博士課程用（甲）

（様式4）

学 位 論 文 の 内 容 の 要 旨

XIEYI ZHANG　　印

（学位論文のタイトル）

Activatable fluorescence detection of epidermal growth factor receptor positive mediastinal lymph nodes in murine lung cancer model

activatable蛍光イメージングを用いたマウス肺癌モデルの
EGFR陽性縦隔リンパ節転移検出の検討

(学位論文の要旨)2,000字程度、A4判

INTRODUCTION

Purpose: We investigated whether lymph node metastases of lung cancer can be detected using the activatable method, by examining epidermal growth factor receptor (EGFR)-positive lung squamous cell carcinoma in murine models.

MATERIALS AND METHODS

Synthesis of fluorophore conjugated antibody

Panitumumab (Pan) (1 mg, 6.8 nmol), was incubated with ICG-Sulfo-OSu (66.8 µg, 34.2 nmol, 5 mmol l^{-1} in dimethyl sulfoxide, Dojindo, Inc., Japan) in 0.1 M Na2HPO4 (pH 8.5) at room temperature for 30 min. The reaction molecule ratio of Pan and ICG-Sulfo-OSu was 1 to 8.

Flow cytometry

EGFR-positive and EGFR-negative human squamous cell carcinoma cell lines were employed for the EGFR targeting studies (H226 and H520, respectively). H226 or H520 cells (1×10^5 cell/well) with Pan-ICG were incubated at 37°C for either 1 or 6 hours. To validate the specific binding of the antibody, excess Pan (50 µg) was used to block 0.5 µg of Pan-ICG conjugate. The fluorescence signal from H226 and H520 cells after incubation with the Pan-ICG conjugate was measured by flow cytometry (BD Biosciences, San Jose, CA).

In vitro fluorescence microscopy studies

The H226 or H520 cells (1×10^4) were incubated for 24 hr. The Pan-ICG was added to the medium (5 µg/mL) and the cells were incubated for either 1 or 6 hr. The cells were then washed once with phosphate-buffered saline, and were subjected to fluorescence microscopy using a BZ-X700 microscope (Keyence, Osaka, Japan) equipped with filters meeting the following criteria: excitation wavelength 672.5–747.5 nm and emission wavelength 765–855 nm.

Animal tumor model

Ten female, athymic nu/nu mice (Japan SLC, Inc., Shizuoka, Japan) were randomly segregated into two groups of five mice each, and H226 and H520 cells suspended in phosphate-buffered saline (1–3 × 10^6 cells/50 µl) were injected into the lung tissue directly in each group, respectively.

Tumor monitored by computed tomography

After injecting the tumor cells, tumor growth was monitored by animal computed tomography (CT; LaTheta LCT-200; Hitachi-Aloka, Tokyo, Japan) every week. Animal models were thought to be established for this in vivo study when the longest diameter of the lung tumor lesion exceeded 8 mm or atelectasis occupied more than half of the lateral lung fields.

In vivo EGFR-targeted imaging studies

A dose of 50 μg Pan-ICG was injected via the tail vein into the tumor-bearing mice. Forty-eight hours after conjugate administration, all the mice were sacrificed by cervical dislocation. The regions of interest were drawn for the five lymph node metastases with the highest fluorescent signal intensities per mouse, and the fluorescence signal intensity was calculated. The ratio of the fluorescence signal intensity between lymph node metastases and the liver was used for statistical comparison. The correlation between the lesion size and the signal ratio was analyzed.

Ex vivo EGFR-targeted imaging studies

To confirm the presence of lymph node metastases and EGFR expression, lymph nodes were embedded in paraffin and stained with hematoxylin and eosin and the Pan-ICG conjugate. Fluorescence microscopy and optical images (hematoxylin and eosin staining) were acquired.

RESULTS

Flow cytometry

Flow cytometry using the Pan-ICG showed strong fluorescence from the EGFR-positive cells and weak fluorescence from the EGFR-negative cells after incubation with the Pan-ICG for 1 or 6 hours.

In vitro fluorescence microscopy

In vitro fluorescence microscopy produced similar results to those obtained for flow cytometry. Images after 1 and 6 hours' incubation showed increased signal intensity in the EGFR-positive tumor cells.

Tumor monitored by CT

The imaging experiments were performed 56 to 98 days (74.9 ± 13.6 days) after cell injection, when the CT criteria were met.

