detection of malignant cells from malignant effusion is very crucial to establish or adjust therapies of patients with cancer. The conventional qualitative detecting mean in malignant pleuroperitoneal effusion is cytological analysis, which is time-consuming and complicated. Therefore, a faster and more convenient detecting strategy is urgently needed. In this study, we reported a rapid method to detect malignant cells from malignant pleuroperitoneal effusion (hydrothorax and ascites) of patients using IR-808, a tumor-targeted near-infrared (NIR) fluorescent heptamethine dye (tNRI dye), which exhibited superior labeling efficacy without specific conjugation to biomarkers. The targeted imaging performance to malignant cells using IR-808 was confirmed by comparing with normal cells, and the fluorescent stability assay of IR-808 in malignant effusion was determined from 1 h to 48 h. In order to save time and dose, the incubation time and concentration were optimized to 10 min and 5 μM, which were used to detect malignant cells from 28 clinical samples of malignant pleuroperitoneal effusion. The results revealed that IR-808 could be internalized selectively by malignant cells of samples, and it could distinguish these malignant cells easily from normal cells under fluorescence microscope. The positive rates between cytological analysis and IR-808 staining method were 86 % (24/28) and 79 % (22/28), respectively. An excellent concordance level (Kappa=0.752, P<0.001) was observed between the two methods. Our results indicated that IR-808, a new NIR fluorescent heptamethine dye with unique optical imaging and tumor targeted properties, could provide a fast and simple way to detect a broad spectrum of malignant cells from malignant pleuroperitoneal effusion in malignant cancer patients.

Introduction

Patients with cancer often have abnormal fluid accumulates relevant to the imbalance rate between entry and exit of fluid into the pleuroperitoneal space. [1-3] Malignant pleuroperitoneal effusion (malignant hydrothorax and ascites) is mostly caused by the invasion and metastasis of malignant cells to pleuroperitoneal membranes, which is common in patients in advanced stage and related to a poor prognosis with a short median survival about 9-17 months after first-diagnosis. [4] Since the presentation of malignant pleroperitoneal effusion precludes patients from crative resection, the detection of malignant pleroperitoneal effusion has great influence on the clinical decision.

In clinical practice, the malignant pleuroperitoneal effusion is determined based on the detection of malignant cells from effusion by cytological analysis. [5-8] Cytological features of malignant cells are characterized by the presentation of nuclear atypia, multinucleation, inclusion body and cell cluster formation. [9, 10] However, the similarity of mesothelial cells with malignant cells in morphology often makes the distinction difficult, especially when the number of cells in effusion is small. [11-13] Furthermore, the procedure of cytological analysis is complicated, and its result is highly dependent on the operator's experience, which makes this method time-consuming and vulnerable to subjective bias. [14] Therefore, a faster and much simpler method to detect malignant cells from pleuroperitoneal effusion is urgently needed.

A new class of tumor-targeted near-infrared (NIR) fluorescent heptamethine dyes (tNRI dyes) has attracted great interest in biodetection and biomedical imaging. [15, 16] These tNRI dyes may preferentially accumulated in the mitochondria of malignant cells, but not in normal, dead or apoptotic cells. [17] And the NIR emission (700-900 nm) of these dyes significantly increases the sensitivity of detection due to the low background from autofluorescence of effusion in this wavelength range. Typically, the contrast index value of cells stained by tNRI dyes can exceed 20, while a contrast index more than 2.5 is regarded as substantial accumulation in malignant cells. [18, 19] In addition, the fluorescence of tNIR dyes keep stable during the preparation of effusion samples, indicating that these heptamethine dyes may be a promising candidate for malignant cells targeting and detection. [20-25]

Recently, Yang et al. found that malignant cells could be tested by tNIR dye IR-783 after mixing human prostate malignant cells with human blood cells. This dye was sufficiently sensitive to detect as few as 10 malignant cells per milliliter in whole blood, indicating that this group of dyes has the potential to detect malignant cells in circulation. [26] However, to the best of our knowledge, the fluorescence detecting ability of tNRI dyes have not been investigated in malignant pleuroperitoneal effusion. Therefore, we herein exploited the ability of tNRI dyes to detect malignant cells from clinical malignant effusion as a fast and simple complementary tool to cytological analysis

In this research, a new tNIR dye with 808 nm emission (IR-808) was chosen to detect malignant cells from cultured cell lines and 28 clinical malignant pleuroperitoneal effusion samples. A conventional NIR dye Cy 7 was taken as control to compare the imaging performance. And the positive rates and concordance level between cytological analysis and IR-808 staining method were proceeded to compare the sensitivity to malignant cells in clinical samples.

