Microscopic lymph node tumor burden quantified by macroscopic dual-tracer molecular imaging

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Lymph node biopsy is employed in many cancer surgeries to identify metastatic disease and to determine cancer stage, yet morbidity and diagnostic delays associated with lymph node biopsy could be avoided if noninvasive imaging of nodal involvement were reliable. Molecular imaging has potential in this regard; however, variable delivery and nonspecific uptake of imaging tracers have made conventional approaches ineffective clinically. Here we present a method of correcting for nonspecific uptake with injection of a second untargeted tracer that allows for quantification of tumor burden in lymph nodes. We confirmed the approach in an athymic mouse model of metastatic human breast cancer by targeting epidermal growth factor receptor, a cell surface receptor overexpressed by many cancers. We observed a significant correlation between in vivo (dual-tracer) and ex vivo measures of tumor burden (r = 0.97, P < 0.01), with an ultimate sensitivity of approximately 200 cells (potentially more sensitive than conventional lymph node biopsy).

The presence or absence of cancer in tumor-draining lymph nodes is recognized as a key element for determining the stage of many cancers, and as a result, node localization and biopsy have become standards of care, most notably for breast cancer and cutaneous melanoma¹. Although helpful for determining cancer stage², lymph node dissections can be associated with overtreatment and considerable morbidity (for example, surgical nerve damage and post-surgical lymphedema)³, and histological analysis of lymph nodes can be time-consuming and may delay subsequent procedures. Motivated by these deficiencies in conventional biopsy, many have attempted to estimate cancer burden noninvasively in animal models and clinical studies, typically employing molecular imaging of cancer-targeted imaging tracers, which has been comprehensively reviewed⁴. Although groups working with these tracers have demonstrated promising results, over 30 years of effort in this area, and at least ten antibody-based imaging tracer clinical trials in the 1980s and 1990s, have failed to result in any widespread use of targeted imaging tracers to detect lymph node metastases. Many factors may affect the success of such approaches, but it is thought that high interstitial variability in tracer delivery to lymph nodes and nonspecific tracer uptake in healthy nodes are the major problems obfuscating the ability to relate tracer uptake directly to tumor burden⁵.

One proposed method to account for variability in tracer delivery has been the simultaneous injection of a second ‘untargeted’ tracer that can be used to account for binding-independent characteristics of a targeted tracer’s uptake⁶–⁸. Our group has recently advanced these dual-tracer approaches to quantify cell-surface cancer receptor concentrations in primary tumors using planar fluorescence imaging⁹ and fluorescence tomography¹⁰. Here, we adapted these principles to lymph imaging and evaluated them in a mouse model of breast cancer metastasis. We used an epidermal growth factor receptor (EGFR)-specific antibody labeled with a near-infrared fluorophore as the targeted tracer and an isotype control antibody labeled with a different near-infrared fluorophore as the untargeted tracer.

RESULTS

In total, we imaged and analyzed tumor burden in the left and right axillary lymph nodes of 22 mice (18 tumor-bearing mice and 4 controls). Six additional tumor-bearing mice had to be euthanized owing to complications with their primary tumor resection surgery. We carried out all imaging with mice in a supine position with forepaws taped above their heads (Fig. 1a). Bioluminescence imaging (Fig. 1b) of tumor spread to lymph nodes was detectable in only 5 of 36 lymph nodes imaged, corresponding to the largest levels of tumor burden (>2,000 cells). We quantified signal from lymph nodes in 5-mm-diameter regions of interest drawn around each axillary region (Fig. 1c). In select cases, corresponding H&E histological staining to verify tumor metastasis to the lymph nodes (Fig. 1d,e) and a quantitative PCR calibration curve (Fig. 1f) were used to directly measure the number of cancer cells present in each lymph node. We observed metastases in both the right and left axillary lymph nodes. Thirteen of the tumor-bearing mice had >200 tumor cells in either the right or left axillary lymph node or both, as measured by quantitative PCR (qPCR) (six on only the right, four on only the left and three in both). We focused on the 200-tumor-cell threshold, as it was two s.d. above the detection limit of the qPCR approach: 100 ± 50 cells.

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Figure 1 Animal model. (a) A white-light image of a mouse positioned for axillary lymph node imaging (1 of 22 mice imaged: 18 tumor-bearing, 4 controls). (b) A bioluminescence image demonstrating metastasis of cancer cells to the axillary lymph node using the MDA-MB-231-luc-D3H2LN cell line (image is representative of 6 of the 18 tumor-bearing mice). (c) White light (left) and EGFR receptor concentration (right) images depicting typical regions of interest used for calculating the average EGFR concentration in a lymph node (representative of 8 mice with >200 tumor cell burden). (d,e) H&E stains of lymph node tissue from a healthy (d) and tumor-bearing (e) mouse (representative of 4 control mice and 8 mice with >200 tumor cell burden, respectively). White arrows in (e) locate a tumor cell cluster. (f) A standard curve relating qPCR numbers to the number of tumor cells. The slope of this line was 257 tumor cells/PCR# (related to PCR cycle number). Scale bars, 1 cm (a–c) and 500 μm (d,e).

We were unable to identify the affected side of the tumor-bearing mice from 3-h fluorescence uptake images of either the targeted or untargeted tracers on their own in any of the tumor-bearing mice (Fig. 2). However, once we corrected for nonspecific and variable delivery of the tracer using the untargeted tracer uptake maps (with the lymph node molecular concentration imaging (LN-MCI) algorithm), we could use the resultant images showing EGFR concentration to clearly identify the affected side of the mouse; there was little appreciable EGFR expression in control mice (Fig. 2).

In all control animal lymph nodes, the average targeted tracer uptake was not significantly different from the average untargeted tracer uptake at any time point (P > 0.05). Conversely, targeted tracer retention was significantly higher than untargeted tracer retention in tumor-bearing lymph nodes (P < 0.001). Quantification of this difference through the LN-MCI approach at multiple time points following tracer injection (using the equation (targeted tracer – untargeted tracer)/untargeted tracer) demonstrated an obvious correlation of EGFR concentration with the extent of tumor burden by 3 h after injection (Fig. 3a). The time it took for this ratio to stabilize was inversely correlated with the measured lymph flow (Fig. 3b and Online Methods).

