Heterogeneous Drug Efficacy of an Antibody-Drug Conjugate Visualized Using Simultaneous Imaging of Its Delivery and Intracellular Damage in Living Tumor Tissues

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Introduction

Recently, clinical trials for approximately 70 various antibody-drug conjugate (ADC) candidates have been conducted [1]. ADCs are humanized monoclonal antibodies with a high affinity for the extracellular membrane proteins of their target tumor cells and are covalently bound to small molecular compounds with high cytotoxicity [1–3]. Over 60% of the low-molecular weight compounds used in ADCs are inhibitors of microtubule function [1,4]. Microtubules elongate and shorten via tubulin polymerization and depolymerization and regulate a variety of cellular processes, including cell division, intracellular transport, and cell polarity [5,6].

ADCs containing microtubule inhibitors exert two types of effects: anti-tumor effects induced by the binding of ADCs to target proteins on the tumor cell membrane after drug delivery and intracellular cytotoxic effects via microtubule inhibitors [2]. During the former type, the binding of the antibody portion of the ADC to the target protein mediates functional inhibition of the target molecule(s) and/or antibody-dependent cell cytotoxicity. On the other hand, the cytotoxic effects during the latter type occur when the ADCs bound to target proteins are incorporated into the cell via endocytosis [7–9]. After endocytosis, the ADC is broken down in the endosome or lysosome, and the microtubule inhibitor is released from the vesicles into the cytoplasm. This process results in inhibition of microtubule function, which induces tumor cell apoptosis. Thus, the important factors for the development of ADCs containing microtubule inhibitors are the specificity of the antibody used in the ADC, the extracellular stability of the linker used to bind the antibody to the low molecular weight drug, the timely breakdown of the conjugate once inside the cell, and the effectiveness of the drug in inhibiting microtubules [1,4]. To evaluate the efficacy of ADCs containing microtubule inhibitors, it is important to quantitatively assess the delivery of ADCs to tumor cells and the effects

Anticancer drug efficacy varies because the delivery of drugs within tumors and tumor responses are heterogeneous; however, these features are often more homogenous in vitro. This difference makes it difficult to accurately determine drug efficacy. Therefore, it is important to use living tumor tissues in preclinical trials to observe the heterogeneity in drug distribution and cell characteristics in tumors. In the present study, to accurately evaluate the efficacy of an antibody-drug conjugate (ADC) containing a microtubule inhibitor, we established a cell line that expresses a fusion of end-binding protein 1 and enhanced green fluorescent protein that serves as a microtubule plus-end-tracking protein allowing the visualization of microtubule dynamics. This cell line was xenografted into mice to create a model of living tumor tissue. The tumor cells possessed a greater number of microtubules with plus-ends, a greater number of meandering microtubules, and a slower rate of microtubule polymerization than the in vitro cells. In tumor tissues treated with fluorescent dye-labeled ADCs, heterogeneity was observed in the delivery of the drug to tumor cells, and microtubule dynamics were inhibited in a concentration-dependent manner. Moreover, a difference in drug sensitivity was observed between in vitro cells and tumor cells; compared with in vitro cells, tumor cells were more sensitive to changes in the concentration of the ADC. This study is the first to simultaneously evaluate the delivery and intracellular efficacy of ADCs in living tumor tissue. Accurate evaluation of the efficacy of ADCs is important for the development of effective anticancer drugs.
on microtubule inhibition by the drug once it is inside living tumor cells. However, during most ADC development processes, drug activity is usually analyzed via in vitro culture to investigate the ability of the drug to shrink tumors using tumor size measurements, to determine the accumulation of drugs in different organs, and to investigate drug retention in the blood [10–13]. However, there have been no investigations that quantitatively evaluated the correlation between the effects of both ADC delivery and microtubule inhibition in living tumor tissues. Due to the inability to directly visualize drug efficacy in tumor cells during the ADC developmental process, the anticancer effect of ADCs varies for each case of cancer that shows expression of the target molecules of the ADCs. For example, an ADC called trastuzumab emtansine (T-DM1) is created by linking the humanized anti-epidermal growth factor receptor type 2 (HER2) antibody trastuzumab to the microtubule inhibitor emtansine (DM1) via a thioether linker [3,14]. This ADC was approved for use in HER2-positive malignant anti-human epidermal growth factor receptor type 2 (HER2) antibody drugs and its effect on microtubule inhibition by the drug once it is inside living tumor cells. After observing these features of microtubules in living tumors, we visualized the delivery of T-DM1 to tumors and its effect on the inhibition of microtubule elongation inside single tumor cells (Figure 1).

