3

CHAPTER 3

Anti-EGFR antibody cetuximab enhances the cytolytic activity of Natural Killer cells toward osteosarcoma

Jens H.W. Pahl, S. Eriaty N. Ruslan, Emilie P. Buddingh, Susy J. Santo, Karoly Szuhai, Massimo Serra, Hans Gelderblom, Pancras C.W. Hogendoorn, R. Maarten Egeler, Marco W. Schilham, and Arjan C. Lankester

Clinical Cancer Research 18 (2012) 432-441

ABSTRACT

Osteosarcoma and Ewing sarcoma are the most common bone tumors in children and adolescents. Despite intensive chemotherapy, patients with advanced disease have a poor prognosis, illustrating the need for alternative therapies. Sarcoma cells are susceptible to the cytolytic activity of resting natural killer (NK) cells which can be improved by interleukin(IL)–15 stimulation. In this study, we explored whether the cytolytic function of resting NK cells can be augmented and specifically directed toward sarcoma cells by antibody-dependent cellular cytotoxicity (ADCC).

Epidermal growth factor receptor (EGFR) expression was examined on osteosarcoma and Ewing sarcoma cell lines by flow cytometry and in osteosarcoma biopsy and resection specimens by immunohistochemistry. Cetuximab-mediated ADCC by NKcells from osteosarcoma patients and healthy controls was measured with 4-hour ^{51}Cr release assays.

EGFR surface expression was shown on chemotherapy-sensitive and chemotherapy-resistant osteosarcoma cells (12/12), most primary osteosarcoma cultures (4/5), and few Ewing sarcoma cell lines (2/7). In the presence of cetuximab, the cytolytic activity of resting NK cells against all EGFR-expressing sarcoma cells was substantially increased and comparable with that of IL-15–activated NK cells. Surface EGFR expression on primary osteosarcoma cultures correlated with EGFR expression in the original tumor. The cytolytic activity of osteosarcoma patient-derived NK cells against autologous tumor cells was as efficient as that of NK cells from healthy donors.

Our data show that the cytolytic potential of resting NK cells can be potentiated and directed toward osteosarcoma cells with cetuximab. Therefore, cetuximab-mediated immunotherapy may be considered a novel treatment modality in the management of advanced osteosarcoma.

INTRODUCTION

Osteosarcoma and Ewing sarcoma most frequently arise in adolescents and young adults and represents the majority of all malignant primary bone tumors in this patient group [1–3]. The current treatment consists of a combination of systemic multi-drug chemotherapy and complete surgical resection [3–5]. In cases with localized disease, up to 70% of the patients achieve persistent remission. In contrast, patients with advanced, metastasized, and recurrent disease experience a very poor prognosis, which has not improved during the last decades despite intensification of chemotherapy regimens. Therefore, novel treatment strategies with a favorable toxicity profile are warranted. In this perspective, we and others have recently reported on the potential exploitation of cellular immunotherapy against sarcomas by natural killer (NK) cells [6–8].

NK cells can respond to and kill cells undergoing cellular stress due to virus infection or malignant transformation. The cytotoxic activity of NK cells is regulated by the equilibrium of inhibiting and activating signals conveyed by target cells. On tumor cells, MHC class I expression (NK cell inhibitory signal) may be down-regulated to evade cytotoxic T-cell recognition. Conversely, the expression of stress ligands (NK cell–activating signal) may be upregulated on tumor cells. Both of these processes may lead to increased sensitivity of tumor cells to NK cells [9,10]. In addition, the interplay with other immune cells and the pro- or anti-inflammatory microenvironment may modulate the function and activity of NK cells [10,11]. Recently, we and others have shown that sarcoma cell lines are moderately susceptible to the cytolytic potential of resting NK cells [6–8]. The cytolytic activity of NK cells can be directly potentiated by activating cytokines, such as interleukin(IL)–15, leading to increased lysis of sarcoma cells [6–8].

In this study, we set out to explore whether the cytolytic activity of resting NK cells can be improved and directed specifically toward sarcoma cells. Therefore, we intended using a monoclonal antibody (mAb) of the human IgG1 subtype which recognizes an antigen expressed on sarcoma cells and is able to induce antibody-dependent cellular cytotoxicity (ADCC) by NK cells. Osteosarcoma has previously been shown to express the epidermal growth factor receptor (EGFR, erbB1/Her1 [12]), which is recognized by the clinically-approved, chimeric IgG1 type mAb cetuximab [13]. So far, the application of anti-EGFR mAb–targeted therapy in bone sarcomas has not been reported. We focused on exploring whether the cytotoxic potential of allogeneic and autologous NK cells can be specifically directed toward sarcoma cells with cetuximab.

