RESEARCH ARTICLE

Axl-Targeted Cancer Imaging with Humanized Antibody h173

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Abstract

Purpose: The tyrosine kinase receptor Axl is overexpressed in various types of cancer and correlated with cancer malignancy. Selective Axl blockade reduces tumor growth and metastasis. The purpose of this study was to examine whether the humanized anti-Axl antibody humanized 173 (h173) labeled with near-infrared fluorescence (NIRF) dye Cy5.5 could be applied as a molecular imaging probe for NIRF imaging of Axl expression in tumor models.

Procedures: NIRF dye Cy5.5 was conjugated to h173 or human normal immunoglobulin G (hIgG) control through amino groups. The resulting probes were evaluated in both A549 (Axl positive) and NCI-H249 (Axl negative) lung cancer xenografts through *in vivo* NIRF imaging. *Ex vivo* imaging and probe distribution assay were also carried out to confirm the *in vivo* imaging results.

Results: After conjugation, binding activity of h173-Cy5.5 was determined to be 97.75 % ± 2.09 % of the unmodified h173. *In vitro* fluorescence-activated cell sorting (FACS) and fluorescence microscopy analysis validated the specific binding of h173 toward Axl-positive A549 cells. h173-Cy5.5 was then applied to image Axl expression *in vivo*. In A549 (Axl positive) cancer xenografts, the tumor uptake of h173-Cy5.5 was significantly higher than that of the hIgG-Cy5.5 control ($P < 0.05$) at late time points (1, 2, 3, 4, and 7 days). On the contrary, in NCI-H249 (Axl negative) cancer xenografts, the tumor uptake of both hIgG-Cy5.5 and h173-Cy5.5 was low and showed no significant difference ($P > 0.05$) at all time points examined. *Ex vivo* imaging and immunofluorescence staining analysis further validated the *in vivo* imaging results.

Conclusions: Collectively, all *in vitro*, *in vivo*, and *ex vivo* data suggested that h173-Cy5.5 could serve as a valid probe for Axl-targeted cancer imaging, which could therefore aid in tumor diagnosis, prognosis, and treatment monitoring.

Key words: Axl receptor, Near-infrared fluorescence (NIRF) imaging, Antibody, Cancer

Introduction

Axl belongs to the TAM (Tyro3, Axl, and Mer) receptor tyrosine kinase (RTK) subfamily and was initially

identified as a transforming gene in chronic myelogenous leukemia [1] and chronic myeloproliferative disorder [2] patients. Protein S and growth arrest-specific 6 (Gas6) are the vitamin K-dependent ligands for Axl, whereas only the latter has high affinity to Axl and activates downstream signaling [3, 4]. Under normal conditions, Axl, together with other TAM members, play important roles in macrophage/dendritic cell clearance of apoptotic cells, cytokine secretion,

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platelet aggregation, natural killer (NK) cell differentiation, neuron function, spermatogenesis, and so on [5]. Axl is also overexpressed in a variety of cancer types, including colorectal cancer [6], esophageal cancer [7], liver cancer [8], thyroid cancer [9], breast cancer [10], ovarian cancer [11], lung cancer [12, 13], glioma [14], osteosarcoma [15], and Kaposi sarcoma [16]. Axl plays multiple roles in tumorigenesis. In lung cancer, for example, the adherence of lung cancer cell lines was found to correlate with the expression of Axl receptor [13]. It is expressed in around 60 % of adherently grown non-small-cell lung cancer (NSCLC) cell lines but is absent in small cell lung cancer (SCLC) cell lines, which grow in suspension [13]. The expression of Axl is clearly associated with tumor invasiveness [12, 17] and drug resistance [17, 18] indicating that Axl expression is a poor prognostic factor in NSCLC. Preclinical studies on selective Axl blockade also confer the preliminary validation of Axl as a cancer therapy target [16, 18–20]. Based on the extreme importance of Axl, noninvasive imaging of Axl could be valuable for early tumor diagnosis, prognosis, optimization of patient selection, treatment planning, and outcome monitoring.

Previously, m173, a mouse-originated monoclonal antibody, which specifically targets the first fibronectin type III (FNIII) domain of human Axl, has been developed [16]. Humanized 173 (h173) was also produced for clinical translation. In this research, we explore whether h173 can be applied for the molecular imaging of Axl expression on the lung cancer xenograft models after conjugating with near-infrared fluorescence (NIRF) dyes.