When evaluating targeted fluorescence uptake alone, we observed very high variability in the efficiency of tracer delivery from the site of injection to the axillary lymph nodes, with an average targeted fluorescence of 0.1 ± 0.1 at 3 h (mean ± s.d. in 36 nodes; range: 0.03–0.18). We did not observe any appreciable or statistical (P > 0.05) differences amongst any of the lymph node groups: controls, mice with <200 cells and mice with >200 cells (Fig. 3c). When we applied the same boxplot analysis to the average EGFR concentration in each lymph node determined by the LN-MCI algorithm (Fig. 3e,f), lymph nodes with >200 tumor cells (16 nodes), as identified by qPCR, had a significantly higher EGFR concentration (P < 0.05) than both the control group (8 nodes) and the <200 cells group (20 nodes), with mean ± s.d. EGFR concentrations of 1.6 ± 1.0 nM, 0.008 ± 0.005 nM, and 0.02 ± 0.02 nM for the >200 cell, control and <200 cell groups, respectively. We did not observe a statistically significant difference between controls and the <200 cell group with Bonferroni correction and a two-tailed analysis (P > 0.05); however, further investigations may yield significance, as we observed P < 0.05 in a simple one-tailed t-test.

Figure 2 LN-MCI. In each set of two images, the left image is a white-light image of a mouse with 30%-threshold overlays of the 3-h post-injection signal from targeted fluorescence (top row), the 3-h post-injection signal from untargeted fluorescence (middle row) or the EGFR concentration map (bottom row). Fluorescence from the injection sites of the forelegs of the mice was also removed in the overlay images. The image on the right of each pair shows the image alone with no thresholds. Each column corresponds to images from a representative mouse from the study: a control mouse (left; 1 of 4 in the imaging study), a tumor-bearing mouse with verified tumor burden in the right axillary lymph node (middle) and a tumor-bearing mouse with verified tumor burden in the left axillary lymph node (right), where the yellow arrows denote the side with confirmed tumor burden (middle and right columns; 2 tumor-bearing mice of 18 included in the imaging study). The units of fluorescence are in percent of the dynamic range of the Pearl Imaging System (i.e., a unit of 100 would be the level of saturation), and the units of the EGFR concentration maps are in nM. Scale bars, 1 cm.
In addition to these findings, we observed a statistically significant correlation ($r = 0.97, P < 0.01$) between the EGFR concentration measured and the number of cells detected in lymph nodes (Fig. 4). The slope of this correlation was 0.4 pM cell$^{-1}$ cm$^{-2}$. Including results from all lymph nodes excised from tumor-bearing mice still resulted in a correlation in lymph nodes with various levels of tumor burden determined by qPCR ($n = 4$ lymph nodes of 36 imaged; dashed black line shows the mean ± s.d. of 8 lymph nodes from 4 control mice). (b) A correlation between the time to plateau of the curves in a and the average lymph flow rates for all tumor-bearing lymph nodes. The dashed line represents the linear regression of the data. (c) Average fluorescence signal measured from the uptake of the EGFR targeted tracer at 3 h after injection in the axillary lymph nodes of control mice ($n = 4$; 8 nodes), nodes from tumor-bearing mice with <200 tumor cells detected by qPCR ($n = 8$; 16 nodes) presented in a boxplot. The boundaries of the box represent the upper and lower quartiles (25% of data greater or less than these boundaries), the horizontal line represents the median and the whiskers represent the maximum and minimum data points excluding outliers (more or less than 1.5 times the upper or lower quartile, respectively). The units of fluorescence correspond to percentage of the dynamic range of the charge-coupled device camera used to detect the fluorescence in the Pearl Imaging System. (d) Log-scale plots of e to illuminate group differences. (e) The same boxplot format is used to compare the average EGFR concentrations measured in vivo in each lymph node using the single time point LN-MCI model. *$P < 0.001$ compared to control and <200 cell groups using a two-tailed one-way ANOVA. $P < 0.05$ compared to the control group using a one-tailed t-test and Tamhane correction for multiple comparisons with unequal variance. Groups in the LN-MCI plots were non-normal by Shapiro-Wilk so Kruskal-Wallis nonparametric test was used to determine significance rather than one-way analysis of variance. (f) Log-scale plots of e to illustrate group differences. Error bars are s.d.

**DISCUSSION**

The novelty of the LN-MCI approach lies in the use of a second untargeted tracer to account for nonspecific uptake of a cancer-targeted imaging tracer. We demonstrated that the approach is capable of quantifying targeted molecule concentrations, as a surrogate of tumor burden, without requiring the tracer uptake images to be quantitative on their own. The approach has the potential to be applied for any cellsurface cancer cell receptor–targeted imaging agent using any molecular imaging modality (or combination of modalities), allowing for simultaneous monitoring of more than one tracer. In the present study, we explored the potential of this LN-MCI approach for imaging EGFR using planar fluorescence imaging, comparing the results to ex vivo qPCR measurements of axillary lymph node tumor burden.

Though any cancer cell marker could theoretically be targeted with LN-MCI, we chose EGFR because antibodies for EGFR are already in clinical use, which could improve the potential for clinical approval of afferent lymph node vessel fluorescence curves (Fig. 5b) as input functions. For a simulated EGFR concentration of 1 nM, the average estimated EGFR concentrations using the LN-MCI approach were 0.14 ± 0.08 nM, 0.52 ± 0.04 nM, 0.84 ± 0.04 nM, and 0.95 ± 0.04 nM when employed at 20, 60, 120, and 180 min after tracer injection, respectively (Fig. 5c,d).