MATERIALS AND METHODS

Patient samples

Formalin-fixed, paraffin-embedded tumor samples were obtained from one biopsy (obtained at the time of diagnosis, prechemotherapy) and 4 resections of local recurrent or metastatic tumors (postchemotherapy) from 4 high-grade osteosarcoma patients (diagnosed between 2008 and 2010) by the Department of Pathology, Leiden University Medical Center. Five short term grown primary osteosarcoma cell cultures (between passage 2 and 13) were generated from the tumor material as previously described [6, 14]. Clinicopathologic details of these patients and samples are summarized in Table 1. Peripheral blood samples from these patients were collected

Table 1: Clinicopathologic details of patient material

Osteosarcoma patient	Sex	Age	Tumor origin	Tumor site	Histologic subtype	Primary cell line
369	Female	31	Local relapsed	Proximal humerus	Fibroblastic	L2792 p3
398	Female	14	Prechemotherapy diagnostic biopsy	Distal femur	Conventional	L2635 p6
404	Male	46	Postchemotherapy resection	Humerus	Conventional	L2826 p9
404	Male	46	Local relapse	Humerus	Conventional	L3312 p13
407	Male	9	Postchemotherapy resection	Proximal tibia	Chondroblastic	L2857 p7

prior to the initiation of chemotherapy after written informed consent approved by the Review Board on Medical Ethics of the Leiden University Medical Center and used for cytotoxicity experiments (Table 1). Tumor specimens were obtained and analyzed according to the ethical guidelines of the national organization of scientific societies (FEDERA, http://www.federa. org/gedragscodes-codes-conduct-en).

Cell lines

The following extensively characterized sarcoma cell lines were included in this study: osteosarcoma cell lines OHS, OSA (SJSA-1), SAOS-2, U2OS, ZK-58 [15] and the Ewing sarcoma cell lines A673, CADO-ES, SK-ES-1, SK-NMC, STA-ET2.1, TC71 [15] and L1062 [14]. All sarcoma cell lines were obtained from the EuroBoNeT cell line repository (by 2007) and were confirmed for their identity by short-tandem repeat DNA fingerprinting in 2011. The cell line TC71 was maintained in IMDM medium (Invitrogen). All other cell lines were grown in RPMI-1640 medium (Invitrogen). Both media were supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 mg/ml streptomycin (all Invitrogen). All Ewing sarcoma cell lines were grown in 0.1% gelatin-coated tissue culture flasks. The chemotherapy-resistant variant cell lines of SAOS-2 and U2OS [16–18] were cultured in IMDM medium with 10% FCS and penicillin/streptomycin and maintained in chemotherapeutic drugs as follows: SAOS-2–DX580 and U2OS–DX580 with 580 mg/ml of doxorubicin (DX); SAOS-2–MTX1000 and U2OS–MTX300 with 1,000 and 300 ng/ml of methotrexate (MTX), respectively; SAOS-2–CDDP6 (SAOS-2–CDDP6mg) and U2OS–CDDP4 (U2OS–CDDP4μg) with 6 and 4 mg/ml of cisplatin (cis-diamminedichloroplatinum, CDDP), respectively. Drug sensitivities of each cell line were calculated from the drug dose–response curves and expressed as IC50 (drug concentration resulting in 50% inhibition of cell growth after 96 hours of *in vitro* treatment). Fold increases in drug resistance, quantified as the ratio between IC50 of each drug-resistant variant to that of its corresponding parental cell line, were as follows: 315 for U2OS–DX580, 328 for SAOS-2–DX580, 135 for U2OS–MTX300, 281 for SAOS-2–MTX1mg, 63 for U2OS–CDDP4mg, and 112 for SAOS-2–CDDP6mg. All cell lines were negative for Mycoplasma infection as regularly checked by PCR. The primary osteosarcoma cultures were maintained in RPMI-1640 medium supplemented with 20% FCS and penicillin/streptomycin in gelatin-coated culture tissue culture flasks.