Experimental section

Heptamethine dye and samples

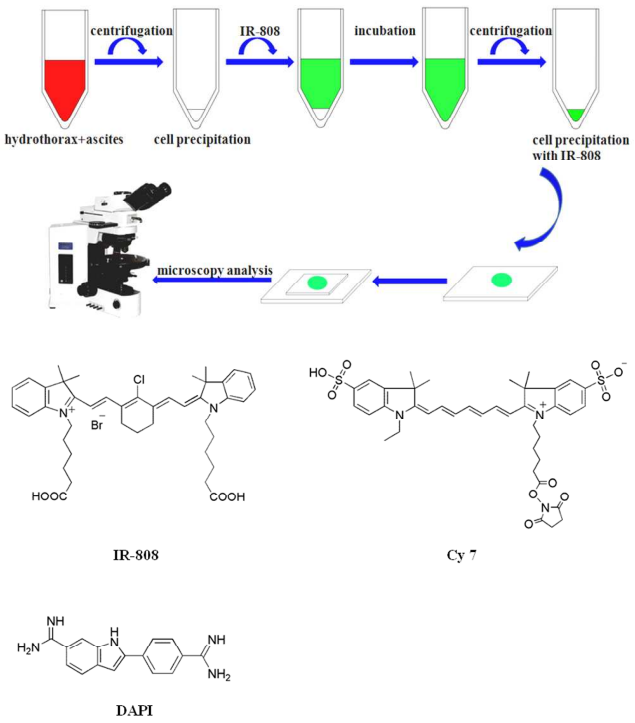
The heptamethine dye IR-808 was kindly provided by Dr. Chunmeng Shi from the Third Military Medical University at Chongqing of China, which was dissolved easily in water at 10 mM and stored at -20 °C before use. All the samples of the malignant pleuroperitoneal effusion in this study were collected from the Pathology Department of Jinling Hospital. The specimens examined in our study were also diagnosed pathologically.

Cell lines and cell culture

All human malignant cell lines were obtained from American Type Culture Collection (ATCC) and were maintained in ATCC recommended media supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), and 10 % fetal bovine serum (GIBCO, USA) at 37 °C with 5 % CO_2, including lung cancer (NCI-H460, A549), breast cancer (MDA-MB-231, MCF-7), colon cancer (HCT-116), glioma cancer (U87), renal cancer (786-O), cervical cancer (Hela), Osteosarcoma (U-2OS), and human normal cells including embryonic kidney cells (293T), vascular endothelial cells (VE), embryonic lung fibroblast (HELF), umbilical vein endothelial cells (HUVEC) were used as control.

Uptake and accumulation of IR-808 in normal and malignant cells in vitro

All cells were cultured at a density of 2×10^6 in a 35 mm petri dish with cover slips, and incubated at 37 °C with 5 % CO_2 for 24 h. The cover slips were incubated with IR-808 at a concentration of 20 μM for 20 min at 37 °C after the cells had reached a confluence of 70 %. Then the cells were stained with 4′, 6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, USA) of cell nuclei after phosphate-buffered saline solution (PBS) washing according to the kit and fixed with 4 % formaldehyde at 4 °C after PBS washing again. The cover slips were covered with microscope slides by an aqueous mounting medium. Images were recorded by a fluorescence microscope (Olympus IX71, Japan) equipped with fluorescent filter sets (excitation, 684-719 nm; emission, 740-950 nm).