### Materials and Methods

#### Construction and Culture of EB1-EGFP Gene-Expressing Cancer Cells

To generate an EB1-EGFP gene construct, the open reading frame of human EB1 cDNA was amplified from a human cDNA pool and inserted into a pEGFP cloning vector (TaKaRa Bio Inc.). The excised EB1-EGFP cDNA sequence was inserted into a pLNCX2 retroviral vector (BD Bioscience). The human KPL-4 breast cancer cell line was kindly provided by Dr. J. Kurebayashi (Kawasaki Medical School, Japan) [31]. EB1-EGFP-expressing KPL-4 cells (EB1-EGFP-KPL cells) were created by transducing KPL-4 cells using the pLNCX2 retroviral vector system containing the EB1-EGFP gene as the insert, and the cells were then cloned. The cloned EB1-EGFP-KPL cells were cultured in Dulbecco’s modified Eagle medium (DMEM) (Thermo Fisher Scientific) containing 10% fetal bovine serum and 400 μg/ml G418 (Thermo Fisher Scientific) at 37°C with 5% CO₂.

#### Purification of Antibody Drugs and Their Fluorescence Labeling

Kadycya (Thermo Fisher Scientific, Japan) and Herceptin (trastuzumab) (Chugai Pharmaceutical, Japan) were used as antibody drugs. T-DM1 and trastuzumab were diluted in 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer containing 30% glycerol and purified using ultrafiltration filters (NanoSep 30K; Pall). Purified T-DM1 and trastuzumab concentrations were measured at a wavelength of 280 nm using an absorption spectrometer (NanoDrop; Thermo Fisher Scientific).

The Cy5 labeling of antibodies was performed using an amine biotin kit (DOJINDO, Japan), with Cy5 mono N-hydroxysuccinimide (NHS) ester (GE Healthcare) used in place of the NHS biotin included in the kit. A 10-μl sample of Cy5 mono NHS ester diluted to a concentration of 8.5 mM was added to 100 μg (1 mg/ml) of antibody drug in dimethyl sulfoxide and incubated at 37°C for 15 minutes. The concentrations of the labeled T-DM1 and trastuzumab, as well as the labelling efficiencies (mole of Cy5/mole of antibody), were determined using the NanoDrop spectrophotometer. Protein absorbance was measured at 280 nm, and Cy5 absorbance was measured at 640 nm to calculate their concentrations by using the molar absorption coefficients. We prepared a sample with a Cy5/antibody molar ratio of 3.0 ± 0.3 (mean ± SD). T-DM1 and trastuzumab labeled with Cy5 were named Cy5-T-DM1 and Cy5-trastuzumab, respectively.

#### Effect of Antibody Drugs on Cell Viability

The effect of T-DM1 and trastuzumab on EB1-EGFP-KPL cell viability was measured using the commercial methyl thiazolyl tetrazolium (MTT) assay (Thermo Fisher Scientific) performed according to the manufacturer’s instructions. Briefly, 1.0 × 10⁴ EB1-EGFP-KPL cells were seeded per well in 96-well plates. Purified T-DM1 or trastuzumab was diluted to an arbitrary concentration with FluoroBrite DMEM. Next, the cells in 96-well plates were treated with concentration gradients of the diluted T-DM1 or trastuzumab (10⁻⁴ to 10⁻⁶ μg/ml) and allowed to grow for the next 72 hours prior to the measurement of cell viability using the MTT assay in a FlexStation 3 Multi-Mode Microplate Reader ( Molecular Devices, Sunnyvale, CA).

#### In Vitro Imaging

The subcultured EB1-EGFP-KPL cells were seeded at a density of 2 × 10⁴ cells/dish on No. 1.5 35-mm glass-bottomed dishes (MatTek) with a thin coating of Matrigel (Corning) and cultured at 37°C in 5% CO₂. The cells were washed with FluoroBrite DMEM (Thermo Fisher Scientific), a
culture medium suited to in vitro imaging, and the medium was then changed to FluoroBrite DMEM. EB1-EGFP-KPL cells were observed using an A1R confocal laser microscope system (Nikon). A 60 × (NA = 1.40) apochromatic lens was used as the objective lens. A 488-nm laser set to 0.5% output was used as the excitation light source for the observation of EB1-EGFP comet movement. The laser-excited EGFP fluorescence was filtered with a 500-500-nm bandpass filter. A resonant scanner was used as the detector, with an HV of 75 arbitrary units (a.u.), resolution of 512 × 512, and a pixel size of 0.104 μm. Time-lapse imaging was conducted at a scan speed of 1.07 s/frame continuously over 20 seconds.