Cell isolations and stimulations

Peripheral blood mononuclear cells (PBMC) were isolated from osteosarcoma patients' blood samples (autologous) or buffy coats of healthy adult donors (allogeneic; Sanquin Blood bank, Region Southwest, Rotterdam, the Netherlands) by Ficoll-Hypaque density gradient centrifugation. NK cells were purified by negative selection, depleting non-NK cells through a combination of biotin-conjugated monoclonal anti-human antibodies and MicroBeads using the "Human NK cell Isolation Kit" (Miltenyi Biotech); NK cell purity was more than 95% as determined by flow cytometry, analyzing NK cells as $CD56^+$, $CD3^-$, $CD14^-$, and $CD19^-$ cells. NK cells were depleted from PBMC (NKD PBMC) by positive selection using anti-CD56 MicroBeads (Miltenyi Biotech); NKD PBMC contained less than 0.1% of NK cells as analyzed by flow cytometry. IL-15–activated NK cells were obtained by incubating purified NK cells in AIM-V medium with 2 mM of glutamine (Invitrogen) supplemented with 10% of pooled human AB serum (Sanquin), penicillin/streptomycin and 10 ng/ml of IL-15 (Peprotech) for 2 to 3 weeks in 24-well format tissue culture plates without feeder cells. To measure NK cell activation after cetuximab crosslinking, upregulation of CD69 expression on NK cells (300,000) was measured after coculture with STA-ET2.1 (150,000), L1062 (80,000), and OSA (75,000) cells for 48 hours in 24-well plates in the absence or presence of cetuximab (10 mg/ml).

Flow cytometry

Determination of NK cell percentages in PBMC, validation of NK cell purity, and expression of the NK cell activation marker (CD69) was analyzed phenotypically by staining with fluorescently labeled antibodies followed by fluorescence-activated cell sorting (FACS). The following antibodies were applied according to the manufacturer's instructions: $CD3^{FITC}$ (SK7), $CD3^{PerCP\text{-}Cy5.5}$ (SK7), $CD14^{PerCP\text{-}Cy5.5}$ (M5E2), $CD19^{PE}$ (4G7), $CD69^{FITC}$ (L78; Becton Dickinson); $CD56^{APC}$ (N901 NKH1), $NKG2D^{PE}$ (ON72; IOTEST Immunotech). Expression of EGFR on the surface of sarcoma cell lines and primary osteosarcoma cultures was measured using the anti-EGFR mAb cetuximab (Erbitux; Merck KGaA) followed by the Alexa Fluor 647 goat anti-human IgG secondary antibody (A21445; Invitrogen). The anti-CD20 mAb rituximab (MabThera; Roche) was used as an IgG1 isotype-matched negative control for cetuximab. FACS measurements were carried out with the FACSCalibur (BD Biosciences) and analyzed with the "BD Cell Quest Pro" software (version 5.2.1).

^{51}chromium release assay

The cytolytic activity of PBMC, NKD PBMC, and purified NK cells against sarcoma cell lines and primary osteosarcoma cultures was measured in 4-hour ^{51}chromium (^{51}Cr) release assays. Target cells were labeled with 100 ml Na-chromate (^{51}Cr, 3.7 MBq) for 1 hour. After washing, $2.5x10^3$ target cells were added to the effector cells in duplicate or triplicate at the indicated effector–target (E:T) ratios and incubated in the presence or absence of cetuximab (10^{-7} to 10 mg/ml as indicated) or the control mAb rituximab (10 mg/ml) for 4 hours at 37°C. Supernatants were collected, and the release of ^{51}Cr was measured with a beta-counter (Wallac/PerkinElmer). Spontaneous and total release were obtained by incubation with medium and Triton X-100 (2.5%; Merck Chemicals), respectively. The specific lysis was calculated by the following

formula: percentage of specific lysis = 100 – (experimental release – spontaneous release/total release – spontaneous release).

Immunohistochemistry

Sections of 4 mm of representative tumor sections were deparaffinized and pepsin antigen retrieval was done. Expression of EGFR was assessed using a mouse monoclonal anti-EGFR antibody (31G7, 1:10; Zymed; Invitrogen) followed by a polyclonal goat anti-mouse/rabbit/rat IgG HRP linker antibody conjugate (DPVO-110HRP; Immunologic) and DAB+Substrate Chromogen System (Dako) detection. The sections were examined with a Leica DM5000 fluorescence microscope and LAS-AF acquisition program (Leica).

Statistical analysis

Statistical analyses were carried out with GraphPad Prism version 5.04 or SPSS version 16.0 (IBM) using paired student t tests, comparing means between groups of samples and linear regression analysis. Error bars represent the SEM. A P value less than 0.05 was considered statistically significant. ns, not statistically significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001.

