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Tumor Cell Purging by Ex Vivo Expansion of Hemopoietic Stem Cells from Breast Cancer Patients Combined with Targeting ErbB Receptors

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ABSTRACT

Tumor cell contamination might induce relapse after autologous transplantation in breast cancer patients. We used an ex vivo purging strategy to decrease the number of contaminating breast tumor cells in leukapheresis without altering the engraftment potential of the hemopoietic progenitor cells. This method is based on immunoselection of $CD34^+$ cells derived from mobilized peripheral blood of patients with metastatic breast cancer and expansion in the presence of flt3 ligand, stem cell factor, interleukin 6, and thrombopoietin. Tumor contamination before and after culture was monitored by mammaglobin messenger RNA amplification by quantitative polymerase chain reaction. We analyzed both adherent and suspended cells obtained after 2 weeks of culture. Hemopoietic progenitors were increased among suspended cells. In this fraction, tumor cell contamination was decreased, whereas it increased within the adherent cell fraction. Experimental models using $CD34^+$ cells from healthy donors spiked with breast cancer cells were also constructed to investigate whether treatment with anti–ErbB-receptor drugs could further reduce the tumor load without affecting the clonogenic potential of hemopoietic cells. For this purpose, we successfully assayed trastuzumab, a monoclonal antibody against ErbB-2, and gefitinib, an epidermal growth factor receptor tyrosine kinase receptor inhibitor. These results suggest that positively selected $CD34^+$ cells from cancer patients contain tumor cells and that ex vivo expansion can reduce the tumor load of the suspended fraction. Target-based agents against ErbB-2, epidermal growth factor receptor, or both—such as trastuzumab or gefitinib—might increase the efficiency of purging.

KEY WORDS

Ex vivo expansion • Stem cell transplantation • Breast cancer cell purging • Anti-ErbB targeted therapy • Real-time quantitative PCR • Mammaglobin mRNA

INTRODUCTION

The clinical benefit of high-dose chemotherapy programs with autologous stem cell transplantation for the treatment of metastatic breast cancer is a matter of discussion [1,2]. Studies on the prognostic significance of occult tumor cell contamination in apheresis products show contradictory results and are still not conclusive [3-10].

Gene-marking studies have shown that in patients with acute or chronic myelogenous leukemia and neuroblastoma, tumor cell reinfusion contributes to relapses after autologous transplantation [11,12]. Contaminating breast cancer cells in leukapheresis products might be the origin of tumor relapse. Purging of tumor cells from leukapheresis may contribute to increased patient survival. In vitro studies showed that tumor cells in mobilized peripheral blood (MPB) collections are viable and retain clonogenic potential [13-16]. Ex vivo expansion of progenitor cells might produce a passive elimination of contaminating tumor cells [17]. Recently, we showed that the culture conditions that allow extensive self-maintenance of cord blood–derived hemopoietic progenitors [18] can be extended to MPB in the presence of flt3 ligand (FL), stem cell factor (SCF), thrombopoietin (TPO),interleukin (IL)–6, and

serum. Under such culture conditions, transplantable precursor and stem cells still retain their proliferation and multilineage differentiation capacity [19].

ErbB-2 and epidermal growth factor receptor (EGFR) are expressed in human breast carcinoma [20,21]; they represent ideal targets of anti-ErbB therapies. The humanized anti–ErbB-2 monoclonal antibody trastuzumab has been approved for the treatment of metastatic breast cancer [22-25]. Similarly, the EGFR tyrosine kinase inhibitor gefitinib (ZR1839) is currently in phase III clinical studies [26-29].

In this work, we set up a small-scale culture system of MPB from breast cancer patients to obtain the expansion of hemopoietic progenitors and the selective elimination of contaminating tumor cells by targeting ErbB receptors. Because mammaglobin (MAM) is the most specific marker for the detection of epithelial cell contamination in hematologic samples [10,30], we used MAM messenger RNA (mRNA) amplification by quantitative polymerase chain reaction (PCR) to monitor tumor contamination before and after culture.