The effect of antibody drugs without Cy5 on microtubule elongation activity in EB1-EGFP-KPL cells was observed via time-lapse imaging using the optical conditions described above for in vitro imaging. When Cy5-T-DM1 and Cy5-trastuzumab were used to examine the effect of these drugs on the microtubule elongation activity in EB1-EGFP-KPL cells, imaging of EB1-EGFP was performed using the same optical method described above. In this case, the Cy5-labeled antibody drug was observed using a 640-nm laser. The laser-excited Cy5 fluorescence was filtered through a 633-738-nm bandpass filter. The resonant scanner was used as the detector for imaging, with an HV of 80 a.u., resolution of 512 × 512, and a pixel size of 0.104 μm. The fluorescence intensity of Cy5 was analyzed using Fiji/ ImageJ. To analyze the Cy5 fluorescence intensity in individual cells, the average signal value from the autofluorescence of tumors that were not treated with the Cy5-labeled antibody drug was subtracted from the signal value obtained from the images.

Analysis of EB1-EGFP Comet Movement

To measure the movement of EB1-EGFP comets, the time-lapse images obtained using confocal microscopy were analyzed using Imaris Version 9.1 (Bitplane). The comet-recognition algorithm used was the “track spots (over time)” function. The parameters used were “diameter” of 0.5 μm and “quality” of 10 a.u.. “Autoregressive motion” was used for the tracking algorithm, with the “max distance” set to 0.8 μm and the “max gap size” set to three time points. To avoid misrecognition due to image noise in the tracking data, only spots that could be tracked for three or more time points were analyzed.

Animal Experiments

Five- to 6-week-old female immunodeficient mice (BALB-c nu/nu) from Charles River (Japan) were used. To prepare the tumor-bearing mice, 2 × 10⁷ EB1-EGFP-KPL cells were subcutaneously injected into the flanks of mice. EB1-EGFP-KPL xenografted mice were housed for 4-5 weeks until the tumor size was 5-10 mm, at which point the specimens were harvested. The animal experiment protocol used in this study was approved by the Tohoku University Animal Ethics Committee.

Administration of Antibody Drugs to Mice and Ex Vivo Imaging

A 200-μl sample of Cy5-labeled antibody-drug dilute to 1.5 mg/ml with saline solution was injected into the tail vein of mice under anesthesia. The mice were euthanized 24 hours after the antibody-drug injection, and the subcutaneous tumors were excised. The excised tumors were washed with FluoroBrite DMEM and placed in a 37°C environment. Next, samples were embedded in low-melting point agarose (PrimeGel 1-20 K, TaKaRa Bio) diluted to 1.5% (w/v) in saline solution. Sections 200 μm in size were prepared using the linear slicer PRO 7 (Dosaka EM, Japan) from the agarose blocks containing the embedded...
tumors. The sections were washed using FluoroBrite DMEM and main-
tained at 37°C. Later, the living tumor tissue sections were placed on a
No. 1.5 35-mm glass-bottomed dish with adequate FluoroBrite DMEM
and covered with a circular coverslip.

Ex vivo whole tumor tissue imaging of EB1-EGFP and Cy5-T-DM1 or
Cy5-trastuzumab was basically performed under the same optical condi-
tions used for the in vitro imaging. The pixel size was modi-
fi ed from 0.104 μm to 0.416 μm since we needed many images to obtain a tiling
image of the whole tissues. The images for tiling were taken in moving
steps, where the images overlapped with each other by 20%. After the im-
ages were obtained, they were connected into a single image of whole
tumor tissue by using the A1R confocal laser microscope system. Enlarged
tumor tissue imaging of EB1-EGFP and Cy5-T-DM1 or Cy5-trastuzumab
was performed under identical optical conditions used for in vitro imaging.

**Statistical Analyses**

All data are presented as the mean ± SD. Statistical analyses were per-
formed using Microsoft Excel software. Statistical significance was deter-
mined using two-tailed t tests or analysis of variance, where appropriate.
*P* values of 0.05 were considered significant and are indicated by “*”;
*P* values of 0.01 are indicated by “**”.