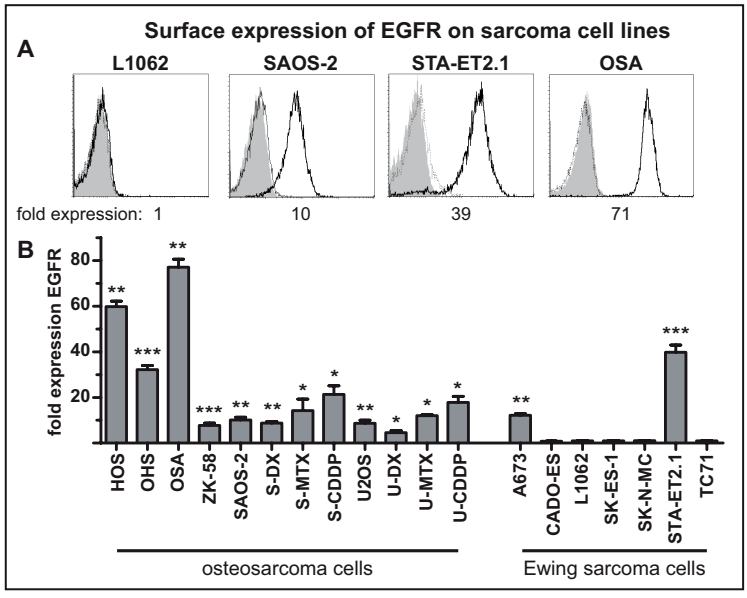

Figure 1: EGFR is expressed on the surface of sarcoma cell lines

(A) Surface expression of EGFR on osteosarcoma and Ewing sarcoma cell lines was measured by flow cytometry using the anti-EGFR mAb cetuximab followed by the Alexa Fluor-647 goat anti-human IgG secondary antibody. A, representative FACS overlay plots, detecting EGFR by cetuximab (bold solid line) and CD20 by the isotype-matched, negative control mAb rituximab (solid line), both followed by secondary antibody and secondary antibody only (grey area). The indicated fold expression of EGFR was calculated by dividing the geometric mean fluorescence intensity of EGFR by the geometric mean fluorescence intensity of the control CD20. **(B)** combined data of the fold change of EGFR expression on all tested sarcoma cell lines of multiple experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

RESULTS

EGFR is expressed on the surface of osteosarcoma cell lines

ADCC by NK cells requires antibody binding to an antigen expressed on the tumor cell surface. Therefore, surface expression of EGFR, as detected by cetuximab, was measured on a panel of osteosarcoma (n=12) cell lines by flow cytometry. All osteosarcoma cell lines expressed EGFR on the cell surface, with the highest expression on the cell lines HOS, OHS, and OSA (Figure 1). The chemoresistant variants of SAOS-2 and U2OS also expressed EGFR. Previously, EGFR has been reported undetectable in Ewing sarcoma cell lines (n=3; [19]). To extend these findings, surface EGFR expression was assessed on a panel of Ewing sarcoma cell lines (n=7). EGFR expression was not detectable on 5 of 7 Ewing sarcoma cell lines. Correspondingly, EGFR expression was not detectable in Ewing sarcoma biopsy and resection specimens, as determined by immunohistochemistry (data not shown).

