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Research Article

Molecular Cancer Therapeutics

Effective Therapeutic Targeting of the Overexpressed HER-2 Receptor in a Highly Metastatic Orthotopic Model of Esophageal Carcinoma

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Abstract

This study aimed to determine the targeted efficacy of trastuzumab (Herceptin) on human epidermal growth factor receptor 2 (HER-2)-overexpressing metastatic esophageal cancer in an orthotopic mouse model. HER-2 overexpression and amplification of human esophageal primary and metastatic tumors were shown with HER-2–fluorescence *in situ* hybridization analysis and HER-2 immunostaining. Following orthotopic implantation with the HER-2–overexpressing OE19 human esophageal cancer cell line, mice were treated with trastuzumab. Sequential magnetic resonance imaging was used to monitor primary tumor and metastasis during treatment. After six weeks, a significant inhibition of primary tumor development was imaged in trastuzumab-treated animals in comparison with the control group. Trastuzumab treatment also led to a reduction of lymphatic metastasis. Thus, HER-2 targeted therapy with trastuzumab resulted in a significant primary tumor growth reduction as well as a decrease of lymph node metastases in the orthotopic model of metastatic esophageal carcinoma. The results of the present study suggest the clinical use of trastuzumab for HER-2–overexpressing esophageal cancer, which is a significant fraction of the patient population. Treatment of this highly treatment-resistant disease with trastuzumab in the adjuvant setting to prevent lymph node metastasis after primary tumor resection is suggested by the data in this report. *Mol Cancer Ther; 9(7); 2037–45. ©2010 AACR.*

Introduction

Human epidermal growth factor receptor 2 (HER-2) overexpression has been found in breast and other types of human cancer and has been developed as a therapeutic target (1–3). *HER-2* gene amplification and protein overexpression are observed in about 20% of breast cancers (4) and are associated with a poor prognosis for these patients (5).

Antibody-based therapy with trastuzumab (Herceptin) is used clinically for targeting HER-2–positive breast cancer (6–8). Trastuzumab is most effective in *HER-2*–positive breast cancer patients when used as adjuvant therapy (9). There is also evidence for the possible efficacy of trastuzumab in HER-2–overexpressing cancers other than breast (10–12).

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HER-2 overexpression was reported in esophageal cancer, with a tendency towards higher rates of positivity in adenocarcinoma (13–27) compared with squamous cell carcinomas (16, 19, 28–33).

A study of 110 esophageal adenocarcinoma patients has shown a strong concordance of HER-2 overexpression in primary and metastatic cancers with high-level *HER-2* gene amplification. These data suggest esophageal cancer patients with HER-2–overexpressing primary tumors as candidates for trastuzumab therapy (34).

We had previously established an esophageal cancer surgical orthotopic implantation nude mouse model with organ and lymph node metastasis (35). In the present study, the surgical orthotopic implantation of the OE19 human esophageal cancer that overexpresses HER-2 was used. This nude mouse model exhibits the patterns of local and metastatic behavior occurring in clinical human esophageal carcinoma. With its high tumor take and metastatic frequency, it is a relevant model for investigating targeted therapies against primary tumor progression as well as metastatic spread. Small-animal magnetic resonance imaging (MRI) was used for real-time *in vivo* imaging of primary tumor and metastatic progression (35).

The aim of the present study was to evaluate the response to targeted trastuzumab therapy in this orthotopic esophageal adenocarcinoma model.

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Materials and Methods

Cell line

The human esophageal carcinoma cell line OE19 was obtained from the European Collection of Cell Cultures, Health Protection Agency. The cells were cultured in RPMI 1640 medium (Biochrome KG) containing 10% fetal bovine serum (Linaris), penicillin/streptomycin (Biochrome KG), transferrin (Sigma-Aldrich), insulin (Sigma-Aldrich), basic fibroblast growth factor (Boehringer), and epidermal growth factor (Boehringer).