**Results**

**The Inhibitory Effect of T-DM1 on Microtubule Dynamics In Vitro**

To visualize the efficacy of T-DM1, we used a retroviral vector to stably
integrate the EB1-EGFP gene into KPL-4 cell chromosomal DNA. KPL-4 cells
are human breast cancer cells with high expression of HER2, the antigen for trastuzumab [10,31,32]. Multiple molecules of EB1-EGFP will bind directly to the plus-end of a microtubule and move in a comet-like fashion during microtubule elongation [29,30,33,34]. As a control experiment, we incubated and observed cloned EB1-EGFP–expressing KPL-4 (EB1-EGFP-KPL) cells at 37°C in the absence of T-DM1. EB1-EGFP accumulated at the ends of elongating microtubules (Figure 2A, left image). Next, using an exposure time of 1.07 s/frame with no delay, we performed a 20-second time-lapse image sequence and then overlaid all imaging data. EB1-EGFP comets moving straight towards the cell membrane were observed (Figure 2A, middle image; Supplementary Movie S1; Figure 2B). The comets shown in the continuous images from the 20-second time-lapse sequence could be tracked for differing lengths of time. Using the analysis software (Imaris), we conducted a bright spot tracking analysis of EB1-EGFP comets and created an overlaid image using all the tracking data (Figure 2A, colored portion of the right image), which demonstrated the same results as the fluorescence images in the middle of Figure 2A. This finding indicated that our analysis accurately measured EB1-EGFP comets. In 15 arbitrary cells, measurement of the movements of EB1-EGFP comets, as determined using Imaris, demonstrated that for 20 seconds of continuous observation, the total number of comets was 1938.0 ± 557.5/cell (mean ± SD; Figure 2D). The comets were visualized at a specific time point (\( t \)) were compared with those measured at \( t + 1 \). Upon joining comets that were within 0.8 \mu m of each other, 280.7 ± 92.5 tracks/cell were observed for \( \geq 2 \) seconds (mean ± SD; Figure 2D). Based on the tracking data, the average movement speed of all the comets that we tracked for \( \geq 2 \) seconds was 0.19 ± 0.02 \mu m/s (mean ± SD; Figure 2B and E). These EB1-EGFP localization patterns and movement speeds were similar to those previously measured [34]. Furthermore, for the comets that could be tracked for \( \geq 2 \) seconds within the 15 arbitrary cells, the total distance traveled was 1.21 ± 0.18 \mu m/comet (mean ± SD; Figure 2F). The track linearity while moving was 0.83 ± 0.05/comet (mean ± SD; Figure 2G). The track linearity was calculated by dividing the total linear distance traveled by the distance actually moved; this measurement indicates the degree of meandering in the direction of microtubule elongation (Figure 2B, right image). Additionally, the total track displacement over the 20 seconds was 332.3 ± 85.3 \mu m/cell (mean ± SD; Figure 2H).

Next, we treated EB1-EGFP-KPL cells with 1 \mu g/ml T-DM1 for 8 or 24 hours and then captured 20 seconds of time-lapse images of the cells to evaluate the effect of T-DM1 on microtubule elongation activity in EB1-EGFP-KPL cells in vitro. Many EB1-EGFP comets were observed in the control experiment with no T-DM1 treatment (Figure 3A; Supplementary Movie S2). However, after treatment with T-DM1 (8 hours, Figure 3B; 24 hours, Figure 3C), the number of EB1-EGFP comets decreased over time (left images in Figure 3, B and C), and movement halted (middle images in Figure 3, B and C; Supplementary Movie S3 and S4). Additionally, a bright spot tracking analysis of EB1-EGFP comets was conducted using Imaris (right images in Figure 3, B and C). To our knowledge, this is the first study to directly visualize microtubule elongation in cells in response to treatment with a microtubule inhibitor-containing ADC using a microtubule plus-end tracking protein. Furthermore, to analyze the effects of T-DM1 on microtubule elongation in detail, EB1-EGFP-KPL cells were cultured for 2, 8, or 24 hours in the presence of 0, 10^{-2}, 10^{-1}, 1, 10, or 10^{5} \mu g/ml T-DM1, and the EB1-EGFP comet movements in these cells were quantitatively analyzed. Five arbitrarily selected cells in each treatment group were continuously imaged with a 20-second time-lapse image sequence, and the movement of EB1-EGFP comets...
that could be tracked for ≥2 seconds was analyzed in a manner as shown in Figure 2. At all concentrations, the results revealed a time-dependent decrease in the number of comets (Figure 3E), the speed of comets (Figure 3F), and the average track displacement (Figure 3G) in each cell. Furthermore, the total track displacement over 20 seconds also decreased in a T-DM1 concentration-dependent manner; however, trastuzumab did not (Supplementary Figure 3). Therefore, these data suggested that the Cy5 label did not significantly alter track linearity (Figure 3D). These results indicated that T-DM1 decreased the appearance of microtubules with elongating ends and the elongation speed of these microtubules throughout the whole cell.