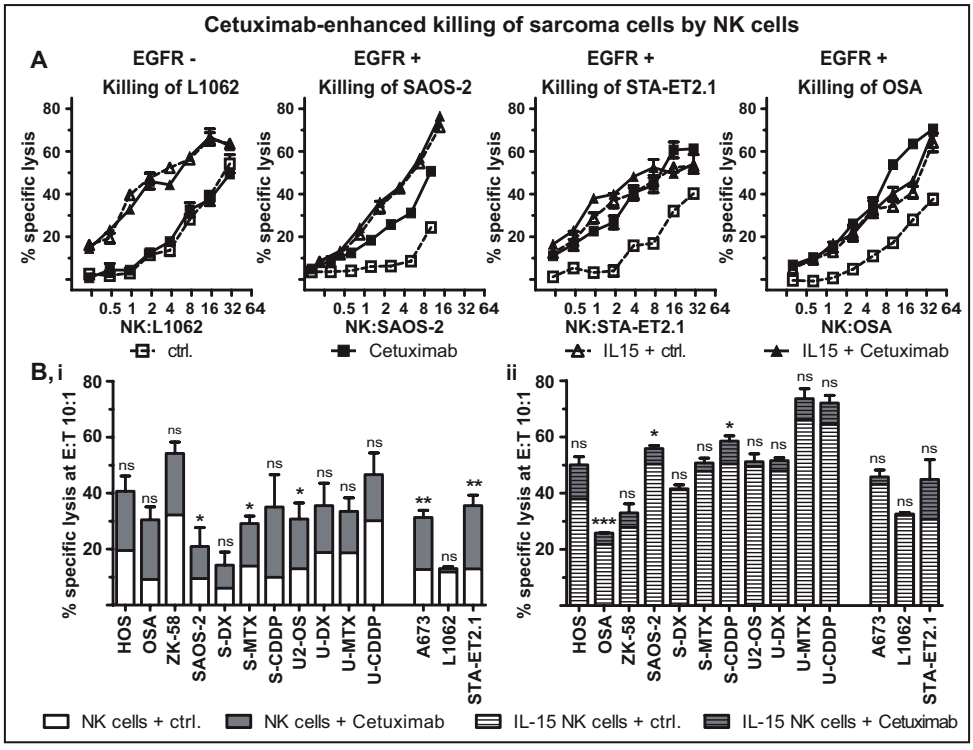

Figure 2: Cetuximab-enhanced killing of sarcoma cell lines by NK cells

Lysis of EGFR$^+$ and EGFR$^-$ sarcoma cell lines by purified, resting NK cells and IL-15–activated NK cells was measured in triplicate in a 4-hour ^{51}Cr release assay in the presence of cetuximab or the isotype-matched, negative control mAb rituximab. The specific lysis (%) of sarcoma cells in the presence of the control mAb was comparable with killing without mAb addition. **(A)** representative data of the specific lysis of sarcoma cell lines (ranked by increasing EGFR density) by resting NK cells (squares) and IL-15–activated NK cells (triangles) in the presence of cetuximab (filled symbols) and the control mAb (open symbols). **(B)** combined data of the specific lysis of sarcoma cells by resting NK cells (i, no pattern) and IL-15–activated NK cells (ii, horizontal pattern) calculated at a 10:1 E:T ratio. Data represent 3 independent experiments done in triplicate, showing lysis in the presence of the control mAb (open bars) and the respective extra lysis induced by the presence of cetuximab (filled bars, SEM). Significance of cetuximab-induced lysis was calculated by comparing whether the total lysis in the presence of cetuximab (filled bar plus open bar) was statistically different to the lysis in the presence of the control antibody (open bar). ns, not significant. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

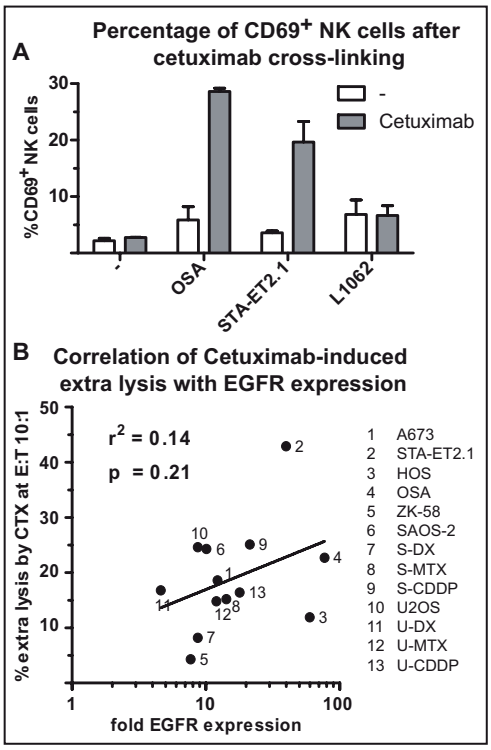

Figure 3: Cetuximab-induced activation of NK cells

(A) EGFR-expressing (OSA and STA-ET2.1) and EGFR-negative cell lines (L1062) were cocultured with purified NK cells in the presence of cetuximab for 48 hours and CD69 expression on NK cells was assessed. The percentage of CD69-positive NK cells is indicated. **(B)** The extra lysis induced by cetuximab, as calculated by subtracting the lysis by the control mAb from the lysis by cetuximab, was correlated with EGFR expression levels on the cell lines. Lysis of $EGFR^+$ sarcoma cell lines (n = 13) by resting NK cells at a 10:1 E:T ratio was measured in the presence of cetuximab or the negative control mAb (Fig. 2). The extra lysis was calculated by subtracting the specific lysis in the presence of the control mAb from the total lysis in the presence of cetuximab, and plotted against the log of the fold change of EGFR expression (Figure 1). The regression coefficient (*r2*) between extra lysis and EGFR expression was 0.14 (*P* = 0.21), as calculated by linear regression analysis.

Cetuximab enhanced cytolysis of EGFR-expressing osteosarcoma cell lines by NK cells

To investigate whether cetuximab can enhance cytolysis of EGFR-expressing osteosarcoma and Ewing sarcoma cell lines by NK cells, resting NK cells were incubated with one $EGFR^-$ and several EGFR* cell lines in the presence of cetuximab or the isotype-matched, nonbinding anti-CD20 control mAb in a 4-hour ^{51}Cr release assay. As compared with cytolysis in the absence of mAb, the negative control mAb did not alter killing of sarcoma cells by NK cells (data now shown). In contrast, the addition of cetuximab increased the lysis of EGFR-expressing sarcoma cells but not of EGFR-negative cell lines (Figure 2, panel A and B, i; data not shown). The lysis of the chemotherapy-resistant variant cell lines of SAOS-2 and U2OS was equally enhanced by cetuximab. Cetuximab-enhanced lysis by resting NK cells was comparable with the lysis by IL-15–activated NK cells (Figure 2, panel A and B, ii). The addition of cetuximab to IL-15–activated NK cells hardly led to a further increase of the cytolytic activity. As an alternative parameter for NK cell activation, it was observed that the percentage of CD69- expressing NK cells increased after coculture of NK cells with EGFR-expressing sarcoma cells in the presence of cetuximab (Figure 3, panel A).

