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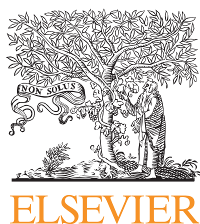

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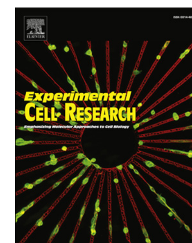

Research Article

# Nanoscale mapping and organization analysis of target proteins on cancer cells from B-cell lymphoma patients

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ABSTRACT

CD20, a membrane protein highly expressed on most B-cell lymphomas, is an effective target demonstrated in clinical practice for treating B-cell non-Hodgkin's lymphoma (NHL). Rituximab is a monoclonal antibody against CD20. In this work, we applied atomic force microscopy (AFM) to map the nanoscale distribution of CD20 molecules on the surface of cancer cells from clinical B-cell NHL patients under the assistance of ROR1 fluorescence recognition (ROR1 is a specific cell surface marker exclusively expressed on cancer cells). First, the ROR1 fluorescence labeling experiments showed that ROR1 was expressed on cancer cells from B-cell lymphoma patients, but not on normal cells from healthy volunteers. Next, under the guidance of ROR1 fluorescence, the rituximab-conjugated AFM tips were moved to cancer cells to image the cellular morphologies and detect the CD20-rituximab interactions on the cell surfaces. The distribution maps of CD20 on cancer cells were constructed by obtaining arrays of ($16 \times 16$) force curves in local areas ($500 \times 500$ nm$^2$) on the cell surfaces. The experimental results provide a new approach to directly investigate the nanoscale distribution of target protein on single clinical cancer cells.
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## 1. Introduction

The integral membrane protein CD20 is 297 amino acids long with a molecule weight about 33 kDa [1]. The exact biological function of CD20 is currently unknown [2], partly because it has no known natural ligand and CD20 knockout mice display an almost normal phenotype [3]. Many of the functions of CD20 have been determined using artificial ligands (antibody) [4]. In vitro experiments proposed that CD20 itself functioned as a calcium ion channel, although direct evidence was lacking [5]. CD20 is an ideal target for monoclonal antibodies (mAb), as it is expressed at high levels on most tumor B cells, but does not become internalized or shed from the plasma membrane after being bound by mAb [6]. The first mAb targeting CD20 was rituximab which was approved in 1997 by the US Food and Drug Administration (FDA) to treat B-cell non-Hodgkin's lymphoma (NHL) [7]. The in vivo mechanisms of rituximab's killing effects are still unclear (in vitro experiments indicate that the potential mechanisms include Fc-FcγR-dependent interactions, complement-dependent cytotoxicity, direct induction of apoptosis, and adaptive cellular

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immunity) [8], but its efficacy is undoubtedly in clinical practice. The use of rituximab, particularly in combination with conventional chemotherapy such as CHOP, has significantly improved the overall response rates and survival of patients with NHL [6,9,10]. The unprecedented success of rituximab makes it become a mainstay in the therapy of a broad variety of B-cell NHL [11,12]. However, there are still many patients who present native or acquired resistance to the mAb treatment [13]. Many new anti-CD20 mAbs with gene-engineered modifications have been developed, but to date none of the newer anti-CD20 antibodies have been shown to be clinically more effective than rituximab in a direct comparison [14]. The knowledge of rituximab's in vivo mechanisms is as yet very scarce, greatly hindering the development of anti-CD20 mAbs. In order to develop new anti-CD20 mAbs with enhanced efficacy, we should have a thorough understanding of the physiological activities involved in rituximab's killing mechanisms, particularly the activities in vivo. Viewed from this aspect, directly investigating the behaviors of tumor cells from B-cell NHL patients will be of significantly clinical impact.