Fluorescence *in situ* hybridization

For proteolytic slide pretreatment, a commercial kit was utilized (Paraffin pretreatment reagent kit, Vysis). Spectrum Orange–labeled *HER-2* probes were used together with Spectrum Green–labeled centromer 17 reference probes (PathVysiont, Vysis-Abbott). Before hybridization, sections were deparaffinized, air dried, dehydrated, and then denatured for 5 minutes at 74°C in 70% formamide-2× SSC solution. After overnight hybridization at 37°C in a humidified chamber, slides were washed and counterstained with 0.2 mmol/L 4′, 6-diamidino-2-phenylindole in an antifade solution. The mean numbers of *HER-2* and centromer 17 signals were estimated for each tumor sample as previously described (36, 37). Our criterion for *HER-2* gene amplification was a *HER-2*/centromer 17 signal ratio Z2. Low-level amplification ratio was defined as *HER-2*/centromer 17 at Z2 to 3. High-level amplification was defined as *HER-2*/centromer 17 ratio of Z3.

Immunohistochemistry of HER-2 expression

The HercepTest (DAKO) was used according to the manufacturer's protocol. Antigen retrieval of the deparaffinized tissue sections was done in a waterbath at 95°C to 99°C for 50 minutes followed by peroxidase blocking and incubation with the prediluted primary antibody. Cell line test slides provided by the manufacturer were used as positive and negative controls. Immunostaining was scored by one pathologist (U.R.), following a four-step scale (0, 1þ, 2þ, 3þ) according to the manufacturer's directions.

RNA extraction and HER-2 amplification

OE19 cancer cells were harvested after trypsinization and washed three times, and 5×10^6 cells were used for RNA extraction using the PARIS Kit (Ambion). This was repeated for MDA-MB-231 and SKBr-3 cells as positive controls. cDNA was obtained with an Invitrogen reverse transcriptase-PCR kit. Amplification of *HER-2* DNA was done with a Thermocycler (Biometra) with Taq-Polymerase (Roche) and *HER-2*–specific primers (GAGCCGCGAGCACCCAAGT, TCCATTGTCTAGCACGGCCA) as well as GAPDH primers as controls (ACCACAGTCCATGCCATCAC, TCCACCACCCTGTTGCTGTA). PCR products were run on a 2% agarose gel. Imaging was done with a Biodoc II (Biometra). For size reference, a 100 bp DNA ladder (New England Biolabs Inc.) was used.

Cell proliferation assay

The effect of trastuzumab on tumor cell growth was examined with a nonradioactive cell proliferation assay (MTT, Promega). OE19 cells were cultured in 96-well plates. Each well contained 10,000 cells in 100 μL RPMI 1640 medium (Linaris) with 10% FCS (Linaris). After overnight incubation, the medium was removed and replaced with RPMI 1640 plus 10% FCS for control and 20 μg/mL trastuzumab (Roche) in RPMI 1640 plus 10% FCS. One plate was used for determination of the starting concentration. MTT reagent (20 μL) was added. The extinction was measured in an enzyme-linked immunosorbent assay reader (Microplate Reader, Dynatech MR500) after 2 hours of incubation at 37°C. The remaining plates were incubated for 72 hours and the extinction was measured as mentioned above.

Orthotopic esophageal carcinoma mouse model

NMRI/*nu/nu* (U.S. Naval Medical Research Institute) nude mice were obtained from Charles River Deutschland at 10 weeks of age and housed in the animal facility of the University Medical Center Hamburg-Eppendorf. All animal procedures were done in accordance with a protocol approved by the Behörde für Wissenschaft und Gesundheit (Freie und Hansestadt Hamburg, Germany). OE19 cells (5×10^6), in a 200 μL suspension, were s.c. injected into the flanks of nude mice with a 1 mL syringe (BD Plastipak, Becton Dickinson S.A.) with a 27-gauge hypodermic needle (Sterican) within 40 minutes of harvesting. The mice were weighed and examined for tumor development every other day. When tumor growth reached 5 to 7 mm, tumors were excised. Esophageal tumor fragments (1 mm^3), derived from the s.c. tumors, were orthotopically implanted to the abdominal esophagus (38). Mice were anaesthetized with ketamine hydrochloride (Graeub)/xylazine hydrochloride (Bayer) mixture (12 mg/mL) and i.p. injected at 10 ml/kg body weight. A 0.8-cm transverse incision in the skin of the epigastric abdomen was made. The abdominal muscles and peritoneum were separated by sharp dissection, and the abdomen was opened. The great curvature of the stomach was held by forceps, and the liver was raised to expose the abdominal esophagus. A lesion of the esophageal serosa was made with sharp forceps. Four tumor fragments were sutured to this lesion with 8.0 prolene sutures (Ethicon). The incision of the abdominal wall was closed using 6.0 vicryl sutures (Ethicon). All procedures of the operation, as described above, were done under an operating dissecting microscope (Carl Zeiss). Postoperative analgesia was achieved by novamine sulfone (1 mg/mL) in drinking water. The mice were weighed and examined for tumor development every other day.