MATERIALS AND METHODS

Reagents and Recombinant Human Cytokines

Trastuzumab (Herceptin) was obtained from Genentech Inc. (San Francisco, CA). ZD1839 (gefitinib) was kindly provided by AstraZeneca (Macclesfield, UK). Recombinant human (rh) SCF was a generous gift from Amgen (Thousand Oaks, CA), rhIL-3 was obtained from Novartis (Basel, Switzerland), rhIL-6 was obtained from PeproTech (Rocky Hill, NJ), rh-erythropoietin (Eprex) was obtained from Cilag (Milan, Italy), rhFL was kindly provided by S.D. Lyman (Immunex Corp., Seattle, WA), and rhTPO was a generous gift from Kirin Brewery (Tokyo, Japan).

Patients and Samples

We studied samples from 10 patients with metastatic breast cancer. Informed consent was obtained from all patients according to the institutional guidelines. Patients were evaluated for secondary metastatic lesions by means of chest and abdomen computed tomography and bone scintigraphy.

The characteristics of breast cancer patients are summarized in Table 1. All patients were female. MPB collections from patients with metastatic breast cancer and MPB collections from healthy donors were harvested from the leftovers of leukapheresis procedures. In breast cancer patients, peripheral blood stem cells were collected after recovery from the third course of conventional-dose chemotherapy with paclitaxel (Taxol; Bristol-Myers Squibb, Rome, Italy) 175 mg/m^2 and vinorelbine (Navelbine; Pierre Fabre, Rome, Italy) 30 mg/m^2. Human recombinant granulocyte colony-stimulating factor (Granocyte 34; Aventis Pharma, Milan, Italy) 10 μg/kg was administered from day +5 until the completion of stem cell harvest. Healthy donors had received only granulocyte colony-stimulating factor for 5 consecutive days. For the reconstitution experiments of tumor contamination, 12 MPB samples derived from healthy donors were collected after informed consent was given.

Table 1. *Characteristics of Enrolled Breast Cancer Patients*

Patient No.	Age (y)	Visceral Metastases	Bone Metastases
1	30	Liver	No
2	42	Lung	No
3	51	Pleural	No
4	44	Lymph nodes	No
5	53	Lung	No
6	56	Liver	No
7	49	Liver	No
8	37	Lymph nodes, liver	No
9	58	Lung, pleural	No
10	50	Lung	No

Patients were evaluated for secondary metastases by means of chest and abdomen computed tomography and bone scintigraphy.

Cell Lines

The SKBR3 human breast cancer cell line was routinely maintained in Dulbecco modified Eagle medium (Invitrogen Life Technologies, Gaithersburg, MD) with 10% fetal calf serum (Hyclone, Logan, UT). We selected this cell line on the basis of MAM expression [30], ErbB-2 genomic amplification, and EGFR overexpression [31].

Experimental Contamination Assay

To set up the contamination model, we performed a reconstitution experiment by using peripheral blood mononuclear cells derived from healthy donors spiked with SKBR3 in limiting dilutions from 1 SKBR3 cell in 10 mononuclear cells to 1 SKBR3 cell in 10^6 mononuclear cells. We used this experimental model to establish the degree of tumor contamination of leukapheresis from patients.

Purification of CD34$^+$ Cells

CD34$^+$ cells were isolated from MPB mononuclear cells with a superparamagnetic microbead selection system by using a high-gradient magnetic field and MiniMACS column (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Purification efficiency was verified by flow cytometry counterstaining with a CD34 phycoerythrin (Becton Dickinson, San Jose, CA) antibody. In the cell fraction containing purified cells, the percentage of CD34$^+$ cells ranged from 90% to 98%.

Cell Culture Expansion System

Expansion culture was performed in 24-well tissue culture plates with 5×10^5 $CD34^+$ cells per well in 1 mL of Iscove modified Dulbecco medium containing 10% calf serum plus SCF (50 ng/mL), FL (50 ng/mL), TPO (10 ng/mL), and IL-6 (10 ng/mL). Total cell number, $CD34^+$ cell production, colony-forming cells (CFC), long-term culture-initiating cell (LTC-IC) content, and tumor cell presence were evaluated at the start of culture and after 2 weeks.

To reproduce tumor-contaminated leukapheresis, we performed coculture by spiking 100 SKBR3 cell with 10^6 $CD34^+$ cells derived from healthy donors in the presence of the same growth factors and cultured with or without 30 μg/mL trastuzumab or 2 μmol/L ZR1839 (gefitinib). Fresh antibody and inhibitor were added every 72 hours; growth factors were added every week. After 2 weeks of culture, the cells were analyzed for total cell number, CFC, LTC-IC, $CD34^+$, and tumor cell content.