Scheme 1 A schematic representation for explaining the whole process of fluorescent heptamethine dye IR-808 to diagnose malignant cells from malignant pleuroperitoneal effusion (hydrothorax and ascites), and the chemical structures of IR-808, Cy 7 and DAPI.

To further optimize the dose and incubation time, a group of 2×10^6 MCF-7 cells were chosen to incubate with IR-808 at the final concentrations of 1.25, 2.5, 5, 10, 20 μM for 20 min, and another group of MCF-7 cells were incubated with a gradual times (5 min, 10 min, 30 min, 1 h, 8 h, 24 h), then all cells were stained with DAPI, 4 % formaldehyde in turn, and imaged with the fluorescence microscope at the same exposure time. The mean fluorescent intensities of the groups were statistically analyzed. Then MCF-7 cells were chosen to co-incubate with IR-808 and DAPI at the best concentration and time, the pictures were got subsequently.

Fluorescent stability assay of IR-808 in malignant pleuroperitoneal effusion

The fluorescent stability of IR-808 in ultra-pure water, malignant pleuroperitoneal effusion cell precipitation mixture with a concentration of 5 μM was performed at 37 °C for 1 h, 2 h, 4 h, 8 h, 12 h, 24 h, 48 h using an IVIS Lumina XR (Xenogen Corporation-Caliper, Alameda, CA, USA) with NIR wavelength (745 nm excitation, 830 nm emission) respectively. Eventually the cell precipitations after centrifugation were dropped on microscope slides and imaged with the same NIR imaging method.

Assessments of IR-808 in the malignant pleuroperitoneal effusion samples

All the samples of the malignant pleuroperitoneal effusion were collected after centrifugation at 1000 rpm for 5 min, leaving cell precipitations only. The cells were co-incubated with IR-808 and DAPI at a concentration of 5 μM for 10 min, and incubated with

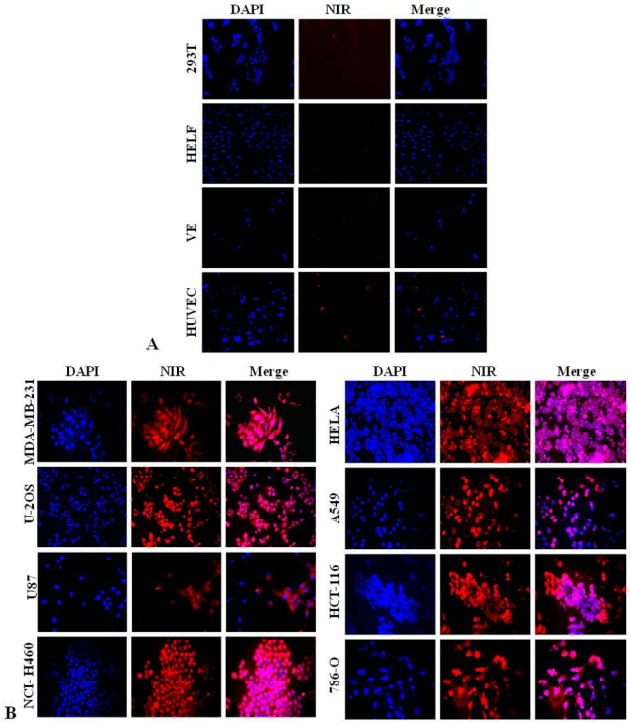


Fig. 1 (A) Normal human cells (293T, HELF, VE, HUVEC) showed no uptake of IR-808 in culture. (B) Human malignant cell lines (MDA-MB-231, U-2OS, U87, NCI-H460, Hela, A549, HCT-116, 786-O) showed significant uptake of IR-808 under similar staining and imaging conditions. Results are shown with images obtained from cells stained with 4′, 6-diamidino-2-phenylindole (DAPI) of cell nuclei, IR-808 staining (NIR), and a merger of the two images (Merge). All images were acquired at 100×magnification.

formaldehyde for 20 min, then smeared on microscope slides and observed according to the procedures described above. Malignant hydrothorax samples were chosen to incubate with IR-808 and cyanine dye Cy 7, another common NIR heptamethine cyanine, in order to compare the imaging performance to malignant cells. Images and cells count were recorded by the special NIR fluorescence microscope. The processes were consistent with above. The whole diagnosis process was shown briefly in scheme 1 with the chemical structures of IR-808, Cy 7 and DAPI.