Materials and Methods

Materials

Monoclonal antibodies to Axl (h173, recognizes human Axl) and Axl-alkaline phosphatase (AP) were kindly provided as gifts by Vasgene Therapeutics Inc. (Los Angeles, CA). PD-10 disposable columns and Cy5.5 monofunctional N-hydroxysuccinimide ester (Cy5.5-NHS) were obtained from GE Healthcare Life Sciences (Piscataway, NJ), 5(6)-Carboxyfluorescein (FAM) was obtained from AnaSpec Inc. (San Jose, CA), and human normal immunoglobulin G (hIgG) was obtained from Rockland (Gilbertsville, PA).

Synthesis of h173-Cy5.5, hIgG-Cy5.5, h173-FAM, and hIgG-FAM

h173-Cy5.5 and hIgG-Cy5.5 were synthesized according to our previously reported procedure [21, 22]. The molar reaction ratio of h173 or hIgG to Cy5.5-NHS was 1:1. The ratio of Cy5.5 to h173 or hIgG was determined by measuring the optical density (OD) 680 nm and OD 280 nm with Beckman DU 530 spectrophotometer (Beckman Instruments Inc., Fullerton, CA). Molar concentrations of Cy5.5 and an antibody were calculated using molar extinction coefficients of $250,000 \text{ M}^{-1} \text{ cm}^{-1}$ for the Cy5.5 dye and $170,000 \text{ M}^{-1} \text{ cm}^{-1}$ for the antibody.

h173-FAM and hIgG-FAM were also synthesized using the same procedure. OD 495 nm was measured to evaluate the fluorescence intensity of these probes.

Binding Activity Assay

Axl binding activity assay of h173-Cy5.5 and hIgG-Cy5.5 was evaluated through Protein G-Agarose bead-based binding assay with Axl-AP as reported previously [21]. Each measurement was repeated in triplicate.

Cell Culture and Tumor Xenografts

The human lung cancer cell lines (A549 and NCI-H249) were obtained from American Type Culture Collection (Manassas, VA) and cultured in RPMI-1640 and 10 % fetal bovine serum (Omega Scientific, Tarzana, CA).

All animal procedures undertaken were approved by the University of Southern California Institutional Animal Care and Use Committee. A lung tumor xenograft model was established by injecting 2×10^6 of A549 or NCI-H249 cells subcutaneously in the right shoulder of nude mice as described previously [21–23].

Immunofluorescence Staining

Immunofluorescence staining was performed as described previously [21]. Axl antibody (Cat #8661) from CST Inc. (Danvers, MA) was used as the primary antibody, and goat anti-rabbit Alexa Fluor 568 from Invitrogen (Paisley, Scotland) was used as the secondary antibody. Images were acquired with a Nikon Eclipse 80i fluorescence microscope (Tokyo, Japan).

Fluorescence-Activated Cell Sorting (FACS) and Fluorescence Microscopy Analysis

For FACS analysis, the attached A549 cells were detached by incubation with citric saline buffer (0.135-M potassium chloride and 0.015-M sodium citrate) as described previously [24]. NCI-H249 cells grow as floating aggregates. Harvested A549 and NCI-H249 cells were aliquoted to 1×10^6 cells/tube and blocked with 10 % normal goat serum on ice. Cells were then incubated with 10 $\mu\text{g/ml}$ hIgG-FAM or h173-FAM, respectively, for 30 min on ice. Subsequently, cells were washed twice with cold phosphate-buffered saline (PBS) and stained with 100- μl 4'-6-diamidino-2-phenylindole (DAPI, 1 $\mu\text{g/ml}$) diluted in PBS. For the quantification of fluorescence by flow cytometry (CyAn analyzer, Beckman Coulter), 10,000 viable cells (DAPI negative) were counted and analyzed. Each sample was repeated in triplicate.

For fluorescence microscopy analysis, A549 cells were planted on a BD Falcon 4-well chamber slide at 5×10^4 cells/well for 24 h. Cells were incubated with 300- μl complete culture medium containing 10 $\mu\text{g/ml}$ hIgG-FAM or h173-FAM, respectively, for 0.5 h at 37 °C. After incubation, cells were washed with PBS and fixed in 4 % paraformaldehyde for 20 min at room temperature. Images were then acquired after mounting.

In Vivo and Ex Vivo NIRF Imaging

In vivo and *ex vivo* fluorescence imaging was performed as reported previously [21, 22]. Five tumors in each group were size-matched. After tail vein injection of 30- μ g hIgG-Cy5.5 or h173-Cy5.5, each mouse was imaged at various time points. *Ex vivo* fluorescence imaging of the tumor and major organs was performed at 2 days postinjection (p.i.).

