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Tumor invasion unit in gastric cancer revealed by QDs-based *in situ* molecular imaging and multispectral analysis

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ABSTRACT

In tumor tissues, cancer cells, tumor infiltrating macrophages and tumor neo-vessels in close spatial vicinity with one another form tumor invasion unit, which is a biologically important tumor microenvironment of metastasis to facilitate cancer invasion and metastasis. Establishing an *in situ* molecular imaging technology to simultaneously reveal these three components is essential for the in-depth investigation of tumor invasion unit. In this report, we have developed a computer-aided algorithm by quantum dots (QDs)-based multiplexed molecular imaging technique for such purpose. A series of studies on gastric cancer tumor tissues demonstrated that the tumor invasion unit was correlated with major unfavorable pathological features and worse clinical outcomes, which illustrated the significantly negative impacts and predictive power of tumor invasion unit on patient overall survival. This study confirmed the technical advantages of QDs-based *in situ* and simultaneous molecular imaging of key cancer molecules to gain deeper insights into the biology of cancer invasion.

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1. Introduction

Gastric cancer (GC) is the fourth most common cancer worldwide [1], and the third leading cancer cause in China [2]. Despite recent progresses in the early diagnosis and the surgery-centered multidisciplinary treatments for GC, the overall clinical outcome of such patients is still far from satisfactory, mainly due to the post-treatment occurrence and metastasis, via blood circulation, lymphatic channels or direct cancer cells escape and seeding [3]. To tackle this problem, many efforts focusing on cancer cells have been made [4,5]. And eventually, the oncology community has come to the understanding that cancer is a disease of imbalance, *i.e.*, not merely a disease of rogue cells but the body's mismanagement of those cells, the fundamental importance of such theoretical changes is that we have to pay particular attention to tumor microenvironment, in addition to cancer cells, because tumor microenvironment plays an important role via the co-evolution of cancer cells and stroma [3,6]. Thus, exploring the co-evolution of cancer cells and surrounding stroma is a new frontier to investigate the complex mechanisms of tumor progression.

In GC, tumor microenvironment is a complex and dynamic community, which is undergoing constant evolutions during cancer invasion, involving tumor cells escape from primary sites into vasculature (blood circulation and lymphatic channel), reside and adhere to endothelial cells, penetrate from vasculature into other organs and reside in them, accompanied with tumor neo-vessels growth [7]. Many important components in tumor microenvironment work together to contribute to cancer invasion, involving inflammatory cells such as macrophages, immune cells such as T and B lymphocytes, stromal cells such as fibroblasts, and neo-blood vessels of various stages of maturity [8]. These players must be in an appropriate anatomic proximity and spatial vicinity with the tumor cells in order to facilitate cancer invasion. And indeed, recent studies have shown that tumor cells, macrophages and tumor neo-vessels in close vicinity with one another form a unique structure called tumor microenvironment of metastasis (TMEM) [9,10], or in

Abbreviations: GC, gastric cancer; TMEM, tumor microenvironment of metastasis; QDs, quantum dots; OS, overall survival; Mo, months.

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more easily understandable terms, called 'tumor invasion unit' (Fig. 1). Therefore, the simultaneous recognition and analysis of all the components in the tumor invasion unit is very important to understanding the new perspective of cancer invasion.

There are few techniques that can simultaneously image multiple components in a complex tumor microenvironment of the same tissue section. Thus, it is urgent to develop a more holistic method to image the complex interactions of stromal components *in situ*. Quantum dots (QDs), with its unique size and surface effects, have shown great potential in biomedical application, especially in multiplexed imaging *in situ* [11]. In this study, taking the advantages of established QDs-based multiplexed imaging *in situ*, we analyzed on the interactions between macrophages, tumor neovessels and cancer cells, and developed a computer-based algorithm of tumor invasion unit.

2. Materials and methods

2.1. Patients and specimens

Tissue sections (4 μm thickness) of 90 human GC cases were selected from the central database on GC established at our cancer center, including 30 with detailed pathological information, and 60 with complete clinico-pathological and survival information available on the patients. This database has been the source of information for several published studies [8,12,13]. Tumor tissues from the first set of 30 patients were used for a trial study, to explore the correlation of tumor invasion unit with classical pathological features, in order to test if there was any relationship between tumor invasion unit with unfavorable pathological features. Tumor tissues from the second set of 60 patients with detailed survival information were used for validation study, in order to further verify if tumor invasion unit could predict the overall survival (OS). The study protocol was approved by the Institutional Ethics Committee of the hospitals. Written informed consent was obtained from the patients before surgery, with permission to use the specimens for scientific research purposes, as well as clinical pathological studies.

2.2. QDs-based double molecular imaging

For QDs-based double molecular imaging, the primary antibodies were mouse anti-human monoclonal antibody against macrophages (MA1-38069, ABR, USA; dilution 1/300) and goat anti-human polyclonal antibody against CD105 (sc-20072, Santa Cruz, USA; dilution 1/300). The secondary antibodies were QDs-525 goat $F(ab')_2$ anti-mouse IgG conjugate (Invitrogen, USA; dilution 1/300, emitting green light) and QDs-655 rabbit $F(ab')_2$ anti-goat IgG conjugate (Invitrogen, USA; dilution 1/1000, emitting red light). The QDs-based *in situ* molecular imaging procedures were performed according to our previously established technical processes [11,14,15], with the following major steps: tissue slides de-paraffinizing → antigen retrieval → blocking → primary antibody for macrophages and CD105 → washing and blocking → staining with QDs-525 and QDs-655 → washing → detection and acquisition. The detailed protocol was illustrated in Fig. 2A.