Hemopoietic Cell Assay

Assays for multilineage granulocyte-macrophage megakaryocyte colony-forming units were performed in plasma clots, as previously described [19]. Briefly, 2×10^3 $CD34^+$ cells were seeded in plasma clots (4 dishes per point) in the presence of IL-3 (20 ng/mL), SCF (50 ng/mL), and erythropoietin (3 U/mL). Colony scoring was performed on day 14 with an optical microscope at ×4 magnitude by counting all the colonies (>50 cells) grown in the plate.

Long-Term Culture-Initiating Cells

The LTC-IC content of unmanipulated and expanded $CD34^+$ cells was analyzed as previously described [19]. Briefly, 2×10^3 $CD34^+$ cells were seeded onto a pre-established irradiated human bone marrow stromal layer and maintained at 37°C for 6 weeks. After the culture period, all cells were harvested and plated for CFC quantitation.

MAM Real-Time Quantitative PCR

The detection of tumor contamination of MPB collection has been described in detail previously [30]. Briefly, total RNA was used as a template for complementary DNA synthesis, and MAM real-time quantitative PCR was performed with specific primers and TaqMan probes (Perkin Elmer, Foster City, CA) (MAM forward primer, 5′-CAGCGGCTTCCTTGATCCT-3′; MAM reverse primer, 5′-TCATGGTGAGGCTGCTGCT-3′; MAM detection probe, carboxyfluorescein-5′-CCACCCGCGACTGAACACCGA-3′-tetramethylrhodamine). The glyceraldehyde phosphate dehydrogenase (GAPDH) gene was amplified as a control (forward GAPDH primer, 5′-GAAGGTCGGAGTC-3′; reverse GAPDH primer, 5′-GAAGATGGTGATGGGATTTC-3′; GAPDH detection probe, carboxyfluorescein-5′-CAAGCTTCCCGTTCTCAGCC-3′-tetramethylrhodamine). All PCR reactions were performed in triplicate in a 96-well microtiter plate format on an ABI PRISM 7700 Sequence Detector System (Perkin Elmer), and amplification curves were analyzed as previously described [30].

Statistical Analysis

Each CFC assay was performed in quadruplicate. The results were analyzed by using the Mann-Whitney *U* nonparametric test. All *P* values are 2 tailed, with significance set at $P < .05$. -Fold increases of total cells, $CD34^+$ cells, CFCs, and LTC-ICs were calculated, and their means and standard deviations were analyzed.

RESULTS

Ex Vivo Expansion of Purified $CD34^+$ Cells from MPB Derived from Patients with Metastatic Breast Cancer

We verified the ex vivo expansion potential of our culture system on purified $CD34^+$ cells derived from 5 patients with metastatic breast cancer. We analyzed the total cell number, $CD34^+$ cell production, and CFC and LTC-IC output after 2 weeks of expansion in the presence of FL, TPO, SCF, and IL-6. Table 2 shows the relative -fold increases of each parameter.

After 2 weeks of culture, total cells and $CD34^+$ cells were expanded 40.8 ± 9.2-fold and 4.2 ± 1.8-fold, respectively. CFCs (12.9 ± 4.0-fold) and LTC-IC (8.7 ± 5.0-fold) were also expanded in all samples. These results were similar to those obtained with $CD34^+$ cells derived from healthy donors, as previously demonstrated [19], thus suggesting that chemotherapy does not affect the clonogenic capacity of hemopoietic stem cells in patients with metastatic breast cancer.

Table 2. *Expansion Potential of MPB $CD34^+$ Cells Derived from Patients with Metastatic Breast Cancer*

	Fold Increase			
Patient No.	Total Cells	$CD34^+$ Cells	CFC	LTC-IC
1	28.2	5.5	9.8	1.8
2	38.5	2.4	17.8	6.2
3	50.9	2.3	9.4	12.9
4	37.6	6.2	10.8	8.5
5	48.8	4.9	16.9	14
mean ± SD	40.8 ± 9.2	4.2 ± 1.8	12.9 ± 4.0	8.7 ± 5.0

A total of 5×10^5 $CD34^+$ cells from 5 leukaphereses were cultured as described in "Materials and Methods." The fold increase of total cells, $CD34^+$ cells, CFC, and LTC-IC was evaluated after 2 weeks of expansion.