**Figure 3** Dual-tracer compared to single tracer imaging. (a) Ratiometric estimations of EGFR concentration ((targeted – untargeted)/untargeted) as a function of time after tracer injection are presented for lymph nodes with various levels of tumor burden determined by qPCR ($n = 4$ lymph nodes of 36 imaged; dashed black line shows the mean ± s.d. of 8 lymph nodes from 4 control mice). (b) A correlation between the time to plateau of the curves in a and the average lymph flow rates for all tumor-bearing lymph nodes. The dashed line represents the linear regression of the data. (c) Average fluorescence signal measured from the uptake of the EGFR targeted tracer at 3 h after injection in the axillary lymph nodes of control mice ($n = 4$; 8 nodes), nodes from tumor-bearing mice with <200 tumor cells detected by qPCR ($n = 8$; 16 nodes) presented in a boxplot. The boundaries of the box represent the upper and lower quartiles (25% of data greater or less than these boundaries), the horizontal line represents the median and the whiskers represent the maximum and minimum data points excluding outliers (more or less than 1.5 times the upper or lower quartile, respectively). The units of fluorescence correspond to percentage of the dynamic range of the charge-coupled device camera used to detect the fluorescence in the Pearl Imaging System. (d) Log-scale plots of e to illuminate group differences. (e) The same boxplot format is used to compare the average EGFR concentrations measured in vivo in each lymph node using the single time point LN-MCI model. *$P < 0.001$ compared to control and <200 cell groups using a two-tailed one-way ANOVA. $P < 0.05$ compared to the control group using a one-tailed t-test and Tamhane correction for multiple comparisons with unequal variance. Groups in the LN-MCI plots were non-normal by Shapiro-Wilk so Kruskal-Wallis nonparametric test was used to determine significance rather than one-way analysis of variance. (f) Log-scale plots of e to illustrate group differences. Error bars are s.d.

**Figure 4** Estimation of tumor burden. (a) The correlation between dual-tracer imaging estimates of axillary lymph node tumor burden at 3 h after tracer injection and qPCR tumor burden measures: $n = 36$ axillary lymph nodes from 18 mice. (b) A similar plot as in a but for lymph nodes with <1,000 cells to provide a clearer view of the correlation at lower tumor burden. Linear regression (dashed black lines) determined the correlation in a and b to be statistically significant (slope: 0.4 pM cell$^{-1}$ cm$^{-2}$ $r = 0.97, P < 0.01$ in both plots).
Figure 5 Modeling and simulations.
(a) The tracer kinetic compartment model for the untargeted (left) and targeted (right) tracers. $C_t$ represents the concentration of tracer in the upstream lymph vessels feeding the sentinel lymph node, $C_U$ represents the concentration of untargeted tracer in the lymph node, $C_r$ represents the concentration of unbound targeted tracer in the lymph node, $F_I$ represents the concentration of specifically bound targeted tracer in the lymph node, $k_3$ and $k_4$ represent the rates of association and dissociation, respectively, of the targeted tracer with its receptor. (b) Typical curves of average fluorescence at the site of injection for the targeted tracer and the untargeted tracer (representative of all mice). (c) The results of a simulation study noise analysis presented in the form of a histogram, applying the single-time-point receptor concentration estimate at 20, 60, 120 and 180 min after tracer injection for a lymph flow rate of 0.15 min$^{-1}$ and a simulated receptor concentration of 1 nM (results are representative of 2% random Gaussian noise added to simulated tracer uptake curves). (d) The mean ± s.d. of the histograms plotted in c for over a range of theoretical lymph flow rates (results are from 1,000 noise iterations).

Imaging agent–conjugated forms, because it is overexpressed in many cancer types and there is a high concordance between EGFR expression in primary tumor and lymph node metastases in a number of cancer types, so that conventional biopsy of the primary tumor could be used to confirm whether or not EGFR is a suitable target. A potential drawback of targeting EGFR is that it is expressed in a number of other healthy human tissues, such as skin. This could result in substantial background signal if the tracers were injected systemically; however, the localized injection approaches used in lymph node tracking result in the majority of tracer remaining in the lymph node.

The salient findings of this study were that axillary lymph node concentrations of EGFR estimated by the LN-MCI model demonstrated a statistically significant correlation with ex vivo measures of lymph node tumor burden by 3 h after tracer injection and that the ultimate sensitivity of the LN-MCI approach and the ex vivo qPCR method of quantifying lymph node tumor was approximately 100 cells. The 3-h delay before the LN-MCI approach became accurate was supported by simulation studies and was attributable to the fact that it takes time for a significant proportion of signal to come from bound tracer, the length of which was inversely proportional to the lymph flow rate (Figs. 3b and 5d).

With respect to the first finding, the slope of the correlation at 0.4 pM cell$^{-1}$ cm$^{-2}$ matched well with a theoretical slope of 0.6 pM cell$^{-1}$ cm$^{-2}$, assuming $1.5 	imes 10^6$ EGFRs per cell within a 2-mm diameter lymph node. A recent study demonstrated a weak correlation between uptake of a targeted positron emission tomography tracer and lymph node weight, and another study demonstrated a correlation between lymph node tracking result in the majority of tracer remaining in the lymph node sections if they overlap the region where the lymph node was cut; however, they will not be observed if they are located outside this region.

We carried out all experiments in this study in a light-tight imaging system. Our group has recently constructed and validated a multi-wavelength imaging system capable of imaging with room lights on by taking advantage of pulsed fluorescence excitation and time-gated detection, which may pave the way to a more rapid clinical translation of the finding in this study.
To correlate with lymph node tumor burden, LN-MCI requires four suppositions to be true: i) the targeted and untargeted tracers have equivalent delivery to the lymph node; ii) the targeted tracer concentration is negligible compared to the concentration of the targeted receptor; iii) the metastatic cancer cells overexpress the receptor being targeted; and iv) the imaging approach needs to be able to detect fluorescence in the lymph node of interest.

With respect to i), the relative equivalency of the tracer uptake curves in tumor-free axillary lymph nodes suggests similar lymph uptake, flow kinetics, pharmacokinetics, biodistribution and photostability for the two tracers employed and similar negligible autofluorescence and optical properties between the two imaging channels within the 3-h imaging window. The equivalency of tracer delivery and washout to the lymph node was expected, as these parameters are primarily dependent on size\textsuperscript{28}, and both tracers were based on ~150-kDa antibodies labeled with ~1-kDa fluorophores. There was a slight bias, with the average EGFR estimate in control nodes of 0.2 ± 0.2 nM, which could have been a result of background EGFR binding or a slight difference in tracer delivery rate; however, this did not affect the apparent 200-cell sensitivity of the LN-MCI approach. Every new set of tracers should be tested before being used in this dual-tracer imaging approach, and new dual-fluorescence ratiometric tracers may represent the best way forward\textsuperscript{25}.