Antibody Distribution Assay

Antibody distribution assay was performed as described previously [21, 23]. Tumors were dissected at 2 days p.i. Secondary antibody goat anti-human Alexa Fluor 568 was used to detect primary antibodies.

Statistical Analysis

Quantitative data are presented as the mean $\pm$ SD. For statistical analysis, the Student's *t* test was used, with the level of significance at $P < 0.05$.

Results

Axl Expression Analysis

We selected the two human lung cancer cell lines, A549 and NCI-H249, for this study.

A549 is a NSCLC cell line, which grows adherently, while NCI-H249 is a SCLC cell line, which grows in suspension. Immunofluorescence staining of tumor tissues (Fig. 1a, b) clearly demonstrated that A549 tumor cells overexpressed the Axl receptor while NCI-H249 did not. This result was consistent with the former report on the correlation between Axl expression and adherence of lung cancer cell lines [13].

Synthesis of h173-Cy5.5, hIgG-Cy5.5, h173-FAM, and hIgG-FAM

h173 and hIgG were conjugated with NIRF dye Cy5.5 through amino groups, which lead to h173-Cy5.5 and hIgG-Cy5.5, respectively. The molar ratio of Cy5.5 to h173 or hIgG was determined to be 0.977 ± 0.022 and 0.984 ± 0.020 , respectively. Binding activity assay was performed to investigate the influence of Cy5.5 labeling on the Axl-binding ability. h173-Cy5.5 maintained a 97.75 ± 2.09 % Axl-binding activity and hIgG-Cy5.5 had minimal binding (0.014 ± 0.001 %) toward this target.

hIgG-FAM and h173-FAM were also synthesized using the same procedure for a cell uptake study. Fluorescence-activated cell sorting (FACS) analysis was performed on A549 and NCI-H249 cells. As shown in Fig. 2a, both A549

Fig. 1. Axl staining on A549 and NCI-H249 tumor sections. Scale bar, 20 μ m.

Fig. 2. **a** Fluorescence-activated cell sorting (FACS) analysis of A549 and NCI-H249 tumor cells with hIgG-FAM or h173-FAM, respectively. **b** Fluorescence microscopy analysis of A549 tumor cells with hIgG-FAM or h173-FAM, respectively. Scale bar, 20 μ m.

and NCI-H249 cells showed minimal hIgG-FAM uptake (1.66 ± 0.23 %, 1.55 ± 0.40 %, respectively). However, A549 cells demonstrated significantly higher h173-FAM uptake (85.31 ± 4.03 %) than did NCI-H249 cells (2.46 ± 0.46 %). Fluorescence microscopy analysis (Fig. 2b) on A549 cells also showed a much stronger fluorescence signal with h173-FAM than with hIgG-FAM. These results validated the specific binding of h173 to Axl-positive A549 cells.

NIRF Imaging of Axl Expression

h173-Cy5.5 was applied to NIRF imaging of Axl expression in both A549 and NCI-H249 tumor-bearing mice. To validate the targeting specificity of h173-Cy5.5, hIgG-Cy5.5 was used as the control. Figure 3a, b shows typical

NIRF images and quantification of hIgG-Cy5.5 and h173-Cy5.5 in Axl-positive A549 tumor-bearing mice. The tumor uptake of h173-Cy5.5 increased quickly with time and reached the plateau between 2 and 3 days p.i. At 6 h p.i., the tumor uptake of hIgG-Cy5.5 and h173-Cy5.5 had no significant difference ($P > 0.05$), indicating the majority of the uptake was caused by passive targeting at this time point. At late time points (1, 2, 3, 4, and 7 days), the tumor uptake of h173-Cy5.5 was significantly higher than that of hIgG-Cy5.5 ($P < 0.05$), which confirmed the target specificity of the tumor uptake. *Ex vivo* imaging was conducted to confirm the *in vivo* NIRF imaging results (2 days p.i.). Similar to the *in vivo* NIRF imaging results, the tumor uptake of h173-Cy5.5 was 1.85 times that of hIgG-Cy5.5 on *ex vivo* imaging as shown in Fig. 3c, d, which consisted with *in vivo* NIRF imaging results. Both probes cleared predominantly via the