To support the results of the T-DM1 inhibition experiments described above, we treated EB1-EGFP-KPL cells with DM1 alone; the cells exhibited a time-dependent decrease in the number of EB1-EGFP comets similar to that observed during T-DM1 treatment (Supplementary Figure 1). The disappearance of comets and the halting of movement were not observed after treatment with trastuzumab alone (Supplementary Figure 2). Additionally, to investigate these antibody drugs on cell viability, T-DM1 or trastuzumab treatment with trastuzumab alone (Supplementary Figure 2). Additionally, appearance of comets and the halting of movement were not observed after that observed during T-DM1 treatment (Supplementary Figure 1). The disappearance of comets and the halting of movement were not observed after treatment with trastuzumab alone (Supplementary Figure 2). Additionally, appearance of comets and the halting of movement were not observed after that observed during T-DM1 treatment (Supplementary Figure 1). The results demonstrated that T-DM1 affects the viability in a concentration-dependent manner; however, trastuzumab did not (Supplementary Figure 3). Therefore, these data suggested that the concentration- and time-dependent inhibition of microtubule elongation by T-DM1 observed in this study resulted due to the DM1 molecule in the T-DM1 complex.

Next, to investigate the relationship between the amount of T-DM1 binding and internalized to the EB1-EGFP-KPL cells and inhibitory effect of T-DM1 on microtubule elongation in the cells, T-DM1 was labeled with a fluorescent dye, Cy5 (Cy5-T-DM1), and incubated with the EB1-EGFP-KPL cells. When the cells were treated with 0.001 μg/ml Cy5-T-DM1 for 24 hours, despite a weak fluorescence signal of Cy5-TDM1 detected in the cells, inhibition of microtubule elongation by T-DM1 was not observed (Figure 4A, four left panels). On the other hand, during incubation with 0.01 μg/ml (Figure 4A, four center-left panels), 0.1 μg/ml (Figure 4A, four center-right panels), or 1 μg/ml Cy5-T-DM1 (Figure 4A, four right panels), the fluorescence signal of Cy5-TDM1 on and in the cells increased (Figure 4B). These results (Figure 4B) were comparable to the data using nonlabeled T-DM1 (Figure 3H, red circles which show the conditions incubated for 24 hours), it was considered that the Cy5 label did not affect T-DM1 activity.

Ex Vivo Imaging of Microtubule Dynamics in Living Tumor Cells Using EB1-EGFP-KPL Cells Transplanted into Tumor-Bearing Mice

The in vitro data obtained on microtubule dynamics in the conditions without T-DM1 were consistent with data from previous studies regarding the number of microtubules with elongating ends, the speed of elongation, and the linearity of the direction of elongation in the absence of an ADC in vitro [33,34]. However, the details of microtubule elongation activity in a living tumor environment have not yet been reported. Tumor vessels are irregularly arranged in tumors, resulting in an uneven localization of their microtubule dynamics; therefore, it is important to analyze...
microtubule elongation in living tumor cells. Thus, we created tumor-bearing mice by xenografting EB-EGFP-KPL cells into mice and analyzed the behavior of EB1-EGFP comets in living tumor cells.

Tumors, 5-10 mm in diameter, developed over a 4- to 5-week period after xenografting EB1-EGFP-KPL cells into mice. After tumor excision, we immediately cut the tumors into 200-μm-thick sections using a Vibratome. Next, we observed the living tumor tissue sections at 37°C using fluorescence imaging with a confocal microscope. EB1-EGFP expression was observed in 50% or more of the whole tumors; however, there were regions with low EB1-EGFP expression and regions with no detectable EB1-EGFP expression due to internal necrosis (Figure 5A). This finding demonstrated the heterogeneity among cancer cells in tumors. We arbitrarily selected 12 tumor cells in which the fluorescence intensity of the EB1-EGFP comets was comparable to that of the in vitro cells and captured 20-second time-lapse images of EB1-EGFP comets in living tumor cells using the same methods described for the in vitro experiments. Accumulation of EB1-EGFP at the plus-ends of elongating microtubules was observed in the ex vivo conditions (Figure 5B, left image). The comets moved at a consistent speed (Supplementary Movie S5). A comparison of the overlay image containing all 20-second time-lapse fluorescence images (Figure 5B, middle image) and the image obtained from the bright spot tracking data of EB1-EGFP comets using Imaris (Figure 5B, right image) demonstrated that these results were consistent, indicating that our analysis method was valid for ex vivo imaging. The ex vivo analysis of EB1-EGFP comet movements showed that the total number of EB1-EGFP comets per cell was 2630.5 ± 516.6 (mean ± SD) over 20 seconds of continuous observation (Figure 5C). Of these visualized comets, the number of comets that could be tracked for ≥2 seconds was 382.2 ± 84.7 per cell (mean ± SD; Figure 5D). Moreover, for the comets that were tracked for ≥2 seconds, the average speed was 0.17 ± 0.01 μm/s (mean ± SD; Figure 5E); the average track displacement, which indicates the distance that a comet traveled from its starting point, was 0.81 ± 0.09 μm per comet (mean ± SD; Figure 5E); the track linearity was 0.69 ± 0.05 per comet (mean ± SD; Figure 5G); and the total track displacement over the 20-second tracking period was 309.3 ± 79.7 μm per cell (mean ± SD; Figure 5H). An analysis of the significant differences between in vitro and ex vivo EB1-EGFP comet