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Therapy with trastuzumab (Herceptin)

After primary tumor growth was determined by MRI on day 14, the mice were randomized into two groups. Group 1 was treated biweekly with an i.p. injection of 20 mg/kg body weight trastuzumab (Roche) in a volume of 100 μL. Group 2 was given sham injections with 100 μL PBS and was used as a control group.

Magnetic resonance imaging

Whole-body MRI of tumor-bearing mice was done to assess the localization and size of tumors under ketamine hydrochloride/xylacine hydrochloride anesthesia as described above. High-resolution magnetic resonance data sets were acquired on a clinical 3 Tesla whole-body magnetic resonance scanner (Intera, Philips Medical Systems) equipped with a standard gradient system (gradient strength = 40 mT/m). A dedicated small-animal solenoid receiver coil (Philips Research) with an inner diameter of 40 mm and an integrated heating system to regulate the body temperature of mice during magnetic resonance scans was used for signal exploitation. The magnetic resonance protocol consisted of a short survey scan and three T2-weighted two-dimensional turbo spin-echo sequences in coronal, sagittal, and axial orientations. Imaging parameters were as follows: repetition time, 2,674 ms; echo time, 90 ms; echo train length, 10; number of excitations, 2. The acquisition matrix and field of view (FOV) were adopted to display the animals in each orientation with an in-plane resolution of 200 × 200 μm. For coronal and sagittal images, a matrix of 400 × 200 pixels and a FOV of 80 × 40 mm were used. Axial images were acquired with a matrix of 160 × 128 pixels and a FOV of 32 × 25.6 mm. Slice parameters were equal for all three sequences with a thickness of 800 μm and 14 slices. The total scan time per animal was 10 minutes and 42 seconds. Image analysis was done on a conventional clinical workstation. Suspicious lesions were considered to be tumors if they appeared hyperintense on the T2-weighted images and had a diameter ≥1 mm.

Examination of esophageal carcinoma at necropsy: primary tumor and metastases

After 6 weeks of treatment, the mice were sacrificed and autopsied. The orthotopic primary tumor as well as lung, liver, and axillary, mediastinal, pancreatic, renal, mesenteric, and inguinal lymph nodes were dissected. Fragments of metastatic liver, lung, and lymph node that were macroscopically visible were placed in Hank's solution for growth of metastatic cell cultures. All other tissues were preserved in formalin and cryopreserved.

Results

HER-2 expression in the OE19 esophageal carcinoma *in vitro* and in the orthotopic model

In comparison with MD-MB-231 and SKBr-3 breast cancer cells, esophageal carcinoma OE19 cells showed high levels of *HER-2* mRNA (Fig. 1A) and thus were chosen for trastuzumab targeted treatment in the orthotopic model. One hundred percent of the mice developed primary tumor growth after implantation of OE19 cell fragments to the abdominal esophagus (Table 1). As we had also previously reported with the human 1590-GFP esophageal carcinoma cell line (35), the primary tumor remained attached to the abdominal esophagus, infiltrated the serosa, but did not cause intraluminal stenosis (Fig. 1B). Immunohistochemical staining of tumor cells showed an intensely positive signal for HER-2 (Fig. 1B). The primary tumor resulted in spontaneous metastases to the liver, lung, and lymph nodes. Macroscopic metastases were recorded by open photography (Fig. 1C).