Fig. 3. **a** *In vivo* near-infrared imaging of A549 tumor-bearing mice after injection of hIgG-Cy5.5 or h173-Cy5.5, respectively. The tumors are indicated by white arrows. **b** Time-fluorescence intensity curves of A549 tumor for hIgG-Cy5.5 and h173-Cy5.5. **c** *Ex vivo* near-infrared imaging of major organs harvested at 2 days p.i. of hIgG-Cy5.5 or h173-Cy5.5. 1 tumor, 2 liver, 3 lung, 4 small intestine, 5 spleen, 6 kidney, and 7 heart. **d** Fluorescence intensity quantification of tumors and major organs at 2 days p.i. of hIgG-Cy5.5 or h173-Cy5.5, respectively. All data in **b** and **d** are presented as the mean $\pm$ SD. The P value was calculated with Student's t test. * $P < 0.05$.

liver, which is in charge of the metabolism of high molecular weight proteins/antibodies. Representative NIRF images and quantification of hIgG-Cy5.5 and h173-Cy5.5 in Axl-negative NCI-H249 tumor-bearing mice were presented in Fig. 4a, b. The tumor uptake of both hIgG-Cy5.5 and h173-Cy5.5 was low and showed no significant difference between them ($P > 0.05$) at all time points examined, indicating the tumor uptake of h173-Cy5.5 was not Axl-mediated. *Ex vivo* imaging further validated the *in vivo* NIRF imaging results (Fig. 4c, d).

Probe Distribution Analysis

The distribution of hIgG-Cy5.5 and h173-Cy5.5 in both A549 and NCI-H249 tumor tissues was further analyzed at 2 days p.i. Immunofluorescence staining (Fig. 5) revealed that a larger amount of h173 accumulated in A549 tumor tissues compared with the hIgG control. Both h173 and hIgG showed minimal accumulation in NCI-H249 tumor tissues, which had a good correlation with the *in vivo* imaging results.

Discussion

This study shows that NIRF dye Cy5.5-labeled h173, a humanized monoclonal antibody against human Axl, exhibits high Axl specificity *in vitro* and *in vivo*. As Axl is a novel target for cancer diagnosis, prognosis, and therapy [5, 25], visualization and quantification of Axl expression through noninvasive imaging methods could be valuable for patient management and in clinical trials.

To avoid the severe reduction of h173 binding activity and the self-quenching effect of Cy5.5, a molar ratio of 1:1 was used for the h173-Cy5.5 conjugation. After conjugation, the binding activity of h173-Cy5.5 was determined to be 97.75 ± 2.09 % of the untouched 173, which indicated that the modification of accessible lysine residues of h173 minimally alter its antigen-binding ability. FACS (Fig. 2a) and fluorescence microscopy analysis (Fig. 2b) further validated the specific binding of h173 to Axl-positive A549 cells.

After *in vitro* characterization, h173-Cy5.5 was further applied to *in vivo* NIRF imaging of Axl expression in both A549 (Axl positive) and NCI-H249 (Axl negative) tumor-

Fig. 4. **a** *In vivo* near-infrared imaging of NCI-H249 tumor-bearing mice after injection of hlgG-Cy5.5 or h173-Cy5.5, respectively. The tumors are indicated by white arrows. **b** Time-fluorescence intensity curves of NCI-H249 tumor for hlgG-Cy5.5 and h173-Cy5.5. **c** *Ex vivo* near-infrared imaging of major organs harvested at 2 days p.i. of hlgG-Cy5.5 or h173-Cy5.5. 1 tumor, 2 liver, 3 lung, 4 small intestine, 5 spleen, 6 kidney, and 7 heart. **d** Fluorescence intensity quantification of tumors and major organs at 2 days p.i. of hlgG-Cy5.5 or h173-Cy5.5, respectively. All data in **b** and **d** are presented as the mean $\pm$ SD.

bearing mice. In order to validate the targeting specificity of h173-Cy5.5, nonbinding hlgG-Cy5.5 was used as the control. In A549 tumor-bearing mice, h173-Cy5.5 reached the tumor site quickly, and the uptake value reached the plateau between 2 and 3 days p.i. (Fig. 3a, b). At 6 h p.i., the tumor uptake of hlgG-Cy5.5 and h173-Cy5.5 had no significant difference ($P>0.05$), indicating that the major fraction of h173-Cy5.5 tumor uptake was nonspecific at an early time point. The enhanced permeability and retention (EPR) effect may contribute to the passive uptake of antibodies in the tumor at this time point. At late time points (1, 2, 3, 4, and 7 days), the tumor uptake of h173-Cy5.5 was significantly higher than that of hlgG-Cy5.5 ($P<0.05$). Clearly, the active uptake of h173-Cy5.5 in the tumor was mainly caused by an h173-Axl specific interaction at these time points. *Ex vivo* imaging (Fig. 3c, d) and immunofluorescent staining analysis (Fig. 5) further validated the *in vivo* imaging data. In NCI-H249 tumor-bearing mice, the tumor uptake of both hlgG-Cy5.5 and h173-Cy5.5 was low and had no significant difference between them ($P>0.05$) at all time points examined (Fig. 4a, b), which was further validated by *ex vivo* imaging (Fig. 4c, d) and