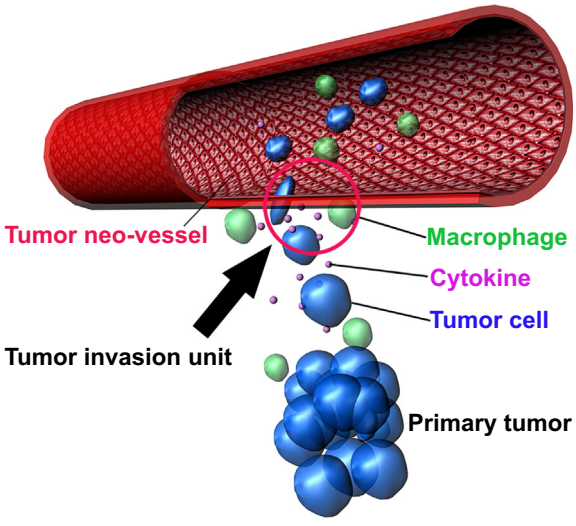

Fig. 1. The concept of tumor invasion unit. Tumor angiogenesis and macrophages infiltration are two important contributors to cancer invasion and metastasis. Tumor cells, macrophages and neo-vessels in close vicinity with one another form a spatially and functionally unique structure called tumor invasion unit to promote cancer cells invasion (Red circle in this illustration). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.3. Evaluation of macrophages and tumor neo-vessels

The QDs stained images were captured by Olympus DP72 camera (Olympus Optical Co., Ltd., Tokyo, Japan) under CRi Nuance multispectral imaging system (Cambridge Research and Instrumentation, Inc., Woburn, MA, USA). The QDs-525 and QDs-655 were excited by UV light (330–385 nm). A spectral cube for each image, which contains the complete spectral information at 10 nm wavelength intervals from 420 to 720 nm, was collected by the CRi Nuance multispectral imaging system. And all the cubes were captured under the same condition at ×200 magnification with the same settings for each image, so as to avoid the selection bias (Fig. 2B). The QDs fluorescence signal unmixing was processed by the software package within the Nuance system as previously described [16]. Then, after obtaining the images of signal unmixing, the macrophages were counted on each image and the total macrophages number was documented as the counts for the patient for further analysis. The same calculation method was used for tumor neovessels counting (Fig. 2C–D).

2.4. Definition and evaluation of 'tumor invasion unit'

After processing QDs images, the images with double signals of both macrophages and tumor neo-vessels at the tumor nest area were acquired (Fig. 2B). As the tumor invasion unit consisted of tumor cells, macrophages and tumor neo-vessels, a circle with the diameter of 60 μm [9] centered on macrophages was chosen, approximately three cell diameters across. With the computer-based algorithm, if there was any red signal in this circle, it was counted as 1, otherwise; it was counted as 0. Then the total counts of five images for each patient was output as the result of tumor invasion unit (Fig. 2D).

2.5. Statistical analysis

Statistical analyses were performed with SPSS software version 21.0 (SPSS Inc. Chicago, IL). For the comparison of individual variables, Fisher's exact test, *t* test and Mann–Whitney Test were conducted as appropriate. The Kaplan–Meier survival curves were plotted to analyze the OS by different study parameters, with log rank test to define the statistical differences between the subgroups. Two sided $P < 0.05$ was judged as statistically significant.

3. Results

3.1. Forms of tumor invasion unit in dynamic changes

With QDs-based *in situ* molecular imaging and multispectral analysis, the tumor infiltrating macrophages and neo-vessels were clearly marked in the easily discernable background of tumor tissue (Fig. 3). Based on the spreading and layout patterns of neo-vessels and the macrophages infiltration steps, two forms of tumor invasion unit in constant dynamic changes could be recognized. In form one (Fig. 3A1–A3), the tumor neo-vessel was seen in longitudinal section as shown in circle 1. With the special longitudinal spreading and irregular morphology, the tumor neo-vessels presented to be multi-angled accompanied with macrophages infiltration at each angle (Fig. 3A2, circle 2). Macrophages undergoing intravasation could also be observed, half in and half out of the blood vessel (Fig. 3A3, circle 3 & 4). In form two (Fig. 3B1–B3), the tumor neovessel is seen in cross section, with macrophages lodging the vessel with membrane processes (Fig. 3B1, circle 5), crossing the vessel wall (Fig. 3B2, circle 6), and in close vicinity to the vessel (Fig. 3B3, circle 7), thus revealing the dynamic interactions between macrophages and neo-vessels to facilitate tumor invasion.

3.2. Correlation of tumor invasion unit with clinico-pathological features

First, we selected a trial set of 30 GC patients with the three most common pathological types, including well differentiated, poorly differentiated and signet-ring cell carcinoma. These patients were classified into 3 groups by different pathological types, 10 cases in each group. The basic clinico-pathological characteristics

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