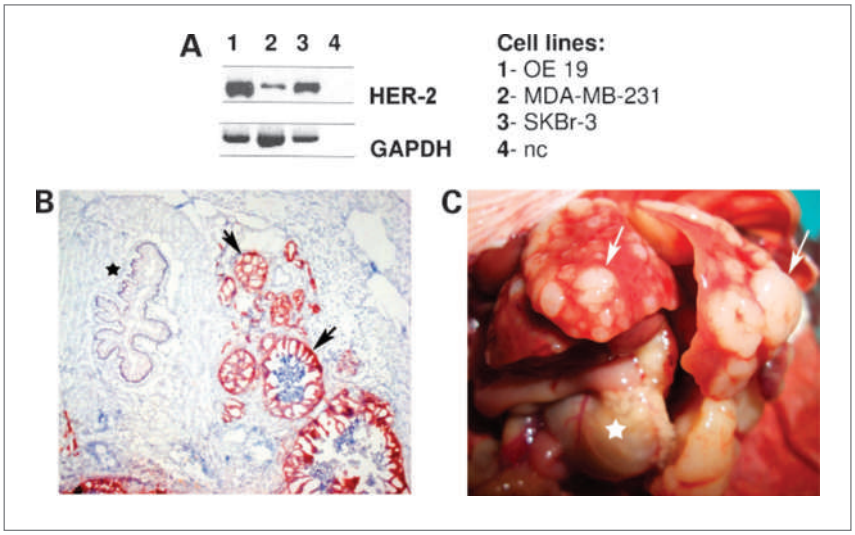

Figure 1. A, *HER-2* mRNA expression in the human esophageal carcinoma cell line OE19 is elevated. B, HER-2–positive primary tumor (arrows) is located at the abdominal esophagus (star) infiltrating the esophageal serosa. C, extensive liver metastases (arrows) can often be identified macroscopically at autopsy. These results are from typical experiments.

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Table 1. Efficacy of trastuzumab on primary and metastatic esophageal cancer

A. Control group

Mouse #	PT weight (g)	PT	PT HER-2$^+$	Metastasis	Metastasis HER-2$^+$	LN metastasis	LN metastasis HER-2$^+$
1	1.7	+	+	+	+	+	+
2	0.7	+	+	–		–	
3	2.4	+	+	+	+	+	+
4	5.4	+	+	+	+	+	+
5	1.8	+	+	+	+	+	+
6	1.9	+	+	+	+	+	+
7	2.6	+	+	–		–	
Overall	2.36	100%	100%	71%	100%	71%	100%

B. Herceptin-treated group

Mouse #	PT weight (g)	PT	PT HER-2$^+$	Metastasis	Metastasis HER-2$^+$	LN metastasis	LN metastasis HER-2$^+$
1	0.6	+	+	+	+	–	
2	0.7	+	+	–		–	
3	1.7	+	+	–		–	
4	1.6	+	+	+	+	+	+
5	1.5	+	+	–		–	
6	0.5	+	+	+	+	+	+
7	0.3	+	+	–		–	
8	1.8	+	+	+	+	+	+
Overall	1.09	100%	100%	50%	100%	37.5%	100%

NOTE: In the control group (A) 100% of mice showed primary tumor (PT) growth and the overall metastatic rate was 71%. In all mice with metastases, lymph node (LN) metastases were found. In the trastuzumab-treated group (B), although 100% of the mice showed primary tumor growth, the tumor weight was significantly lower ($P = 0.044$). The overall metastatic frequency was reduced to 50% and lymph node metastases were reduced to 37.5% in the treated as opposed to 71% in the untreated group.

Inhibition of *in vitro* OE19 cell proliferation by trastuzumab

Trastuzumab inhibited the proliferation of OE19 cells *in vitro*. A significant difference ($P = 0.045$) of relative cell proliferation was observed in cell cultures treated with trastuzumab (Fig. 2A).

Trastuzumab targeting of primary tumor growth

Two weeks after surgical orthotopic implantation of OE19, primary tumor growth was confirmed by MRI. Treatment started at this time. In the treatment group, eight mice were treated with i.p. trastuzumab twice a week. In the control group, eight mice were treated with i.p. PBS of the same volume at the same times as the treatment group. One mouse in the control group died on day 11 after the beginning of treatment due to causes unrelated to tumor growth or therapy and was thus excluded from further analysis. Sequential MRI was done throughout the experiment. At the termination date, after 6 weeks of treatment, the mice were sacrificed.