With respect to ii), for the current study, assuming the cells are roughly 20 μm in diameter, then in the volume surrounding the cell (~4,000 μm\textsuperscript{3}, assuming the cell to be a sphere), there are on average 1.5 × 10\textsuperscript{6} EGFR molecules, yielding a localized EGFR concentration of ~60 nM. If extrapolating from the average fluorescence at the site of injection over time, and assuming all of the fluorescence not in the footpad had accumulated in the lymph node, approximately 0.1% of the injected dose of 0.05 nanomoles at a maximum could be in the axillary lymph node. If the internal diameter of the lymph node is assumed to be about 2 mm, and the tracer is homogeneously distributed throughout the lymph node, this results in a maximum concentration of targeted tracer of ~6 nM, about 10% of the localized EGFR concentration. Moreover, as tracer is constantly flowing out of the lymph node and not all of the tracer will have traveled through the axillary lymph node, the actual concentration of tracer in the lymph node is probably at least an order of magnitude less.

With respect to iii), gene expression can vary from patient to patient and even within a tumor\textsuperscript{29}. Therefore, the optimal receptor to target will be different from patient to patient, and determining which receptor to trace may require previously undescribed approaches. New high-throughput circulating tumor cell gene analysis approaches may be one way of determining the optimal target\textsuperscript{30}.

Finally, with respect to iv), the limited depth sensitivity of the planar fluorescence imaging presented in this study (~1.5–2 mm, determined by Monte Carlo simulations for both targeted and untargeted imaging agents when assuming homogeneous distribution of fluorescence) would not prevent its use intraoperatively in the clinic; however, more noninvasive applications could be achieved using fluorescence tomography approaches with larger source-detector spacing\textsuperscript{21,31}. These approaches are typically associated with poorer spatial resolution, which could be improved using anatomical imaging guidance; for example, ultrasound-guided fluorescence tomography has been shown to provide sensitivity to optical properties at considerable depths (1–4 cm) in breast tissue\textsuperscript{12,32,33}. The anatomical guidance could also be used to avoid analysis of signals arising from tissue surrounding the lymph nodes that could contain background EGFR\textsuperscript{20}. Depth sensitivity beyond a few centimeters will probably require nuclear medicine approaches such as dual-energy single photon emission tomography\textsuperscript{24} in order to image both targeted and untargeted imaging agents simultaneously. This could be ideal, as single photon emission computed tomography tracers and gamma cameras are widely used to locate lymph nodes through lymphoscintigraphy, and the principles demonstrated here could be readily adapted to such deeper depth sensitivity approaches.

METHODS

Methods and any associated references are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

K.M.T. designed the experiments, developed the kinetic modeling methodology, carried out the experiments, analyzed all imaging data and wrote the paper. K.S.S. helped design the experiments and validation procedure and helped write the paper, J.R.G. carried out much of the animal imaging and carried out all qPCR and bioluminescence imaging. S.C.K. carried out photon propagation simulations. P.J.H. analyzed all H&E stains. R.I.B. and P.A.K. provided clinical support for design and direction of the study. T.H. helped supervise the project. B.W.P. provided full support for the project and gave substantial feedback on all aspects of the project.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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References