Thus, the cytotoxic function of resting NK cells toward EGFR-expressing sarcoma cell lines as well as their activation status was substantially augmented in the presence of cetuximab.

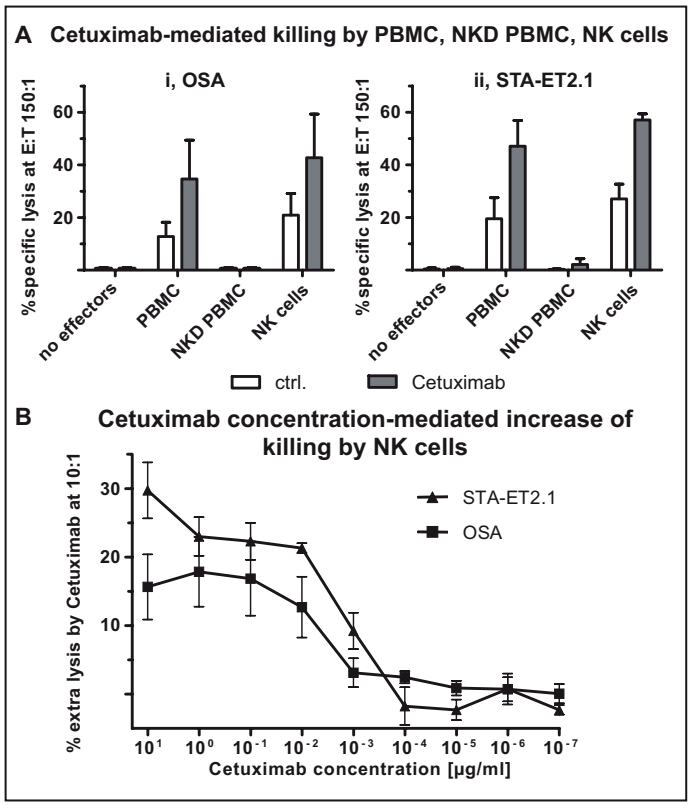

Figure 4: Cetuximab-mediated killing is dependent on NK cells

(A) The specific lysis of the osteosarcoma cell line OSA (i) and the Ewing's sarcoma cell line STA-ET2.1 (ii) in the presence of cetuximab or the control mAb by resting NK cells was compared with the lysis by total PBMCs, NKD PBMCs, and medium (absence of effector cells). Killing by PBMCs was analyzed at a 150:1 E:T ratio. Killing by NKD PBMCs was analyzed at a 150:1 E:T ratio corrected for the absence of NK cells in accordance to the percentage of NK cells in the corresponding PBMCs. Killing by purified NK cells was extrapolated to a 150:1 E:T ratio of PBMCs in accordance with the percentage of NK cells in the corresponding PBMCs. Combined data of 2 experiments. **(B)** The extra lysis of the two $EGFR^+$ cell lines OSA and STA-ET2.1 by resting NK cells was measured in the presence of increasing concentrations of cetuximab, from 10^{-7} to 10 mg/ml at a 10:1 E:T ratio. Combined data of 3 independent experiments.

Cetuximab-mediated lysis is independent of EGFR expression intensity

Despite different sensitivities to NK cell killing, the magnitude of cetuximab-enhanced lysis by resting NK cells was comparable among most EGFR-expressing cell lines (Figure 2, panel B, i). This increase did not correlate significantly with EGFR surface densities (Figure 3, panel B). Thus, even the minimal EGFR expression levels on some of the sarcoma cells were sufficient for the induction of ADCC.

Cetuximab-mediated lysis is dependent on NK cells

In the absence of effector cells, cetuximab did not elicit cytolytic effects on EGFR-expressing cell lines during the 4-hour cytotoxicity assay (Figure 4). FcγRIIIa/CD16 expression is required to elicit ADCC by NK cells. Because FcγRIIIa/CD16 can also be expressed by monocytes, it was studied whether cetuximab-mediated ADCC by PBMC is dependent on NK cells. In the presence of cetuximab, lysis of EGFR-expressing sarcoma cells by PBMC was comparable with the cetuximab- enhanced lysis by purified NK cells (Figure 4, panel A). In contrast, NK cell depletion abolished killing by PBMC both in the absence and presence of cetuximab, indicating that in this *in vitro* system cetuximab-mediated killing was strictly dependent on NK cells present in the PBMC.