One hundred percent of the mice in the treated and the untreated groups had primary tumor growth on the abdominal esophagus. Primary tumors were dissected and weighed. Tumor weights ranged between 0.7 g and 5.4 g with a mean of 2.36 g in the control group and between 0.3 g and 1.8 g with a mean of 1.09 g in the trastuzumab-treated group (Table 1A and B), showing a significant difference ($P = 0.044$) between the groups (Fig. 2B). Trastuzumab targeting resulted in significantly reduced primary tumor size. No adverse effects of trastuzumab therapy were observed.

Trastuzumab targeting of metastasis

Primary tumor, parenchymal organs, and lymph nodes were dissected at necropsy and prepared for further analysis. Upon histologic examination, metastases were seen in 71% of animals in the control group, whereas 50% of animals in the trastuzumab-treated group showed metastases (Table 1A and B). Considering only lymphatic metastasis, 71% of the animals in the control group had lymph node metastases whereas 37.5% of animals in the trastuzumab-treated group had positive lymph nodes (Fig. 2C). Trastuzumab targeting thus also leads to a reduction of lymphatic spread.

Primary tumors and liver, lung, and lymph node metastases showed amplification of *HER-2* in fluorescence *in situ* hybridization analysis. An example is given for primary tumor amplification in Fig. 3A. All of the

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primary tumors and metastases in the treated and the untreated groups, regardless of the metastatic organ (liver, lung, lymph node), expressed high levels of HER-2 as seen by immunostaining (Fig. 3B).

In vivo tumor imaging

Sequential *in vivo* MRI of control and treated animals was done every two weeks after orthotopic implantation. The final imaging was done on the day of sacrifice. Primary tumor growth could be confirmed in all cases two weeks after orthotopic implantation. Before the start of treatment, primary tumor imaging showed comparable tumor sizes in untreated and treated groups (Fig. 4A and C) two weeks after orthotopic implantation. Over the time course of the treatment, slower progression of tumor size could be observed in sequential imaging in the trastuzumab-treated group compared to the untreated control, leading to significant imageable tumor size difference at the time of termination of the experiment (Fig. 4B and D).

Discussion

Orthotopic models are essential in the understanding of primary tumor progression *in vivo* and the biology of metastatic spread (38, 39). The esophageal carcinoma cell line OE19 overexpresses HER-2 and thus is ideal for trastuzumab targeting. The orthotopic model showed overexpression of HER-2 in the primary tumor, as well as liver, lung, and lymph node metastases.

The tumor take rate in this OE19 orthotopic tumor model was 100%. Metastatic frequency in this study was 71% in the untreated group. The high primary tumor take and metastatic frequency allowed evaluation of trastuzumab targeting of the primary tumor as well as metastasis.

Orthotopic models have previously been successfully used for evaluation of trastuzumab therapy for other tumor types (40–42). The efficacy of trastuzumab on *HER-2*–positive breast cancer has been shown *in vitro* and in orthotopic models as well as clinically in breast cancer patients (6–9).

There is also evidence for a possible response of *HER-2*–positive cancers other than breast to trastuzumab (10–12). Several studies have suggested that *HER-2* amplification/overexpression may be relevant for these tumor types, but the potential benefit of trastuzumab in tumors other than breast is largely unknown.

Inhibition of metastasis by trastuzumab has been described in orthotopic human pancreatic cancer xenografts with low-level HER-2/*neu* expression (42). Incidence of metastases was reduced, especially in the liver.

There is evidence that HER-2 overexpression is involved in progression from dysplasia in patients with Barrett's esophagus to adenocarcinoma (43), and targeted treatment with trastuzumab has been attempted (44).

HER-2 overexpression was reported in up to 83% of esophageal cancer, with a tendency towards higher rates of positivity in adenocarcinoma (10–83%; refs. 13–27) compared with squamous cell carcinomas (up to 56%; refs. 16, 19, 28–33). A similar variability was observed in amplification analyses.