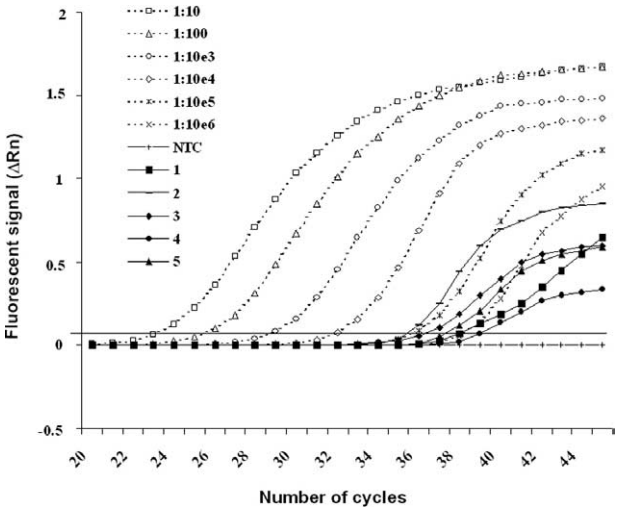

Figure 1. Quantitation of tumor contamination on MPB harvest. Mammaglobin amplification curves on reconstitution experiments with SKBR3 cells and peripheral mononuclear cells (curves from 1 SKBR3/10 mononuclear cells to 1 SKBR3/10^6 mononuclear cells from a healthy donor) and on 5 (from patient 1 to patient 5) MPB collections derived from breast cancer patients are shown. NTC indicates no template control. RN indicates reporter fluorescence normalized to internal control.

Detection of Tumor Cell Contamination in Unmanipulated and Expanded Leukapheresis Collections Derived from Patients with Metastatic Breast Cancer

We assessed the presence of tumor contamination on mononuclear cells derived from 10 leukapheresis of patients with metastatic breast cancer (Table 1) by MAM quantitative RT-PCR. Five of 10 samples were positive for MAM mRNA. To evaluate the degree of tumor contamination, we compared the MAM amplification curves of positive samples with those obtained on reconstitution experiments with SKBR3 cells and peripheral mononuclear cells from healthy donors. The levels of contamination of leukapheresis from patients were comparable to those obtained in the limiting dilution ranging from 1 SKBR3 cell in 10^6 to 1 SKBR3 in 10^5 mononuclear cells of healthy donors (Figure 1).

Leukapheresis from patients were subjected to $CD34^+$ cell purification and cultured. After 2 weeks of expansion, the levels of MAM mRNA were compared with those of unmanipulated mononuclear cells and with those of purified $CD34^+$. After $CD34^+$ selection, our system was unable to detect any level of MAM; this suggests that the tumor load was reduced below the threshold level of detection. During cell culture, 2 different fractions are distinguished: a nonadherent subpopulation, which consists of hemopoietic cells that grow in suspension, and adherent cells containing epithelial tumor cells, monocytes, and endothelial cells. The level of MAM mRNA in these 2 cultured subpopulations was compared with that of unmanipulated mononuclear cells. Tumor marker was increased in the adherent fraction of culture compared with unmanipulated mononuclear cells, thus suggesting that our system expands, also contaminating epithelial tumor cells. Conversely, MAM mRNA was reduced in the suspended fraction in all samples. In 1 sample, tumor cells became undetectable. Table 3 shows the relative amounts of MAM mRNA in each sample (from patient 1 to patient 5) after each step of in vitro manipulation compared with those of a standard calibration curve with SKBR3 and mononuclear cells from healthy donors.