**Figure 5.** Imaging of microtubule elongation in living tumor cells and analysis of elongation characteristics. (A) Typical fluorescence images of 200-μm-thick living tumor tissue sections. The yellow arrowhead presents the location of B, which shows a typical image of tumor cells. Bar, 1 mm. (B) Typical fluorescence image of living tumor cells expressing EB1-EGFP. The image on the left is a still image, the image in the center is a time-lapse fluorescence image overlay, and the image on the right shows an overlay of the digital image data generated using Imaris on top of the center image. The yellow dotted line represents the outline of the cell. Bar, 10 μm. The color scale represents the location of comets at different time points. (C-H) Analysis results of EB1-EGFP comet movement in living tumor cells. EB1-EGFP comet movements tracked for ≥2 seconds during the 20-second observation period were analyzed. We arbitrarily selected 12 living tumor cells with a fluorescence intensity consistent with observed in the in vitro EB1-EGFP cells. We compared the characteristic values of the EB1-EGFP comet movement between the in vitro (orange dots) and ex vivo (blue dots) measurements. The orange dots are identical to the dots shown in Figure 2, C-H. (C) The total number of EB1-EGFP comets per cell. (D) The total number of EB1-EGFP comet tracks per cell. (E) The mean movement speed of EB1-EGFP comets per cell. (F) The mean track displacement of EB1-EGFP comets per cell. (G) The mean track linearity of EB1-EGFP comets per cell. (H) Total track displacement per cell. Black bars and error bars represent the mean values and SD, respectively, of data per cell under in vitro and ex vivo conditions (C-H). Significant differences were determined using the t test (*P < 0.05 and **P < 0.01). Total number of EB1-EGFP comets (C), total number of EB1-EGFP comet tracks (D), mean speed of EB1-EGFP comet (E), mean track displacement of EB1-EGFP comets (F), mean track linearity of EB1-EGFP comets (G), and total track displacement (H) of left and right cell enclosed with yellow dotted line in B are 3095 and 3170 in c, 478 and 496 in D, 0.14 μm/s and 0.18 μm/s in E, 0.64 μm and 0.90 μm in F, 0.69 and 0.79 in G, and 305.9 μm and 447.9 μm in H, respectively.
movements demonstrated no difference in the total track displacement per cell (Figure 5H). However, cancer cells in living tumor tissues have unique characteristics in terms of their microtubule dynamics. Compared to cancer cells in an in vitro environment, the number of microtubules with elongating plus-ends was 1.36 times higher in living tumor cells (Figure 5C), while the microtubule elongation speed was decreased by a factor of 0.84 (Figure 5E).
and the linearity of the elongation was decreased by a factor of 0.90 (Figure 5G). To our knowledge, this is the first to report these characteristics of microtubule elongation in living tumor cells.

Ex Vivo Imaging of Cy5-T-DM1 Drug Delivery and Microtubule Inhibition in Living Tumor Cells

To visualize T-DM1 delivery to tumors, a total of 200 μl of Cy5-T-DM1 at a concentration of 1.35 mg/ml was injected into the tail vein of mice with EB1-EGFP-KPL cell xenografts; the ratio of T-DM1 to body weight in the mice was 15 mg/kg. Twenty-four hours after the T-DM1 injection, the tumors were enucleated, and 200-μm-thick sections were prepared using a Vibratome; the sections were observed at 37°C under a confocal microscope for ex vivo imaging. Additionally, as a control, Cy5-labeled trastuzumab (Cy5-trastuzumab) was prepared, injected into tumor-bearing mice, and observed in the same way as the Cy5-T-DM1. Both Cy5-T-DM1 and Cy5-trastuzumab showed unequal distributions during delivery to the tumor tissue (left images in Figure 6, A and B). The EB1-EGFP expression patterns were also heterogeneous (right images in Figure 6, A and B). We quantified drug delivery and microtubule elongation inhibition simultaneously in areas of dense and sparse drug distribution in the tumor tissue. Based on the fluorescence intensities, the areas with high drug concentrations (Figure 6A, light blue square) had 2.2-fold higher levels of Cy5-T-DM1 in a 2500-μm² area than the areas with low drug concentrations (Figure 6A, white square). A mixture of cells was observed with varying levels of EB1-EGFP comet activity in the areas with low concentrations of Cy5-T-DM1. In areas where cells still indicated some microtubule elongation, Cy5-T-DM1 delivery to the cells was not observed (Figure 6C, upper two cells in the right image; the white arrowhead shows one of the two cells; Supplementary Movie S6). Conversely, Cy5-T-DM1 inhibited most of the microtubule elongation in cells in the areas of high Cy5-T-DM1 concentrations (Figure 6D, right image; Supplementary Movie S7).