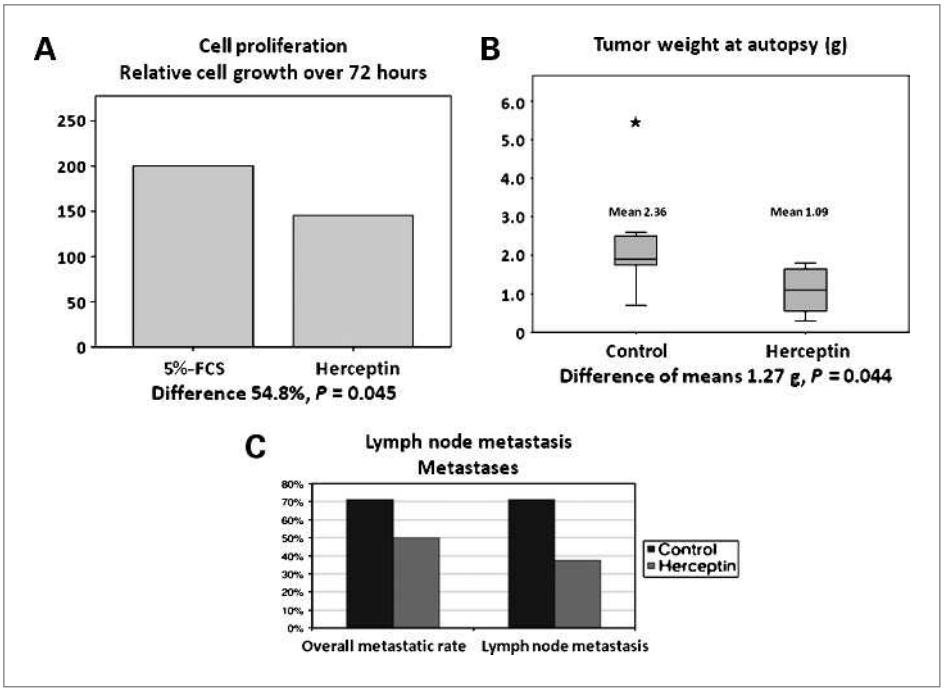

Figure 2. A significant inhibition of cell proliferation as well as primary tumor growth reduction was seen *in vitro* (A) as well as *in vivo* (B) by trastuzumab. HER-2 targeted therapy also led to inhibition of lymph node metastases (C).