1. Chen, S.L., Iddings, D.M., Scheri, R.P. & Bilich, A.J. Lymphatic mapping and sentinel node analysis: current concepts and applications. CA Cancer J. Clin. 56, 292–309; quiz 316–297 (2006).
2. Tobiiter, N.E. & Detmar, M. Tumor and lymph node lymphangioisogenesis—impact on tumor metastasis. J. Leukoc. Biol. 80, 691–696 (2006).
3. Schrenk, P., Rieger, R., Shamiyeh, A. & Wayand, W. Morbidity following sentinel lymph node biopsy versus axillary lymph node dissection for patients with breast carcinoma. Cancer 89, 608–614 (2000).
4. Sampath, L., Wang, W. & Sevick-Muraca, E.M. Near infrared fluorescent optical imaging for nodal staging. J. Biomed. Opt. 13, 041312 (2008).
5. Reilly, R.M. et al. Problems of delivery of monoclonal antibodies. Pharmaceutical and pharmacokinetic solutions. Clin. Pharmacokinet. 28, 126–142 (1995).
6. Little, T.J. et al. Quantifying surface biomarker expression in thick tissues with ratiometric three-dimensional microscopy. Biophys. J. 96, 2405–2414 (2009).
7. Pogue, B.W. et al. Imaging targeted-agent binding in vivo with two probes. J. Biomed. Opt. 15, 030513 (2010).
8. Baeten, J., Haller, J., Shih, H. & Ntziachristos, V. In vivo investigation of breast cancer progression by use of an internal control. Neoplasia 11, 220–227 (2009).
9. Tichauer, K.M. et al. In vivo quantification of tumor receptor binding potential with dual-reporter molecular imaging. Mol. Imaging Biol. 14, 598–592 (2012).
10. Davis, S.C. et al. Dynamic dual-tracer MRI-guided fluorescence tomography to quantify receptor density in vivo. Proc. Natl. Acad. Sci. USA 110, 9025–9030 (2013).
11. Sztitz, L.B. et al. Phase II trial of cetuximab in patients with refractory colorectal cancer that expresses the epidermal growth factor receptor. J. Clin. Oncol. 22, 1201–1208 (2004).
12. Nicholson, R.F., Gee, J.M. & Harper, M.E. EGFR and cancer prognosis. Eur. J. Cancer 37 (Suppl. 4), S59–S515 (2001).
13. van Agthoven, T., Timmermans, M., Dorssers, L. & Henzen-Logmans, S.C. Expression of estrogen, progesterone and epidermal growth factor receptors in primary and metastatic breast cancer. Int. J. Cancer 63, 790–793 (1995).
14. Wei, Q. et al. EGFR, HER2, and HER3 expression in laryngeal primary tumors and corresponding metastases. Ann. Surg. Oncol. 15, 1193–1201 (2008).
15. Wei, Q. et al. EGFR, HER2 and HER3 expression in esophageal primary tumours and corresponding metastases. Int. J. Oncol. 31, 493–499 (2007).
16. Shen, L. et al. EGFR and HER2 expression in primary cervical cancers and corresponding lymph node metastases: implications for targeted radiotherapy. BMC Cancer 8, 232 (2008).
17. Carlson, J., Shen, L., Xiang, J., Xu, J. & Wei, Q. Tendencies for higher co-expression of EGFR and HER2 and downregulation of HER3 in prostate cancer lymph node metastases compared with corresponding primary tumors. Oncology Lett. 5, 208–214 (2013).
18. Bue, P. et al. Expression of epidermal growth factor receptor in urinary bladder cancer metastases. *Int. J. Cancer* **76**, 189–193 (1998).
19. Italiano, A. et al. Epidermal growth factor receptor (EGFR) status in primary colorectal tumors correlates with EGFR expression in related metastatic sites: biological and clinical implications. *Ann. Oncol.* **16**, 1503–1507 (2005).
20. Reali, F.X. et al. Expression of epidermal growth factor receptor in human cultured cells and tissues: relationship to cell lineage and stage of differentiation. *Cancer Res.* **46**, 4726–4731 (1986).
21. Kern, K.A. Sentinel lymph node mapping in breast cancer using subareolar injection of blue dye. *J. Am. Coll. Surg.* **189**, 539–545 (1999).
22. Brader, P. et al. Imaging of lymph node micrometastases using an oncolytic herpes virus and [F]FEAU PET. *PLoS ONE* **4**, e4789 (2009).
23. Tafreshi, N.K. et al. Noninvasive detection of breast cancer lymph node metastasis using carbonic anhydrases IX and XII targeted imaging probes. *Clin. Cancer Res.* **18**, 207–219 (2012).
24. Tafreshi, N.K. et al. A mammaglobin-A targeting agent for noninvasive detection of breast cancer metastasis in lymph nodes. *Cancer Res.* **71**, 1050–1059 (2011).
25. Savarir, E.N. et al. Real-time in vivo molecular detection of primary tumors and metastases with ratiometric activatable cell-penetrating peptides. *Cancer Res.* **73**, 855–864 (2013).
26. Weaver, D.L. Pathology evaluation of sentinel lymph nodes in breast cancer: protocol recommendations and rationale. *Mod. Pathol.* **23** (suppl. 2), S26–S32 (2010).
27. Sexton, K. et al. Pulsed-light imaging for fluorescence guided surgery under normal room lighting. *Opt. Lett.* **38**, 3249–3252 (2013).
28. Porter, C.J. & Charman, S.A. Lymphatic transport of proteins after subcutaneous administration. *J. Pharm. Sci.* **89**, 297–310 (2000).
29. Gerlinger, M. et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. *N. Engl. J. Med.* **366**, 883–892 (2012).
30. Hettiarachchi, K., Kim, H. & Faris, G.W. Optical manipulation and control of real-time PCR in cell encapsulating microdroplets by IR laser. *Microfluid. Nanofluid.* **13**, 967–975 (2012).
31. Liebert, A. et al. Time-resolved multidistance near-infrared spectroscopy of the adult head: intracerebral and extracerebral absorption changes from moments of distribution of times of flight of photons. *Appl. Opt.* **43**, 3037–3047 (2004).
32. Zhu, Q. et al. Benign versus malignant breast masses: optical differentiation with US-guided optical imaging reconstruction. *Radiology* **237**, 57–66 (2005).
33. Flynn, B.P., Dsouza, A.V., Kanick, S.C., Davis, S.C. & Pogue, B.W. White light-informed optical properties improve ultrasound-guided fluorescence tomography of photoactive protoporphyrin IX. *J. Biomed. Opt.* **18**, 046008 (2013).
34. Koral, K.F. et al. SPECT dual-energy-window Compton correction: scatter multiplier required for quantification. *J. Nucl. Med.* **31**, 90–98 (1990).
ONLINE METHODS
Breast cancer lymph metastasis model. All animal procedures were carried out in accordance with the Institutional Animal Care and Use Committee at Dartmouth College under an approved protocol. To evaluate the potential of the lymph node molecular concentration imaging (LN-MCI) algorithm to be used to detect tumor burden in tumor-draining lymph nodes, a mouse model of lymph node metastasis that was as realistic as possible was studied. In all, 28- to 8-week-old athymic nude female mice (C3HLE/NCr-Foxn1nu, Charles River Laboratories, Wilmington, MA) were used in the experiments. 1 × 106 MDA-MB-231-luc-D3H2LN human breast cancer cells (PerkinElmer, Waltham, MA) were injected into the right inferior mammary fat pad of twenty-four of the mice, with four mice randomly selected as controls with no tumor injection. The cells were purchased expressly for this study, confirmed to be pathogen free from the supplier by the IMPACT Profile I (PCR) at the University of Missouri Research Animal Diagnostic and Investigative Laboratory. The number of mice was chosen based on a sample size estimation on the correlation between the imaging method of tumor burden estimation and ex vivo analyses of tumor burden, in addition to a 30% attrition rate owing to complications of tumor surgery. The calculation assumed a probability of Type I Error of 0.05, a Power = 0.9, and a correlation coefficient of 0.8. The primary tumors were allowed to grow for 6 weeks before the primary tumors were surgically removed at a size of 2.4 ± 1.1 cm³. The mice were then returned to their cages for a further 12 weeks to allow metastases time to form before imaging35. Select mice were imaged earlier if primary tumor grew back to a size of 10% of the mass of the mouse. The MDA-MB-231-luc-D3H2LN was chosen for a number of reasons: i) the cell line has been specifically selected from lymph node metastases and therefore has a high likelihood of spreading chosen for a number of reasons: i) the cell line has been specifically selected from lymph node metastases and therefore has a high likelihood of spreading; ii) the cell line has been transfected with a luciferase gene, which can be used to make the cells bioimunose with injection of luciferin36, providing a means of monitoring metastatic tumor growth; and iii) the cells moderately overexpress the cell membrane receptor, epidermal growth factor receptor (EGFR)37, a receptor overexpressed in many cancer types12 and targeted in this study.