Histological and Statistical Analysis

The collected cells of malignant pleuroperitoneal effusion were smeared on microscope slides after centrifugation at 1000 rpm for 5 min, then fixed in 95 % ethanol for 30 min, and stained with hematoxylin and eosin for 2 min. The whole slides needed washing for 1 h and drying for pathological analysis.

All statistical analyses were performed using SPSS 19.0 software program (SPSS, Chicago, IL). The statistical significance of the two detective methods were evaluated via McNemar paired chi-square test and Kappa test from descriptive statistics. Data were expressed as mean ± SD, a two-sided P value of less than 0.05 was considered statistically significant in all of the statistical tests.

Results and Discussion

Preferential accumulation of IR-808 in malignant cells

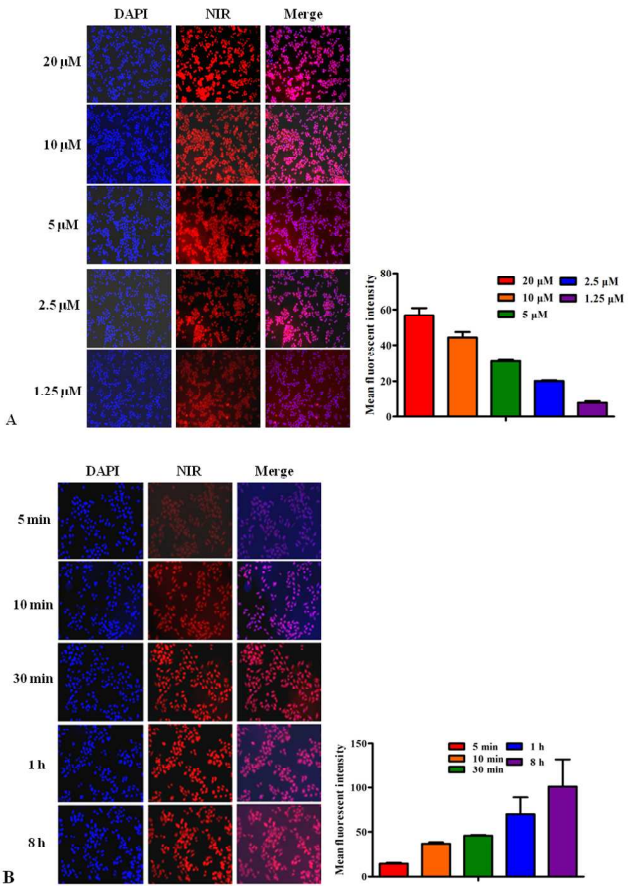


Fig. 2 To further optimize the dose and time, 2×10^6 MCF-7 cells were chosen to incubate with IR-808 at the final concentrations of 1.25, 2.5, 5, 10, 20 μM for 20 min (A), and time points of 5 min, 10 min, 30 min, 1 h, 8 h (B), then stain with DAPI, IR-808 staining (NIR), and a merger of the two images (Merge). The mean fluorescent intensities at different conditions were statistically analyzed. All images were acquired at 100×magnification.

Cells labelled by IR-808 were observed in a fluorescence microscope with special near infrared absorption and emission spectrum. The uptake and accumulation of IR-808 were studied in four human normal cell lines (Fig. 1A) and eight human malignant cell lines (Fig. 1B). In this experiment, we used the compatible incubation time at 20 min and the concentration of 20 μM. The dye was not found to accumulate in human normal embryonic kidney cells (293T), vascular endothelial cells (VE), embryonic lung fibroblast (HELF), umbilical vein endothelial cells (HUVEC), but in human lung cancer (NCI-H460, A549), breast cancer (MDA-MB-231), colon cancer (HCT-116), glioma cancer (U87), renal cancer (786-O), cervical cancer (Hela), osteosarcoma (U-2OS). The images obtained from cells stained with DAPI of cell nucleus and IR- 808 of cytoplasm, which were summarized in Fig. 1. It was clear that only malignant cells could be stained, while normal cells were unstained. There were no apparent differences among all these malignant cells, indicating this dye preferentially accumulated in malignant cells, was not affected by cell different surface receptors or biological factors, which were further confirmed that IR-808 staining method was a broad spectrum technology on detecting malignant cells.