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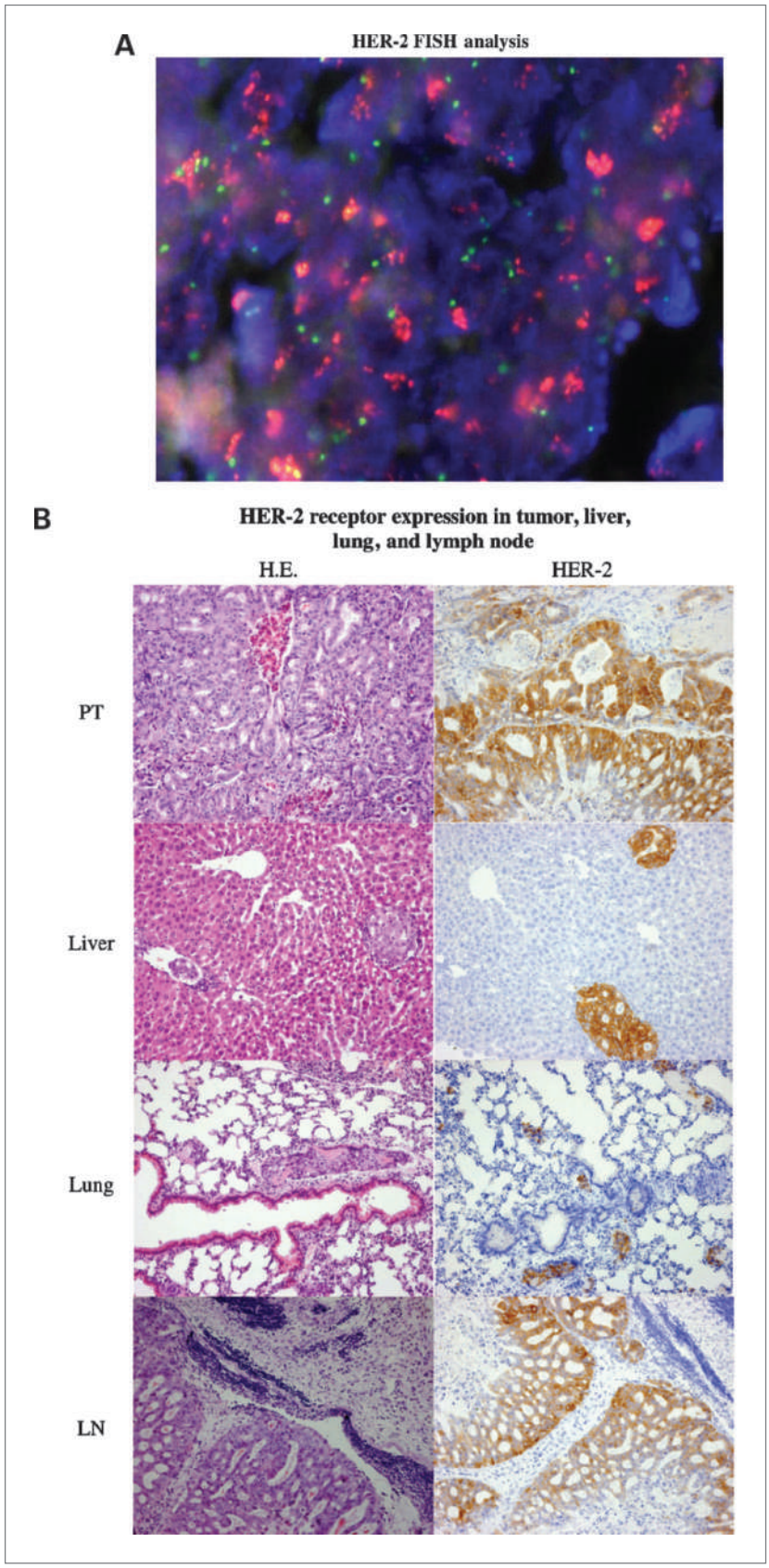

Figure 3. HER-2 expression in esophageal cancer cell line OE19. Orthotopic primary tumor, and liver, lung, and lymph node metastases. OE19 cell line showed high level *HER-2* amplification (A). Orthotopic primary tumor, and metastases to the liver, lung, and lymph node showed high-level *HER-2* overexpression (B) throughout. These results are from typical experiments. FISH, fluorescence *in situ* hybridization; PT, primary tumor; LN, lymph node; H.E., hematoxylin and eosin staining.

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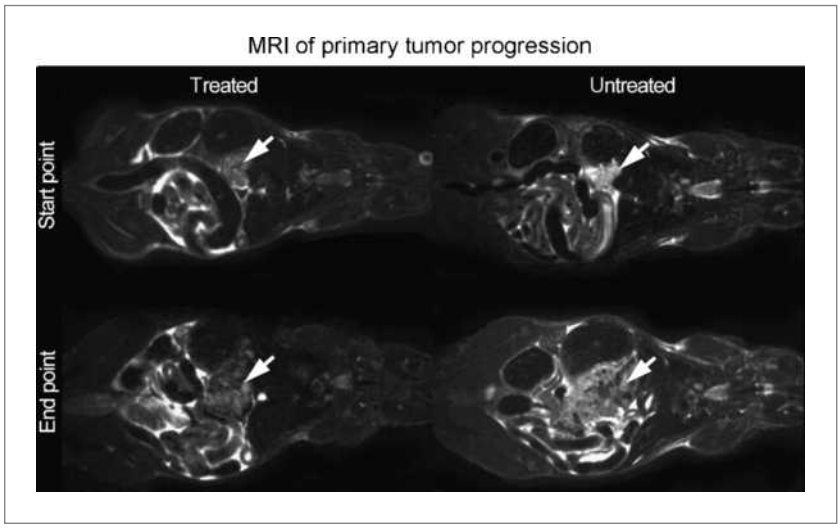

Figure 4. Serial MRI confirmed inhibition of primary tumor progression in trastuzumab targeting at the beginning and end point of treatment in comparison with the untreated group. Arrows, primary tumors.