Fluorescence imaging. Four weeks following the surgical removal of the primary tumor, the mice were anesthetized with 1.5–2% isofluorane and placed in a supine position with forelegs taped above their heads to expose the axillary lymph nodes (Fig. 1a) on the heated (37 °C) bed of a Pearl Imaging System (LICOR Biosciences, Lincoln, NE), a commercial small-animal planar fluorescence imaging system with white-light and two fluorescent imaging channels: a 700-nm channel and an 800-nm channel. The system is based on wide-field illumination and detection using ‘stadium’ lighting for illumination and a 22-bit charge-coupled device camera for detection, providing 83-µm resolution at the focal plane. The 700 nm channel excites fluorescence around 685 nm with an intensity distribution of 2 mW cm⁻² and detects emission about 720 nm, whereas the 800 channel excites fluorescence around 785 nm with an intensity distribution of 4 mW cm⁻² and detects about 820 nm. To capitalize on the wavelength bands of the Pearl's two fluorescent channels for carrying out dual-tracer EGFR concentration imaging, an EGFR-specific antibody was labeled with IRDye-800CW to act as a targeted tracer, and an untargeted antibody was labeled with IRDye-700DX to act as the untargeted tracer (see section on fluorescence tracer synthesis below). Both tracers are known to demonstrate strong photostability at the excitation intensities employed by the Pearl Imaging System39. Once positioned in the Pearl, white-light, 700-nm channel, and 800-nm channel images were acquired before injecting 0.1 nanomoles of each tracer simultaneously intradermally into each front footpad. The front footpad of the mouse was chosen as a site of injection since it has been shown previously that injections carried out here are preferentially drained through the axillary lymph nodes, a superficial node in mice that is easy to image39. Following tracer injection, all three image types were collected sequentially (each image type having a duration of approximately 5 s) at 2-min intervals up to 3 h post-injection, at which point the mice were euthanized and the right and left axillary lymph nodes were excised and analyzed with quantitative real-time PCR for an ex vivo estimate of tumor burden. Temporal images of targeted and untargeted tracers were analyzed with in-house software written in the programming language Matlab (MathWorks, Natick, MA) to apply the LN-MCI algorithm on a pixel-by-pixel basis and calculate a parametric map of EGFR concentration. The average concentration of EGFR in each axillary lymph node, to be used as a surrogate of tumor burden, was calculated from a 5-mm circular region of interest centered on each lymph node (Fig. 1c). The size of the region of interest was chosen based on the largest lymph node measured ex vivo so as not to miss any signal arising from any lymph node.

Quantitative PCR for tumor burden validation. Given that a human breast cancer cell line was grown in a mouse model in this study, it was possible to use genetic markers to determine the ratio of cancer to normal tissue with real-time PCR based on established approaches40,41. For improved sensitivity in this regard, the presence of luciferase gene expression was analyzed instead of a human-specific gene, as the luciferase gene is not expressed naturally by mice but was transfected into the MDA-MB-231-luc-D3H2LN cell line by the manufacturer. Total RNA was isolated from flash-frozen lymph nodes using the RNeasy mini kit and Qiagen PCR (Qiagen, Venlo, Netherlands), and 18-gauge needles were used to homogenize the tissue. To assess gene expression levels, cDNA was prepared from lymph node RNA using the SuperScript III First Strand cDNA Synthesis Kit and oligo dT primers (Life Technologies, Grand Island, NY). Reactions (25 µl) were set up using 2x iQ Supermix (Bio-Rad, Hercules, CA), pre-validated Luciferase primer and probe set (Assays on Demand, Applied Biosystems, Life Technologies), and equivalent amounts of template cDNA. Quantitative real-time PCR was performed with a CFX96 Real-Time PCR Detection System (Bio-Rad). Each sample was assayed in duplicate, and 18s rRNA served as the endogenous control for normalization. The expression levels for each gene were calculated relative to a standard curve of known tumor cells added to naive lymph nodes. For the standard curve, 10, 100, 1,000, 10,000, and 100,000 were added in quadruplicate (Fig. 1f). All procedures and analyses were carried out blinded to the fluorescent imaging results by author J.R.G.

Fluorescence tracer synthesis. The labeling procedures for the targeted and untargeted tracers were essentially the same. The EGFR-specific antibody, Erbitux (clinical grade from Bristol-Myers Squibb, Princeton, NJ) was labeled with the NHS ester form of IRDye-800CW (LICOR Biosciences), and mouse IgG (Athena Research and Technology, Athens, GA), an untargeted isotype control of Erbitux, was labeled with the NHS ester form of IRDye-700DX (LICOR Biosciences). Labeled solutions were concentrated to µM concentrations. The antibodies were initially diluted in phosphate buffer solution (PBS) before being run through a 5-ml polycrylamide desalting column to remove sodium azide. With the antibodies in 500-µl aliquots, 50 µl of 1 M sodium bicarbonate solution was added to increase the pH of the solution for optimal binding to the fluorophore as per manufacturer instructions. The fluorophores were diluted in DMSO and added to the antibody aliquots in a 3:1 dye-to-antibody ratio. These solutions were then left to mix on a magnetic stir plate for 2 h at room temperature. The dye-antibody conjugates were then separated from unassociated antibody and fluorophore with a desalting column using PBS to flush. Presence of the conjugate was confirmed in the resulting aliquots with a fluorimeter and then concentrated with a 50-kDa MW Vivaspin 2 (GE Healthcare).

Flow cytometry. The number of EGFRs per cell of the MDA-MB-231-luc-D3H2LN line and healthy lymph node tissue was quantified by flow cytometry. For MDA-MB-231-luc-D3H2LN, cells were grown in vitro until they reached a confluence of 80%; they were then trypsinized, counted, and washed with PBS. Three sets of 5 × 10⁶ cells were then labeled with 4 µg ml⁻¹ of EGF Biotin (Molecular Probes, Invitrogen, Camarillo, CA), washed, and secondarily labeled with a 1:25 dilution of Cy5-Streptavidin (Invitrogen, Camarillo, CA). Three additional sets of 5 × 10⁶ cells were then stained only with the 1:25 dilution of Cy5-Streptavidin as controls to account for autofluorescence and nonspecific staining. Each set of cells was loaded sequentially into a flow cytometer (FACScan) and data were acquired with Cell Quest Acquisition software (Becton Dickinson, San Jose, CA). In addition, a calibration standard was run using Quantum Cy5 MESF beads as described by the manufacturer (Bangs Laboratory, Fishers, IN) to quantify EGFR expression9. To analyze the expression of EGFR in healthy mouse lymph node cells, axillary lymph nodes of three
mice were dissected upon killing. They were then placed between two general-purpose glass microscope slides, physically minced in order to release the cells, and put into tissue culture media. These solutions were then spun down and three sets of $5 \times 10^6$ cells were selected for flow cytometry analysis as discussed above. All analyses were repeated in triplicate on three different days, and the lymph nodes were taken from three different control mice that were not used in this imaging study. All procedures and analyses were carried out blinded to the group by author J.R.G.