Targeted imaging property of the lipophilic dye was attributed to the higher negative inside mitochondria

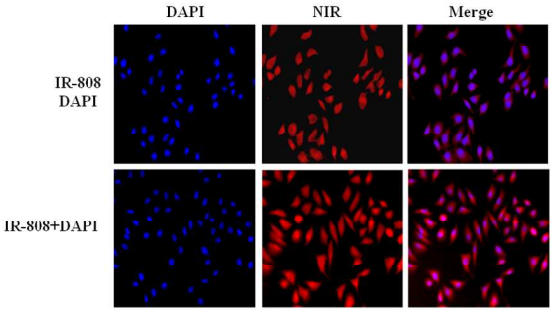


Fig. 3 To further test the effect of co-incubation with DAPI and IR-808 (IR-808 + DAPI), MCF-7 cells were chosen to incubate at the concentrations of 5 μM about 10 min, compared with individual incubation (IR-808, DAPI). All images were acquired at 100×magnification.

transmembrane potentials of malignant cells than their normal counterparts, verified by co-localization with a membrane potential-dependent mitochondrial tracker, rhodamine 123, which made IR-808 as a broad spectrum detection agent because most malignant cells have been identified with higher mitochondrial membrane potentials. [27-30] In addition, viable malignant cells could be distinguished from dead cells or apoptotic cells because the whole reaction pathway was energy-dependent.

There were a few stains in HUVEC cell lines by NIR fluorescence imaging, because a few non-specific staining or spots appeared inevitably on the slides.

Effect of IR-808 on concentration and time test

In order to determine whether IR-808 with low concentration had good imaging ability, we chose a gradient ranging from 20 to 1.25 μM to dye the human breast cancer MCF-7, and other experimental conditions such as the cells count, the treatment temperature and the exposure times were consistent with each group. As shown in Fig. 2A, imaging differences were detected as the change of concentration in these assays, and malignant cells could be dyed even at a concentration of 1.25 μM. The concentration of 5 μM was chosen to the next study because the NIR fluorescence signal at this concentration could reach a contrast value over 20. So low dose of IR-808 could be chosen to incubate, and the optical property of targeted imaging was not apparently affected, so this new method may be cost-saving than clinical practice.

From Fig. 2B, the targeted imaging was differentiated due to the incubation times. It was appropriate and enough to detect malignant cells when the cells were incubated with IR-808 about 10 min, which was less than clinical routine operation. Further performance of co-incubation with DAPI and IR-808 was tested at 5 μM about 10 min, compared with individual incubation. The results revealed that the detection performance was not influenced when samples were co-incubation with IR-808 and DAPI, indicating that the new method was timesaving without losing its accuracy (Fig. 3).

Optical properties of IR-808 in malignant pleuroperitoneal effusion samples

Zhang et al. tested that the fluorescence signal of these dyes was pretty stable after formalin fixation, raising the possibility of developing sensitive means of detecting target sites especially for tumor evaluation. [31] As shown in Fig. 4A, the fluorescence

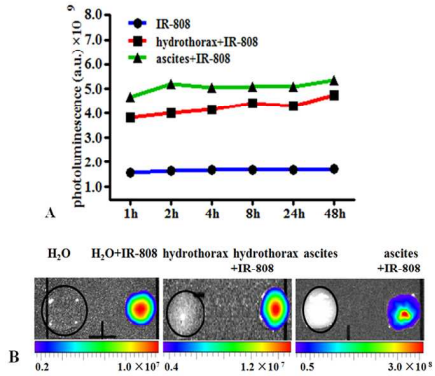


Fig. 4 The fluorescent intensity of malignant pleuroperitoneal effusion (hydrothorax and ascites) with IR-808 at different time points (1 h, 2 h, 4 h, 8 h, 24 h, 48 h) was calculated (A). The cell precipitations from malignant pleuroperitoneal effusion were collected and imaged respectively (B).