In vivo EGFR-targeted imaging studies

The EGFR-positive node metastases had significantly stronger fluorescent signal intensities than the EGFR-negative nodes 48 hours after injection. All the 25 EGFR-positive lymph node metastases were clearly identified with the ICG-specific fluorescence signal, while the EGFR-negative tumor specimens produced a much weaker signal. The lesion-to-liver fluorescence signal ratio was calculated (Fig 5), with a significantly higher signal ratio in the EGFR-positive lymph node metastases ($p < 0.05$). Visually, ex vivo images showed the EGFR-positive lymph node metastases acquired higher fluorescent signals than the EGFR-negative ones (Fig 6). The signal ratio did not correlate with the lesion size in the EGFR-positive lesions (Fig 7, $r = 0.36$, $p = 0.08$).

Ex vivo EGFR-targeted imaging studies

We confirmed the presence of lymph node metastases in all 50 lesions by staining with hematoxylin and eosin. At 30 minutes after incubation with Pan-ICG, the EGFR-positive tumors showed stronger fluorescent signal intensity than the EGFR-negative tumors (Fig 8).

CONCLUSION

We have synthesized an activatable Pan-ICG conjugate and we have shown that this activatable conjugate can detect mediastinal EGFR-positive lymph node metastases of squamous cell carcinoma with such high contrast that this activatable Pan-ICG has a potential in assisting surgeons during endoscopic lung surgery.

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【はじめに】

肺癌患者におけるリンパ節転移の検出は、治療効果や生存率の向上に重要である。今回、蛍光物質であるインドシアニングリーン（ICG）を標識した抗EGFR抗体を用いたactivatable蛍光イメージングの技術を用いて、マウス肺癌モデルのEGFR陽性リンパ節転移の検出を試みた。

【対象と方法】

癌細胞はEGFR陽性細胞であるH226とEFGR陰性細胞であるH520をin vitroおよびin vivo実験に用いた。病変検出に用いた薬剤は、パニツムマブ（Pan）抗体にICGを1:8の割合で標識してactivatable抗体(Pan-ICG)を作成した。

In vitroの実験として、細胞培養液中にPan-ICGを添加した状態で1時間・6時間インキュベーションした後、flowcytometry(FACS)および蛍光顕微鏡でEGFR陽性癌細胞への特異的なPan-ICGの結合を確認した。

In vivo実験では、H226およびH520のいずれかを直接肺に移植して、肺癌マウスを作成した（H226群5匹・H520群5匹）。肺癌モデル確立の確認には毎週CTを撮像することで行った。腫瘍径が8mmを超える・片側肺の1/2以上が無気肺になる、のいずれか一方を満たした段階で安楽死させ、瀉血した後、開胸して蛍光イメージングを行った。蛍光イメージングにはMaestro（BD社製）を用いて、励起光（700nm）・蛍光フィルタ（800nm）を使用した。それぞれのマウスでは縦隔リンパ節の明るいものから上位5つを選択して、蛍光強度を計測した。またリファレンスには肝臓の蛍光強度を使用した。

【結果】

FACSの結果では、EGFR陽性癌細胞のみで蛍光が検出され、EGFR陰性細胞では蛍光が検出されなかった。蛍光顕微鏡では、1時間・6時間後の両者でEGFR陽性癌細胞のみで蛍光が検出された。

肺癌マウスモデルを用いた蛍光イメージングでもEGFR陽性リンパ節25個（5リンパ節/マウス）のすべてで強い蛍光シグナルが得られた。EFGR陰性リンパ節転移ではほとんど蛍光シグナルを得られなかった。蛍光シグナルの病変-肝臓比ではEGFR陽性群と陰性群に統計的な有意差を持って明らかな違いが認められた($p < 0.05$)。病変の大きさと蛍光シグナルには統計的相関を認めなかった（Fig 7, $r = 0.36$, $p = 0.08$）これらの結果については、摘出リンパ節および免疫染色を含めた病理学的検討でも、同様の所見が認められた。

【結語】

私たちはactivatable蛍光物質Pan-ICGを合成し、EGFR陽性癌細胞に対して統計的有意差を持って、強い蛍光シグナルを得ることに成功した。このことは、実際の胸腔鏡を使った手術にも応用が可能であると考えられる。