Next, we investigated the effect of the Cy5-T-DM1 dose administered in tumor-bearing mice to the T-DM1 delivery to tumors or microtubule elongation activity in living tumor cells. The difference in the Cy5-T-DM1 dose administered increased the heterogeneity of the drug delivery to tumors (Supplementary Figure 4). In Figure 6, the ratio of Cy5-T-DM1 to mouse body weight was 15 mg/kg. In tumor-bearing mice administered 3.0 mg/kg Cy5-T-DM1, which is one fifth of 15 mg/kg, low-density areas of Cy5-T-DM1 or areas without Cy5-T-DM1 expanded (Supplementary Figure 4). Furthermore, in tumor-bearing mice administered 0.6 mg/kg Cy5-T-DM1, which is one fifth of 3.0 mg/kg, Cy5-T-DM1 was almost not delivered to most tumor cells (Supplementary Figure 4). The correlation between microtubule elongation based on measurements of EB1-EGFP comet movement and the intracellular concentration of Cy5-labeled T-DM1 in 10 arbitrarily selected cells was calculated in tumor-bearing mice administered 15 mg/kg Cy5-T-DM1; a high coefficient of correlation was observed (Figure 6F, Supplementary Figure S5, R = −0.815). These data are the first to characterize the correlation between ADC delivery and cytotoxicity in living tumor cells.

In the control experiment, an unequal distribution of Cy5-trastuzumab, similar to that of Cy5-T-DM1, was observed (Figure 6B), and in regions of high drug concentration (Figure 6B, orange square), most of the trastuzumab was localized within the tumor cells (Figure 6E). However, the same analysis as that used in Figure 5, C and D showed that regardless of the intracellular concentration of trastuzumab, the microtubule elongation activity was comparable to that in the T-DM1–untreated group (Figure 5; Figure 6, E and G; Supplementary Movie S8). These results demonstrated that the observed inhibition of microtubule elongation in living tumor cells induced by T-DM1 was due to the DM1 contained in the T-DM1 complex, and inhibition of microtubule elongation was due to T-DM1 uptake by cells.

Discussion

Previous studies have provided substantial information from measurements of EB1 comets in the absence of an ADC in vitro; however, no previous study has utilized the measurement of EB1 comet movement to evaluate microtubule elongation in living tumor cells [23,34]. Therefore, the details of microtubule elongation activity in tumor cells are poorly understood. In this study, the results of microtubule elongation analysis in living tumor cells demonstrated that the total displacement over 20 seconds in one cell was similar between in vitro and tumor cells (Figure 5H); however, large differences were observed in the characteristics of microtubule dynamics with regard to the total displacement in both cell types. Living tumor cells contained 1.36-fold more microtubules with an elongating end plus-end than in vitro cells (Figure 5C), while the microtubule elongation speed was decreased by a factor of 0.84 (Figure 5E), and the linearity of elongation was decreased by a factor of 0.90 (Figure 5G). For microtubules under in vitro cell culture conditions, the flat two-dimensional environment facilitates cellular processes such as cell division and intracellular transport, and such an environment may require less microtubule three-dimensional rearrangement than a three-dimensional tumor environment [35]. Conversely, extracellular stimuli may exert greater control over cell division and intracellular transport in a three-dimensional in vivo environment than in an in vitro environment. Therefore, microtubules in living tumor cells are required to regulate their cell polarity in a three-dimensional environment, leading to microtubule elongation in a greater number of directions than in in vitro cells. This may contribute to an increased number of microtubules with elongating ends and a decreased linearity of microtubule tracks in living tumor cells compared to in vitro cells. The decreased microtubule elongation speed in living tumor cells compared to in vitro cells may be attributed to in vitro cells being present in nutrient-rich conditions and exposed to larger amounts of proteins that regulate microtubule structure and elongation speed than cells in a tumor environment, which is substantially less nutrient-rich. Therefore, in vitro and tumor cells measured under the same (37°C) conditions may demonstrate a difference in microtubule elongation speeds. In practice, even in cloned identical cells, cell proliferation is generally faster under in vitro conditions than in a tumor environment. Differences in oxygen and nutrient concentrations may lead to a difference in proliferative ability and microtubule elongation speed in the
two different environments. Furthermore, we failed to observe a large change in the total track displacement, a unified metric for measuring microtubule elongation activity, which may be attributed to the complexity of the characteristics of microtubule elongation activity, as described above.