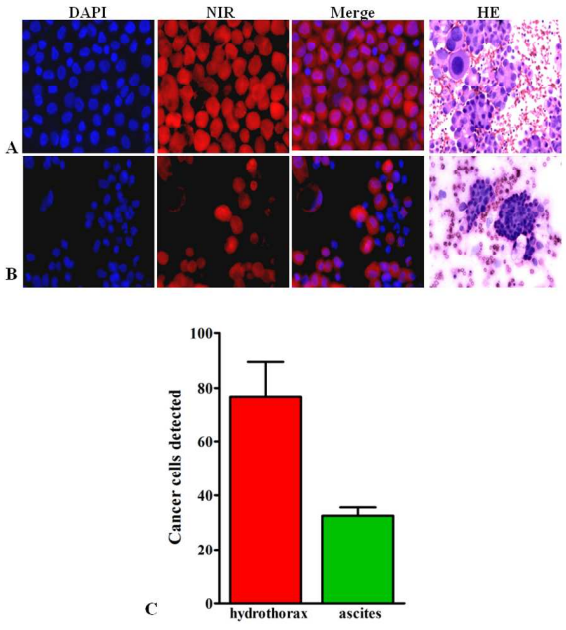


Fig. 5 Malignant cells of hydrothorax (A) and ascites (B) samples were dyed by DAPI, IR-808 staining (NIR), and a merger of the two images (Merge), which could be clearly observed after IR-808 NIR imaging, but other cell types of the samples did not be stained. Cytological analysis (H&E) of malignant cells from malignant pleuroperitoneal effusion was conducted accordingly. All images were acquired at 400×magnification. The percentage of staining malignant cells in all cell types from the two samples was counted respectively (C).

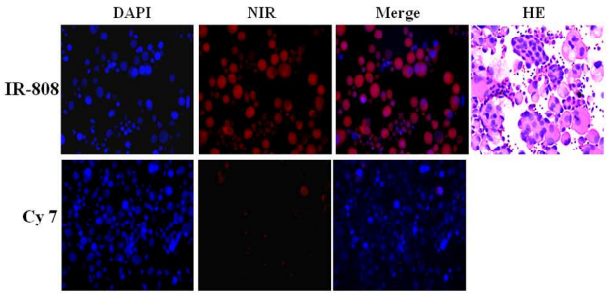


Fig. 6 Malignant cells in hydrothorax samples were dyed by IR-808 and Cy 7. DAPI, IR-808 or Cy 7 staining (NIR), a merger of the two images (Merge) and H&E were conducted in turn. Malignant cells could be clearly observed after IR-808 NIR imaging, but Cy 7 could not. All

images were acquired at 400×magnification.

Table 1 Characteristics of 28 patients and comparison of the two methods

	Case No.	hydrothorax	ascites	
Overall	28	17	11	
Sex				
Male	16	12	4	
Female	12	5	7	
Age (years)				
≤55	5	3	2	
>55	23	19	4	
Cytological analysis	24	17	7	Kappa= 0.752
IR-808 staining	22	15	7	P<0.001

intensity of IR-808 was investigated from 1 h to 48 h by IVIS Lumina XR (745 nm excitation, 830 nm emission), which kept a well, stable fluorescence intensity in malignant pleuroperitoneal effusion (hydrothorax and ascites) at the initial and final test time points, indicating that the fluorescence intensity of IR-808 was very stable for further imaging application. The photoluminescence of IR-808 in malignant pleuroperitoneal effusion showed a higher value, indicating that some large molecular agents in malignant effusion such as albumin, enzymesand carcinoembryonic antigen, may be nonspecific labelled by excess dyes, but this phenomenon could not affect malignant cellular imaging under the microscope. After 48h, cell precipitations in malignant pleuroperitoneal effusion were collected and imaged after centrifugation, leading the result that only the samples including malignant cells showed targeted imaging (Fig. 4B).