**Lymph node molecular concentration imaging.** The lymph node molecular concentration imaging (LN-MCI) approach is based on dual-tracer compartmental modeling that has been described for imaging primary cancer receptor concentrations in detail previously. To adapt the original dual-tracer modeling approach for detecting metastatic cells in the lymph system, slightly modified compartment models were developed for the targeted and untargeted tracer uptake (Fig. 5a). Within the lymph node, the targeted tracer was modeled to be freely associated ($C_f$) or bound ($C_b$) to targeted tumor cell receptors, and the whole system was modeled to be driven by the concentration of tracer in the upstream lymphatic vessels, $C_l$, which enters the lymph node at flow rate, $F_l$. The targeted tracer was modeled to only be able to exit the lymph node into the downstream lymphatics at the same flow rate, $F_o$, assuming flow equilibrium conditions. Rate-constants $k_3$ and $k_4$ were used to describe the rate of tracer–receptor association and dissociation, respectively. By assuming that the unbound (freely associated) concentration of tracer in the lymph node is always homogeneously distributed and that the concentration of targeted tracer in the lymph node is negligible compared to the concentration of targeted receptor, a system of first-order differential equations can be developed to govern the rate of change of tracer concentration in each compartment. For the targeted tracer:

$$\frac{dC_f(t)}{dt} = F_l C_f(t) - F_l C_f(t) - k_3 C_f(t) + k_4 C_b(t)$$

$$\frac{dC_b(t)}{dt} = k_3 C_f(t) - k_4 C_b(t)$$

and $ROI_T(t) = \eta_T \left[ C_f(t) + C_b(t) \right]$ where $ROI_T(t)$ represents the measured fluorescence signal from the uptake of the targeted tracer in the lymph node as a function of time, $t$, and the parameter $\eta_T$ represents the detection efficiency of the imaging system for the targeted tracer. For the untargeted tracer:

$$\frac{dC_l(t)}{dt} = F_l C_l(t) - F_l C_l(t)$$

and $ROI_U(t) = \eta_U C_l(t)$ where $C_l(t)$ is the concentration of untargeted tracer in the lymph node, $ROI_U(t)$ represents the measured fluorescence signal from the uptake of the untargeted tracer in the lymph node as a function of time, and the parameter $\eta_U$ represents the detection efficiency of the imaging system for the untargeted tracer. The compartment model used for the untargeted tracer (Fig. 5a) was very similar to that of the targeted tracer with the exception that there was no bound compartment. The utility of measuring the uptake of the untargeted tracer can be plainly seen in the set of Equations (1) and (2), as its uptake can be used as a surrogate of tracer concentration in the lymph node, $C_f(t)$ or $C_b(t)$, which is problematic/impractical to measure. From the last expressions in Equations (1) and (2), the following expression can be derived:

$$\frac{\eta_U}{\eta_T} \frac{ROI_U(t) - ROI_T(t)}{ROI_U(t)} = \frac{C_f(t) + C_b(t) - C_f(t)}{C_f(t)}$$

for any time point, $t$. Equation (4), which amounts to normalizing a targeted tracer uptake image, subtracting from it an untargeted tracer uptake image, and dividing that difference by the untargeted tracer uptake image, can be shown to be proportional to the receptor concentration if the free concentrations of the targeted and untargeted tracers share roughly the same time course (i.e., if $C_f(t) = C_b(t)$, and assuming that binding is an adiabatic process (i.e., that $C_b(t)/C_f(t)$ remains a constant))49, as follows:

$$\frac{\eta_U}{\eta_T} \frac{ROI_U(t) - ROI_T(t)}{ROI_U(t)} = \frac{C_b(t)}{C_f(t)} = \frac{k_3}{k_4} = BP$$

Equation (4) was used to estimate receptor concentration in simulations and in all animal experiments at multiple time points up to the 3-h imaging window in this study. The ratio of detection efficiencies, $\eta_U/\eta_T$, was estimated by placing the stock solution of targeted and untargeted fluorescence that was injected into the mice into the Pearl Imaging and measuring the fluorescence at both wavelengths, wherein

$$\frac{\eta_U}{\eta_T} \frac{\Phi_U}{\Phi_T}$$

if the optical properties of the tissues imaged are not significantly different between wavelengths, which is approximately true for 700 and 800 nm in biological tissues—where $\Phi_T$ and $\Phi_U$ are equal to the untargeted and targeted tracer fluorescence measured from the stock solution, respectively. All image analyses were carried out blinded to the group and ex vivo analyses of tumor burden by author K.M.T.

**Time-to-plateau of BP estimation.** The ratiometric estimate of BP presented in Equation (4) requires the ratio of bound to free concentrations of the tracer to reach a quasisteady state in order to be accurate, and therefore the plot of Equation (4) over time must reach a plateau before Equation (4) is accurate. To determine the time to plateau of these temporal ratiometric curves, the curves were fit with a fifth-order polynomial to smooth out noise, and the time to plateau was taken as the point the derivative of this fitted polynomial reached 0.

**Lymph flow estimation.** The set of differential equations in Equation (1) and (2) can be solved in a more rigorous sense than in Equation (4) if the temporal uptakes of $ROI_U(t)$ and $ROI_T(t)$ are assumed to be known, into a workable linear precedure that requires the use to measure $C_l$ (ref. 51):

$$ROI_T(t) = F_l \frac{t}{1 + BP} \int_0^t \frac{F_l}{\eta_T} \Phi_T u du + \frac{\eta_U}{\eta_T} F_l \Phi_U \int_0^t \Phi_T u du + \frac{\eta_U}{\eta_T} ROI_U(t)$$

Equation (6) could be used on its own to estimate $F_o$, $\eta_U/\eta_T$, and BP, respectively, with knowledge of $ROI_T(t)$ and $ROI_U(t)$ using linear least-squares. In fact, initial tests have demonstrated a strong correlation between the BP estimated with Equation (4) and that with Equation (6). The problem is that this approach requires continuous measurement of tracer uptake in lymph nodes and therefore may be difficult to employ clinically. In this experiment, however, tracer uptake was measured over time, allowing the lymph flow, $F_o$, to be estimated on a case-by-case basis. A quick unit analysis can show that the units of $F_l$ need to be in inverse time. Conventionally, flow is measured in volume of fluid flowing through a volume of tissue per time (for example, ml. lymph fluid ml. tissue$^{-1}$ min$^{-1}$). In this study, the units were simplified to min$^{-1}$ for simplicity.