There has been no previous study evaluating the in vitro ADC efficacy as measured by changes in the EB1 comet movement. In our in vitro studies, inhibition of microtubule elongation by T-DM1 increased in a concentration- and reaction time-dependent manner. For example, in case of in vitro cells that were treated with Cy5-T-DM1 for 24 hours, the distribution of Cy5-T-DM1 fluorescence intensity on and in the cell was approximately 0.1-1.53 μm/cell and the total track displacement was approximately 223.66-366.8 μm/cell for cells incubated with 0.001 μg/ml Cy5-T-DM1 (Figure 4B, blue dots). Similarly, Cy5-T-DM1 fluorescence intensity was approximately 13.6-59.6 μm/cell and the total track displacement was approximately 34.5-93.7 μm/cell (Figure 4B, yellow dots) in the 0.1-μg/ml Cy5-T-DM1 treatment group. The Cy5-T-DM1 fluorescence intensity was approximately 5.2-62.7 μm/cell (Figure 4B, yellow dots) in the 0.1-μg/ml Cy5-T-DM1 treatment group; the intensity was approximately 54.7-106.5 μm/cell and the displacement was 0.2-52.8 μm/cell (Figure 4B, pink dots) in the 1.0-μg/ml Cy5-T-DM1 treatment group. From these in vitro data under 0.001-1.0 μg/ml Cy5-T-DM1 treatment conditions, the following relational expression was derived: y = −3.1x + 255.8 (Supplementary Figure 5). On the other hand, 24 hours after injecting 15 mg/kg Cy5-T-DM1 into the tail vein during the ex vivo experiments, the distribution of Cy5-T-DM1 fluorescence intensity on and in the tumor cells changed from approximately 3.6 to 34.2 μm/cell in tumor cells, and in response to the change in Cy5-T-DM1 distribution, the total track displacement changed from 2.4 to approximately 421.3 μm/cell (Figure 6F, blue dots; Supplementary Figure 5). From the ex vivo data, the following relational expression was derived: y = −12.0x + 350.4 (Supplementary Figure 5). In a comparison of coefficients of in vitro and ex vivo relational expression, the sensitivity of ex vivo EB1-EGFP-KPL cells to T-DM1 was 3.9-fold higher than the in vitro cells (−12.0/−3.1 ≈ 3.9), although EB1-EGFP-KPL cells in both environments were originally identical. In other words, these data indicate that microtubule elongation may be affected by a substantially smaller change in the amount of drug delivered to the living tumor cells than to cells in an in vitro environment, suggesting that tumor cells are more sensitive to changes in drug concentration than in vitro cells. These results show that it is important to evaluate drug efficacy in living tumor cells to accurately understand the anticancer efficacy of ADCs. The concepts and methods described in this study contribute to improving the accuracy of the preclinical process for developing ADCs.

Conclusion
We developed a quantitative evaluation method at the single tumor cell level in living tumor tissue considering the heterogeneous cellular delivery and inhibitory effects of an ADC-containing microtubule inhibitor. Furthermore, the results of this study suggest that the responsiveness of T-DM1 and the dynamics of microtubule elongation activity differ between in vitro and in vivo conditions. Therefore, this method is presumed to be an effective means to measure the efficacy of ADC in a heterologous tumor microenvironment and is expected to be useful for future ADC development.

Supplementary data to this article can be found on line at https://doi.org/10.1016/j.tranon.2020.100764.

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Author Contributions
K. G., N. F., and Y. N. conceived and designed the project and designed experiments. M. T.-K., N. K., Y. H., M. T., H. T., and T. I. contributed to the development of the concepts through discussion of this study. K. G. and H. H. created the EB1-EGFP-KPL cells. H. N., M. T.-K., and N. K. cultured cells and generated tumor-bearing mice. H. T. and T. I. prepared trastuzumab and T-DM1. H. N. and M. T.-K. performed the animal experiments. H. N. conducted the imaging experiments and obtained the data. K. G., N. F., and Y. N. analyzed the results. K. G. wrote the paper.

Declaration of Competing Interests
The authors declare that they have no conflicts of interest in relation to the contents of this article.

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