Detection of malignant cells from malignant pleuroperitoneal effusion

Encouraged by the above results, we then studied whether the dye could detect malignant cells from malignant pleuroperitoneal effusion of patients with lung cancer, ovarian cancer and breast cancer. Fig. 5 showed that malignant cells from malignant effusion samples could be stained after incubating with IR-808, while other cell types of the samples such as macrophages and mesothelial cells did not be stained. The percentage of the staining malignant cells from the two samples was counted respectively (76.42±13.13; 32.48±3.07), indicating that not all cell types could be visualized except malignant cells. Cytological analysis of malignant pleuroperitoneal effusion was conducted parallel. Prominently, the whole procedure of the new detecting method cost about 1.5 hours including centrifugation, washing, incubation, smearing and imaging, and the diagnostic report for cancerous or non-cancerous effusion could be obtained preliminarily by the detection of labelled cells. While cytological analysis needed much more time to wash and drying. So, the timesaving and convenient operations of the new detection method were more worthy than cytological analysis. But it could not be used to confirm the characteristics of malignant cells morphologically.

We also used Cy 7 as control, it was found that no Cy 7 retained in malignant cells in hydrothorax samples (Fig. 6), suggesting that not all fluorescent heptamethine dyes just like Cy 7 were attached to the malignant cells typically in a broad spectrum range and characterized with simultaneous tumor targeting and NIR imaging capabilities.

Comparison of detection rate between cytological analysis with the new staining method

28 samples (17 hydrothorax and 11 ascites) in this study were collected from definite diagnostic patients (16 males and 12 females; age: 61.44±10.26) from May to September in 2013 (Table 1). Using cytological analysis and IR-808 staining method, the positive rates in the 28 samples with cancer were 86 % (24/28) and 79 % (22/28), respectively. There was a well concordance level (Kappa=0.752, P<0.001) between these two methods. There were two samples detected by cytological technique but not IR-808 staining method. The reason may include 1) the quenching of NIR fluorescence happened with some inevitable factors; 2) the integrities of some cells were destroyed; 3) exposure time of the specimens was too long thus resulted in photo bleach. All IR-808 positively stained samples were confirmed by cytological analysis, indicating its high sensitivity. There were four samples unidentified by these two methods owing to the reason that the malignant pleuroperitoneal effusion may be caused from non-cancerous lesions, such as the imbalance between osmotic pressure and blood pressure, or the stimulation of inflammation in patients with cancer. [32] It is necessary to combine with the two methods to meet the diagnosis needs.

Conclusions

At present, the clinical qualitative detecting means for malignant cells in malignant pleroperitoneal effusion are dependent on routine cytological analysis. However, the operation steps of cytological analysis are complicated and time-consuming. In this study, our fast and convenient diagnosis strategy just needs centrifugation, incubation and washing, but has high accuracy and sensitivity to diagnose malignant cells in malignant pleuroperitoneal effusion. The key of our strategy is the use of multifunctional NIR heptamethine dye IR-808, which presents the malignant cells targeting property of malignant effusion. To our knowledge, this is the first report of IR-808 as a NIR optical imaging agent to label malignant cells in clinical specimens, and this method could provide information for cytological analysis. The present results suggest that IR-808 staining method is sensitive, convenient, low-cost and timesaving; it may be useful as a tool for cytological analysis and other clinical application in future.

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Notes and references

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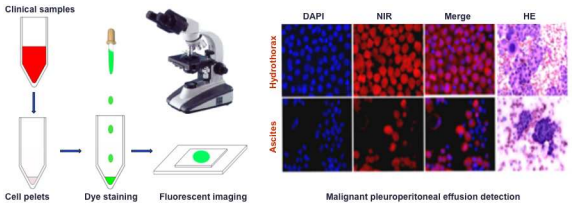

We present a fluorescent-based method to detect malignant cells in clinical pleuroperitoneal effusion samples using NIR heptamethine dye