**Noise analysis.** Theoretical targeted and untargeted tracer uptake curves in a tumor-bearing lymph node were created using full analytical solutions to the system of expressions in Equation (1)$^{52}$ and Equation (2)$^{53}$, respectively. To carry this out, the lymph input function, $C_l$, was estimated by the derivative
of the fluorescence measured in footpad site of injection on the mice in the animal experiment (Fig. 5b), assuming that all fluorescence leaving the site of injection travels through the lymph system and passes through the lymph node of interest. For the targeted tracer, $k_4$ was assumed to be 0.06 min$^{-1}$ from literature values$^{53}$, and $k_5$ was estimated to be 0.16 min$^{-1}$, assuming a target concentration of 1 nM, which was a typical level found in mouse experiments, with a tracer affinity of 2.6 nM$^{-1}$ (affinity of Erbitux for EGFR)34. All simulations were repeated over a range of lymph flow rates, $F_L$, 0.05–0.25 min$^{-1}$, determined from the range of lymph node flow rates measured in the mouse experiments (average $F_L = 0.15 \pm 0.07$ min$^{-1}$) by fitting the analytical solution to Equation (2)$^{33}$ to the untargeted tracer lymph node uptake. 2% random Gaussian noise was added to all simulated curves. The resulting curves were then fit with the models in Equation (3) and Equation (4) using in-house software written in MATLAB (Mathworks, Natick, MA) to evaluate the noise sensitivity and accuracy of each model. Noise addition was repeated $1 \times 10^5$ times.

**Statistical analyses.** All statistics in this study were carried out with the statistical package SPSS (IBM, Armonk, NY). A one-way analysis of variance was used to compare means between tumor-burden groups (control, <200 cells, >200 cells) to compare targeted tracer fluorescence uptake and measured EGFR concentration in each group. Variance equivalency between groups was determined by a Levene statistic: if $P > 0.05$, variance was assumed equivalent, whereas if $P < 0.05$, variance was not assumed equivalent. Bonferroni correction was employed to account for multiple comparisons in equivalent variance data sets, whereas for nonequivalent variance, Tamhane correction was employed. Linear regression was employed to compare in vivo EGFR concentration with ex vivo qPCR evaluations of tumor cell burden. Statistical significance was based on a two-tailed $P < 0.05$ with equal variance assumed unless stated otherwise. All data are presented as mean ± s.d.

35. Jenkins, D.E., Homig, Y.S., Oei, Y., Dusch, J. & Puchio, T. Bioluminescent human breast cancer cell lines that permit rapid and sensitive in vivo detection of mammary tumors and multiple metastases in immune deficient mice. *Breast Cancer Res.* **7**, R444–R454 (2005).

36. Contag, C.H., Jenkins, D., Contag, P.R. & Negrin, R.S. Use of reporter genes for optical measurements of neoplastic disease in vivo. *Neoplasia* **2**, 41–52 (2000).

37. Reilly, R.M. et al. A comparison of EGF and MAb 528 labeled with 111In for imaging human breast cancer. *J. Nucl. Med.* **41**, 903–911 (2000).

38. Peng, X. et al. Phthalocyanine dye as an extremely photostable and highly fluorescent near-infrared labeling agent. *Proc. SPIE* **6097**, 19 (2006).

39. Wu, T. et al. Fluorescence imaging of the lymph node uptake of proteins in mice after subcutaneous injection: molecular weight dependence. *Pharm. Res.* **29**, 1843–1853 (2012).

40. Alcose, S.F. et al. Real-time PCR-based assay to quantify the relative amount of human and mouse tissue present in tumor xenografts. *BMC Biotechnol.* **11**, 124 (2011).

41. Becker, M. et al. Sensitive PCR method for the detection and real-time quantification of human cells in xenotransplantation systems. *Br. J. Cancer* **87**, 1328–1335 (2002).

42. Hamzei, N. et al. Comparison of kinetic models for dual-tracer receptor concentration imaging in tumors. *Austin J. Biomed. Eng.* **1**, 9 (2014).

43. Samkoe, K.S. et al. High vascular delivery of EGF, but low receptor binding rate is observed in AsPC-1 tumors as compared to normal pancreas. *Mol. Imaging Biol.* **14**, 472–479 (2012).

44. Tichauer, K.M. et al. Tumor endothelial marker imaging in melanomas using dual-tracer fluorescence molecular imaging. *Mol. Imaging Biol.* **16**, 372–382 (2014).

45. Tichauer, K.M. et al. Accounting for pharmacokinetic differences in dual-tracer receptor density imaging. *Phys. Med. Biol.* **59**, 2341–2351 (2014).

46. Tichauer, K.M., Samkoe, K.S., Klubben, W.S., Hasan, T. & Pogue, B.W. Advantages of a dual-tracer model over reference tissue models for binding potential measurement in tumors. *Phys. Med. Biol.* **57**, 6647–6659 (2012).

47. Tichauer, K.M. et al. Improved tumor contrast achieved by single time point dual-reporter fluorescence imaging. *J. Biomed. Opt.* **17**, 066001 (2012).

48. Lammertsma, A.A. & Hume, S.P. Simplified reference tissue model for PET receptor studies. *Neuroimage* **4**, 153–158 (1996).

49. Innis, R.S. et al. Consensus nomenclature for in vivo imaging of reversibly binding radioligands. *J. Cereb. Blood Flow Metab.* **27**, 1533–1539 (2007).

50. Jacques, S.L. Optical properties of biological tissues: a review. *Phys. Med. Biol.* **58**, R37–R61 (2013).

51. Ichise, M. et al. Linearized reference tissue parameter imaging methods: application to $^{11}$C-DASB positron emission tomography studies of the serotonin transporter in human brain. *J. Cereb. Blood Flow Metab.* **23**, 1096–1112 (2003).

52. Lammertsma, A.A. et al. Comparison of methods for analysis of clinical $^{11}$C-clozapine studies. *J. Cereb. Blood Flow Metab.* **16**, 42–52 (1996).

53. Kety, S.S. The theory and applications of the exchange of inert gas at the lungs and tissues. *Pharmacol. Rev.* **3**, 1–41 (1951).

54. Patel, D. et al. Monoclonal antibody cetuximab binds to and down-regulates constitutively activated epidermal growth factor receptor vill on the cell surface. *Anticancer Res.* **27**, 3355–3366 (2007).