In the era of personalized treatment, the prerequisite for therapy planning is to have accurate diagnostic tests that identify patients who can benefit from the targeted therapies [15]. This requires we should exactly characterize the pathological properties (such as gene expression [16]) of the patients at single-cell and single-molecule levels [17]. The advent of atomic force microscopy (AFM) provides a nanoscopic window to understand the activities of single molecules on the surface of living cells in aqueous environment [18]. By linking ligands onto the AFM tip, AFM can probe the individual specific receptor-ligand interactions on the cell surface, and this technique is termed single-molecule force spectroscopy(SMFS) [19]. SMFS has now been widely used in life sciences and a broad variety of receptor-ligand interactions have been detected, but these researches were commonly performed on cells cultured in vitro [20–22]. Directly investigating the receptor-ligand interactions on tumor cells from clinical patients is scarce. Here we used AFM to localize CD20 molecules directly on tumor B cells from clinical lymphoma patients. In order to investigate the behaviors of tumor B cells, the prerequisite is to recognize them from healthy cells. It is difficult to discern tumor B cells until recently due to the researches of receptor tyrosine kinase-like orphan receptor 1(ROR1) [23]. Evidence indicates that ROR1 is selectively expressed on the surface of B-cell chronic lymphocytic leukemia (CLL) and on some B-cell lymphomas (including mantle cell lymphoma, marginal zone lymphoma, follicular lymphoma), whereas normal B cells, other normal blood cells, and normal adult tissues do not express ROR1 [24–27]. Hence ROR1 is a suitable marker for distinguishing tumor B cells from healthy cells. In this work, we used ROR1 fluorescence labeling to recognize the tumor cells on the pathological cell samples prepared from B-cell NHL patients, and then detected the distribution of CD20 on the tumor cells by applying SMFS with rituximab-conjugated tips.

## 2. Materials and methods

### 2.1. Sample preparation

The clinical pathological samples were prepared by the medical personnel from Chinese Affiliated Hospital of Military Medical Academy of Sciences according to the standard procedures of bone marrow aspiration. A bone marrow biopsy was obtained from a B-cell NHL patient (marginal zone lymphoma) with bone marrow invasion. Then a drop of bone marrow was placed on a poly-L-lysine-coated glass slide and fixed for 30 min by 4% paraformaldehyde (Solarbio company, Beijing, China). Poly-L-lysine is positively charged and cells are negatively charged. After coating the glass slide with poly-L-lysine, the cells can be attached onto the glass slide by electrostatic adsorption. Next, ROR1 labeling procedures were performed on the chemically fixed cell samples on the glass slide. For ROR1 labeling experiments, goat-anti-human-ROR1 antibody (R&D systems, Minneapolis, USA) and FITC-conjugated donkey-anti-goat IgG secondary antibody (Kang-Chen company, Shanghai, China) were used. There were three main steps involved in the ROR1 labeling procedures. First donkey serum was used as the blocking reagent to avoid the non-specific staining. Then ROR1 antibody was added and incubated for 3 h at room temperature. Finally FITC-conjugated secondary antibody was added and incubated for 30 min at room temperature. At the beginning of each step, the samples were washed 3 times (10 min each time) by using PBS. After the labeling, the samples were placed onto the stage of a fluorescence microscope (Ti, Nikon, Japan) and the fluorescence images of the samples were obtained by using blue light for excitation. For control experiments, the ROR1 labeling procedures were performed on the peripheral blood cells obtained from healthy volunteers.

### 2.2. Tip functionalization

The prerequisite for measuring receptor-ligand interactions by AFM force spectroscopy is linking the ligands onto the AFM tip. For silicon tip, covalent coupling of silanes was the appropriate method for modifying AFM tips for single-molecule recognition studies [28]. The tip used here was silicon nitride, thus we used this method for tip functionalization. The type of the AFM probe was MLCT (Bruker, Santa Barbara, CA, USA). Rituximab was obtained from Chinese Affiliated Hospital of Military Medical Academy of Sciences. The procedure of linking rituximab molecules onto the AFM tips was according to the reference [29,30]. The NHS-PEG3500-MAL (JenKem Technology, Beijing, China) was used as linker molecules. First the tip was coated with a layer of $NH_2$ by the mixed vapor formed by aminopropyltriethoxysilan (Sigma-Aldrich, Saint Louis, MO, USA) and N,N-diisopropylethylamin (Sigma-Aldrich, Saint Louis, MO, USA) which were placed in a glass desiccator under argon gas. Then, the NHS group of the PEG linker can react with the $NH_2$ on the AFM tip. The MAL group of the PEG linker can react with the thiol group (SH) which is absent in antibodies. To make rituximab have SH groups, N-succinimidyl 3-(acetylthio) propionate (SATP) [28] was used. After the treatment of SATP, rituximab had SH groups, and then could react with the MAL group of the PEG linker.

### 2.3. Atomic force microscopy

AFM imaging and measurements were performed in PBS at room temperature using a Bioscope Catalyst AFM (Bruker, Santa Barbara, CA, USA) which was set on an inverted fluorescence microscope (Ti, Nikon, Japan). The normal spring constant of the cantilever was 0.01 N/m and its exact spring constant was calibrated by thermal noise method [31]. The deflection

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