Effect of Trastuzumab and Gefitinib on Tumor Cells in Coculture

It has been demonstrated that trastuzumab, a monoclonal antibody directed against ErbB-2, inhibits the proliferation of ErbB-2–overexpressing cells [22-25,32]. Similarly, ZD1839, a tyrosine kinase inhibitor specific for EGFR, is able to block the growth of EGFR-expressing human cancer cells [26-29]. We analyzed whether these 2 molecules might contribute

Table 3. *Relative Amounts of MAM mRNA in MPB from Breast Cancer Patients (from Patient 1 to Patient 5) Compared with Those of the Calibration Curve*

Sample*	Calibration Curve†	Before CD34 Selection	After CD34 Selection	Adherent Fraction After Expansion	Nonadherent Fraction After Expansion
1		1.25	ND	2.51	0.53
2		9.87	ND	58.26	1.55
3		2.67	ND	4.16	ND
4		0.71	ND	8.56	0.4
5		1.86	ND	4.47	0.09
Calibration					
1:10^6	1				
10:10^6	4.8				
100:10^6	90				

ND indicates not detectable.

*A total of 10^6 cells collected after each step of manipulation were subjected to MAM real-time quantitative PCR.

†Calibration curve was obtained from 1×10^6 normal mononuclear cells spiked with increasing numbers of SKBR3 (1, 10, and 100).

Table 4. *Relative Quantitation of Mammaglobin by Using the Comparative Ct Methods*

Variable	MAM Average Ct	GAPDH Average Ct	ΔCt (MAM−GAPDH)	ΔΔCt (ΔCt− $DCt_{SKBR3\ T0}$)	MAM (Relative to SKBR3 T_0)
SKBR3 T_0	33.65 ± 0.002	28.66 ± 0.12	4.99	0	1
SKBR3 + T	31.23 ± 0.11	28.63 ± 0.44	2.6	−2.39	5.25
SKBR3 − T	25.34 ± 0.46	28.47 ± 0.34	−3.13	−8.12	278.2

Ct indicates the threshold cycle, defined as the PCR cycle at which the reporter fluorescence dye cleaved from the MAM probe generates a target-specific detection signal (ΔRn) that passes above the baseline. The ΔCt value is determined by subtracting the average GAPDH Ct value from the average MAM Ct value. The calculation of ΔΔCt involves subtraction by the ΔCt calibrator value (SKBR3 T_0). The range given for MAM relative to SKBR3 T_0 is determined by evaluating the expression 2^{-DDCt}

SKBR3 T_0 indicates the start of coculture of $CD34^+$ cells obtained from leukapheresis of healthy donors spiked with SKBR3 cells.

SKBR3 + T indicates coculture of $CD34^+$ cells obtained from leukapheresis of healthy donors spiked with SKBR3 cells and grown for 2 weeks in the presence of trastuzumab.

SKBR3 − T indicates coculture of $CD34^+$ cells obtained from leukapheresis of healthy donors spiked with SKBR3 cells and grown for 2 weeks in the absence of trastuzumab.

to purge contaminated stem cell collections during in vitro manipulation.

We performed coculture with $CD34^+$ cells obtained by leukapheresis from healthy donors and the SKBR3 cell line in the presence or absence of trastuzumab or gefitinib. This cell line was selected for the characteristics of ErbB-2 genomic amplification and EGFR overexpression, which allow trastuzumab and gefitinib to inhibit cell growth. After 2 weeks of culture in the presence of trastuzumab, MAM mRNA was undetectable in the suspended fraction (data not shown). Relative quantitation with comparative threshold cycle methods of the tumor marker (Table 4) demonstrated that on adherent fractions, the levels of MAM in the trastuzumab-treated cells (5.25-fold increase of MAM with respect to the start of culture) were lower than those in untreated cultures (278.2-fold increase of MAM with respect to the start of culture), which presented transcript levels 52.9-fold higher than in the presence of the antibody. Figure 2 shows MAM and GAPDH amplification curves obtained after 2 weeks of coculture in reconstitution assays with SKBR3 cells and hemopoietic precursors in the absence or presence of trastuzumab. The same results were obtained upon EGFR tyrosine kinase inhibitor treatment. Moreover, by investigating CFC content, we demonstrated that trastuzumab and gefitinib did not change the clonogenic capacity of hemopoietic cells (Table 5).