Next, the dependence of cetuximab-mediated lysis on the concentration of cetuximab was investigated in a serial dilution experiment. The lysis induced by cetuximab was comparable

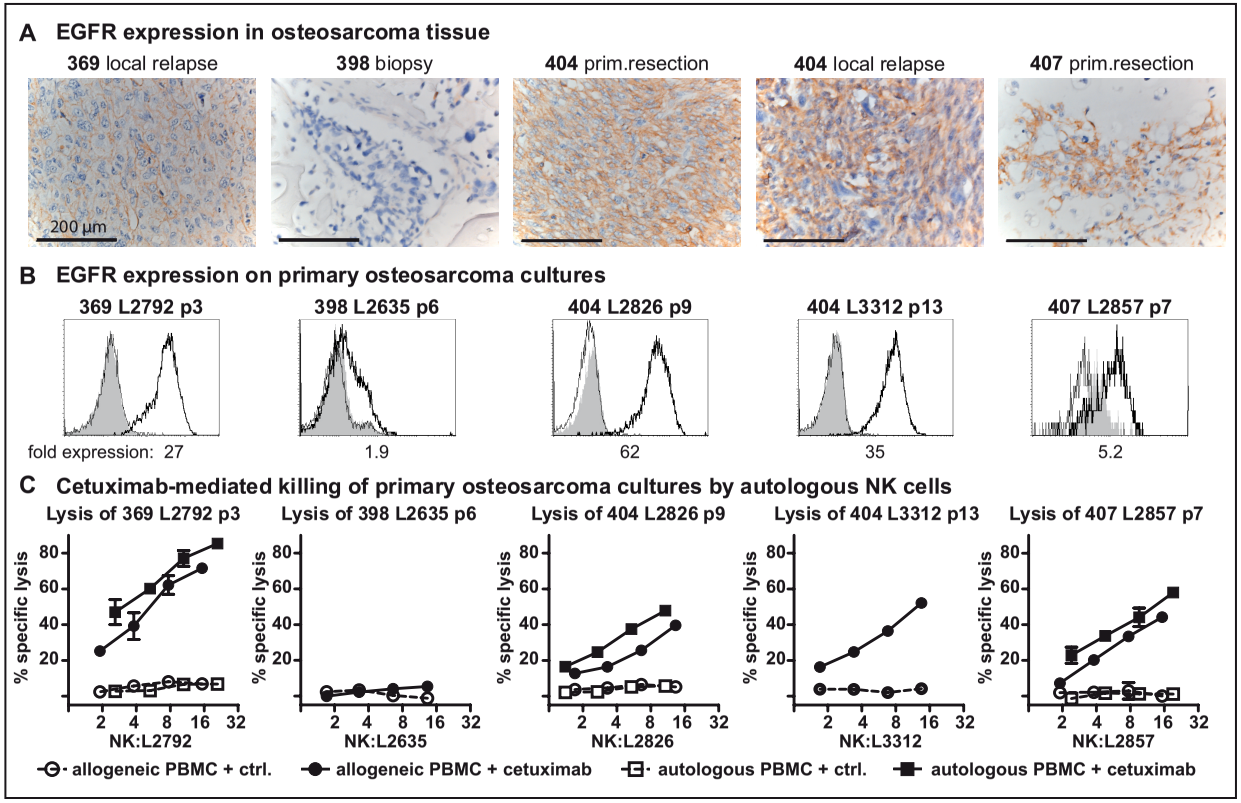

Figure 5: EGFR⁺ primary osteosarcoma cultures, derived from EGFR⁺ osteosarcoma specimens, are highly susceptible to cetuximab-mediated lysis by autologous PBMC

(A) EGFR expression in biopsies and resection specimens from osteosarcoma patients was detected using a mouse monoclonal anti-EGFR antibody followed by a polyclonal goat anti-mouse/rabbit/rat IgG HRP linker antibody conjugate. Brown-colored EGFR staining patterns and counterstaining with Mayer's hematoxylin are displayed at a 40-fold magnification for the respective tumor origin and patient. **(B)** Primary osteosarcoma cultures were generated from EGFR⁺ and EGFR biopsies and resections of osteosarcoma patients as indicated. EGFR expression on the primary osteosarcoma cultures was measured as described in Figure 1 at the indicated passage numbers. **(C)** Killing of primary osteosarcoma cultures by autologous PBMC, derived from the same osteosarcoma patient, and allogeneic PBMC (one representative result from 2 healthy donors) was analyzed in duplicate in the presence of cetuximab or the control mAb at the indicated E:T ratios. p, passage number; prim, primary.

between 10 and $1x10^{-2}$ mg/ml of cetuximab, but it was reduced by at least 50% at a concentration of $1x10^{-3}$ mg/ml (Figure 4, panel B). Lower cetuximab concentrations failed to enhance lysis. Hence, 0.01 mg/ml of cetuximab was the minimal concentration to substantially enhance cytolysis by NK cells.