immunofluorescent staining analysis (Fig. 5). These results indicated that the accumulation of h173-Cy5.5 in NCI-H249 tumors was not Axl-mediated. The liver showed a modest accumulation of the two probes. On the contrary, probe accumulation in other organs (the lung, small intestine, spleen, kidney, and heart) was minimal. All *in vivo*, *ex vivo*, and *in vitro* data suggested that h173-Cy5.5 exhibited as a valid probe for Axl-targeted cancer imaging. To the best of our knowledge, this study demonstrated the noninvasive imaging of Axl for the first time. The imaging probes based on the h173 antibody could be potentially used for patient satisfaction (for Axl-targeted immunotherapy) once the translatable agents are properly designed and developed. However, systematic study is needed before the imaging agents are used for a treatment-monitoring process. Axl-targeted imaging could not be used for therapy-based inhibitors of Axl kinase [18, 20] since they do not down-regulate Axl expression. For antibody-based immunotherapies, the therapeutic antibody should minimally interfere the binding of the imaging antibody in order to eliminate the potential blocking effect from the circulating therapeutic antibody. Moreover, Axl-targeted therapy not only inhibits

Fig. 5. Antibody staining on A549 or NCI-H249 tumor sections 2 days p.i. of hIgG-Cy5.5 or h173-Cy5.5, respectively. Scale bar, 20 μ m.

tumor cell growth but also reduces tumor-associated vascular density [16, 19], which could result in reduced probe uptake. All these factors need to be considered in Axl-targeted treatment-monitoring experiments.

Full antibodies have long circulation $t_{1/2}$, attributed to the interaction of an Fc domain and salvage receptor neonatal Fc receptor (FcRn) [26]. This characteristic is advantageous during treatment because less administered dose is needed to maintain antibody serum levels. In addition, the high h173-Cy5.5 uptake in the Axl-positive A549 cancer cells along with a minimal distribution in the nontargeted organs ensures future study of h173-based cancer therapy efficacy. h173 could be further conjugated with therapeutic radio-nuclides [27], drug delivery nanoparticles [28], or toxins [29] for enhanced therapeutic efficacy. However, the fact that tumor uptake of h173-Cy5.5 does not peak until 2 days p.i. could compromise its imaging application in some situations. This is particularly a drawback when repeated imaging is demanded within short-term intervals, for instance when dynamically monitoring the Axl-expression level during treatment. New forms of engineered antibody fragments, such as F(ab')₂ (bivalent, 110 kD), scFv-Fc (bivalent, 100 kD), minibody (bivalent, 80 kD), triabody

(trivalent, 75 kD), and diabody (bivalent, 55 kD) [30], may have similar or even higher binding affinity than a whole antibody but with reduced molecular weight. Therefore, studies based on these new forms of antibodies are expected to optimize imaging pharmacokinetics. Optical imaging with NIRF probes offers relatively low cost, nonradioactive, and semiquantitative imaging. Because of shallow penetration depth and intense light scattering, optical imaging is relevant for superficial or endoscopy-accessible tissues, as well as intraoperative visualization. Further studies to generate NIRF probes with high quantum yield and deep tissue penetration are expected. Besides, the combination of NIRF imaging with magnetic resonance imaging (MRI), positron emission tomography (PET), or X-ray can further offer anatomical and functional information with accurate location and quantification of the fluorescence signal, as well as integrate diagnosis and treatment [31, 32]. Axl is also expressed by tumor-associated vascular cells, such as endothelial cells and vascular smooth muscle cells [33, 34]. h173 does not recognize murine Axl; it can only target human tumor cells not murine vasculature in our lung cancer xenograft models. Thus, the different tumor uptake level could reflect well the Axl expression in different tumor cells.

We predict that it will be more efficient in Axl-targeted imaging and therapy in humans, targeting directly both tumor cells and tumor vasculature.

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Conflict of Interest. The authors declare the following competing financial interest(s): K.N. is the employee of Vasgene Therapeutics Inc.

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