One phase I-II trial was conducted to assess the effects of trastuzumab in the treatment of patients with esophageal adenocarcinoma overexpressing HER-2 in combination with paclitaxel, cisplatin, and radiation (45). This study was limited by a small number of patients. No increase in toxicity due to trastuzumab was observed.

In an analysis of our patient population, we previously reported a *HER-2* amplification of 15% in esophageal adenocarcinoma patients (16 of 110; ref. 34). We found a strong concordance of the HER-2 status in primary and metastatic esophageal cancer with high-level HER-2 gene amplification. Such patients will be candidates for trastuzumab treatment, preferably in the adjuvant setting, because our results show inhibition of metastasis, especially lymph node metastasis (34).

Our results show a significant reduction of esophageal adenocarcinoma tumor cell proliferation by trastuzumab. *In vitro* a significant reduction of cell proliferation by trastuzumab ($P = 0.045$) was observed. *In vivo* there was a significant reduction of primary tumor growth ($P = 0.044$) in the orthotopic model in the trastuzumab-treated group in comparison with the untreated group. Metastasis was also inhibited. There was no adverse effect to trastuzumab therapy observed *in vivo*.

It has already been shown that trastuzumab blocks cancer cells in the cell cycle (46–49) and induces apoptosis. Furthermore sensitivity of OE19 cells to trastuzumab has previously been shown (50).

In the literature, HER-2 positivity rates in esophageal adenocarcinoma are up to 83% (13–27). Therefore, targeted therapy with trastuzumab in HER-2–positive esophageal carcinoma seems to be an option in the individualized treatment.

There is a discussion on the risk of central nervous system metastases in patients treated with or without trastuzumab. Trastuzumab, which is a large monoclonal antibody, does not penetrate the blood-brain barrier and, thus, may allow the brain to become a sanctuary site for micrometastases. The results of a study by Lai and colleagues did not support an association between trastuzumab therapy and an increased risk of central nervous system metastases (51). However, several other studies report a higher risk in treated patients (52–55).

A Chinese study on the correlation between *HER-2* gene amplification and lymphangiogenesis and their prognostic significance in human breast cancer suggests that by upregulating vascular endothelial growth factor C expression, HER-2 overexpression induces lymphangiogenesis and thus promotes metastasic spread (56).

The efficacy of trastuzumab on tumor angiogenesis will be determined in future experiments.

In our highly metastatic orthotopic model of esophageal carcinoma, the overall metastatic rate *in vivo* was reduced from 71% in the untreated to 50% in the trastuzumab-treated group. Lymph node metastases were seen in 71% of untreated animals, whereas the metastatic rate was reduced to 37.5% in the trastuzumab-treated group. These results show a strong effect of trastuzumab therapy on the primary tumor as well as on lymph node metastases. The results of the present study suggest the clinical use of trastuzumab for HER-2–overexpressing esophageal cancer, which is a significant fraction of the patient population with this disease. In particular, treatment with trastuzumab could be used in the adjuvant setting to prevent lymph node metastasis after primary tumor resection.

Sequential *in vivo* small-animal MRI allows noninvasive imaging of esophageal carcinoma progression over the course of the experiment. After confirmation of primary tumor growth (tumor take rate of 100%), treatment can be started and the effect of treatment monitored over time. Responsiveness to therapeutic treatment can be evaluated in real time with this highly sensitive imaging technique.

In conclusion, HER-2 targeted therapy with trastuzumab (Herceptin) shows a significant primary tumor growth reduction as well as a reduction of lymph node

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metastases in an orthotopic model of metastatic esophageal carcinoma. These preclinical results suggest a role for HER-2 targeted antibody-based treatment of HER-2–overexpressing esophageal carcinoma. The results suggest, in particular, trastuzumab treatment in the adjuvant setting to prevent lymph node metastasis after primary tumor resection.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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