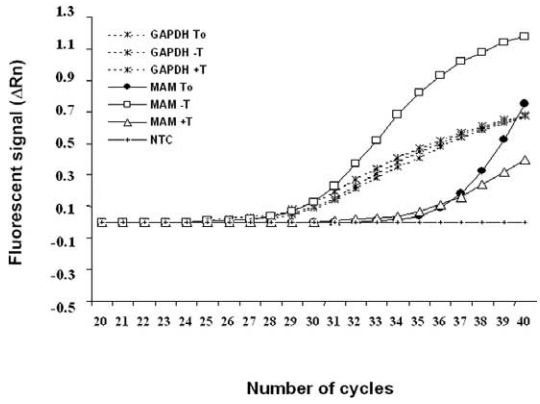

Figure 2. Effect of trastuzumab on cocultures of SKBR3 cells and hemopoietic precursor cells. Representative amplification curves are shown of GAPDH (control gene) and MAM (target gene) obtained by mammaglobin (MAM) quantitative reverse transcriptase-PCR in coculture of SKBR3 tumor cells and normal progenitor cells. Cells were analyzed before (T0) and after 2 weeks in the presence (+T) or absence (−T) of trastuzumab. NTC indicates no template control. RN indicates reporter fluorescence normalized to internal control.

DISCUSSION

This work proposes a novel strategy for tumor cell purging of leukapheresis from patients with metastatic breast cancer scheduled for high-dose chemotherapy programs with hemopoietic stem cell rescue. This system is based on ex vivo expansion of $CD34^+$ cells from patients with metastatic breast cancer in combination with ErbB receptor targeting.

In our laboratory, we previously set up an efficient expansion method of adult $CD34^+$ cells derived from healthy donors [19]. Here we report that this culture condition (FL, TPO, SCF, and IL-6 in the presence of serum) allows the maintenance and even some expansion of $CD34^+$ derived from tumor cell–positive

Table 5. *Colony-Forming Cell (CFC) Content*

Variable	CFC
−Trastuzumab	182 (64–346)
+Trastuzumab	147 (52–233)
−Gefitinib	122 (70–154)
+Gefitinib	136 (78–148)

Tumor-contaminated leukapheresis were reconstituted by spiking 100 SKBR3 cells with 10^6 $CD34^+$ cells derived from healthy donors and expanded with or without 30 μg/mL trastuzumab and 2 μmol/L ZR1839. After the period of culture, CFC content was evaluated. Values indicate the median and range of several experiments (6 experiments with different donors in the presence of trastuzumab and 5 with gefitinib).

collections. The absolute number of $CD34^+$ cells and CFC and LTC-IC content were significantly increased after 2 weeks of culture. The hemopoietic expansion potential in contaminated collections was not different from that of healthy donors [19]. Nonetheless, tumor cells persist and proliferate in vitro, as demonstrated by quantitative reverse transcriptase-PCR at the end of the culture period. However, we observed differences between the adherent and suspended fractions of culture: the adherent cells contained higher levels of MAM mRNA than unmanipulated cells, whereas on the suspended fraction, which contains most hematopoietic progenitors, there was a reduction of the marker level in all cultured samples. The ability of tumor epithelial cells to adhere on plastic could contribute to decreasing the tumor contamination. Positive selection of $CD34^+$ cells from MPB of breast cancer patients is not enough to completely eliminate epithelial tumor cells in hemopoietic autografts, and the ex vivo culture condition does not result in an effective purging of tumor cells. For these reasons, to improve the efficiency of the tumor-purging protocol, we considered additional strategies, such as targeting ErbB family receptors during hemopoietic ex vivo expansion. We investigated whether trastuzumab, the monoclonal antibody against ErbB-2 [22-25,31], and the EGFR tyrosine kinase receptor inhibitor ZD1839 (gefitinib) [26-29] are able to reduce the tumor load without affecting the clonogenic potential of hemopoietic cells.

$CD34^+$ cells obtained from leukaphereses of healthy donors were mixed with the ErbB-2^+ and $EGFR^+$ SKBR3 human breast cancer cell line and cultured for 2 weeks in the presence of trastuzumab and gefitinib. After 2 weeks of culture in the presence of trastuzumab, the tumor marker was undetectable in suspended cells, and there was a 52.9-fold reduction in the adherent fraction. Moreover, clonogenic assays showed that trastuzumab and gefitinib spare hemopoietic cells. These results demonstrate that EGFR targeting does not upset the hemopoietic developmental capacity.

In conclusion, our protocol might be used as an efficient in vitro purging of leukapheresis collections from breast cancer patients with ErbB-2 overexpression or EGFR expression.

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