EGFR$^+$ primary cultures derived from EGFR$^+$ osteosarcoma tumors are highly susceptible to cetuximab-mediated lysis by autologous PBMC

Primary tumor cell cultures (n=5) were generated from osteosarcoma biopsy (n=1) and resection (n=4) specimens derived from 4 different osteosarcoma patients (Table 1). EGFR expression in these osteosarcoma samples was membranous, as determined by immunohistochemistry, and correlated to the EGFR densities on the corresponding primary cultures, as determined by flow cytometry between passages (p) 3 and 13. Except for osteosarcoma patient 398, in which EGFR was not detectable in the biopsy and only weakly detectable on the corresponding primary culture L2635 p6, all osteosarcoma patients (369, 404, and 407) presented EGFR expression both in the original tumor material and the respective primary cultures (L2792 p3; L2826 p9, and L3312 p13; L2857 p7; Figure 5, panel A and B).

The cytolytic activity of NK cells from patient-derived PBMC toward EGFR-expressing autologous osteosarcoma cultures was substantially enhanced by cetuximab (Figure 5, panel C). Notably, patient-derived NK cells lysed the osteosarcoma cultures as efficient as NK cells from healthy donors. Thus, when sarcoma cells are specifically targeted with cetuximab, their lysis by resting NK cells can be enhanced by ADCC.

DISCUSSION

The identification of antigens specifically expressed on tumor cells has fueled the development of tumor-specific, mAb-based targeted therapies. The introduction of anti- CD20 mAb (Rituximab, MabThera) and anti-Her2 mAb (Trastuzumab, Herceptin) against B cell malignancies and breast cancer, respectively, has improved patient prognosis [20,21]. Surface expression of EGFR has been shown in several tumors, such as colorectal cancer, head and neck squamous cell carcinoma, and is specifically recognized by the chimeric IgG1 mAb cetuximab [13,20]. In numerous clinical phase II and III studies, the addition of cetuximab to conventional multi-drug chemotherapy or radiotherapy has led to a significant improvement of clinical response rates, progression-free survival, and overall survival. Therefore, cetuximab therapy was approved for the treatment of recurrent, refractory, and metastatic colorectal cancer [22–25] as well as head and neck squamous cell carcinoma [26,27] by the U.S. Food and Drugs Administration (FDA).

Previously, we and others have shown the cytolytic potential of NK cells against sarcoma cells [6–8]. In this study, we sought to explore whether this cytolytic activity can be more specifically targeted toward sarcoma cells using a sarcoma-reactive mAb, with a human Fc portion that can bind to FcγRIIIa/CD16 on human NK cells. As several studies have described surface EGFR expression in osteosarcoma tumors and on osteosarcoma cell lines [12,19,28–31], we explored the potential of the anti-EGFR mAb cetuximab to specifically direct NK cell–mediated killing to sarcoma cell lines. In agreement with previous studies on other tumor types, cetuximab induced NK cell–dependent ADCC against EGFR-expressing sarcoma cells. Similar to previous

studies [32–35], we show that 0.01 mg/ml of cetuximab was the minimal concentration to induce cetuximab-mediated lysis by NK cells. These concentrations have been reported in sera of patients treated with cetuximab and in the tumor environment in a xenograft model [36,37], indicating that cetuximab-mediated ADCC could be a functional anticancer mechanism *in vivo*.

Although in other studies the magnitude of cetuximab-induced ADCC correlated with the level of EGFR expression [34,35,38], this correlation was not evident in osteosarcoma, despite the use of highly comparable methods to assess ADCC [32,39]. In fact, minimal EGFR densities were sufficient for the cetuximab-induced lysis of sarcoma cells [32,39]. Cetuximab-induced ADCC was comparable with the maximal killing achieved by IL-15–activated NK cells. In contrast to some other models [32,34,40,41], we did not observe an additive effect of cetuximab on the lysis by cytokine (IL-15)–activated NK cells.

Multiple mechanisms may account for the antitumor effect of cetuximab in patients. Masking of the EGFR extracellular binding site from its natural ligand EGF inhibits the activation of the receptor tyrosine kinase and downstream signaling pathways [13,42]. EGFR blockage has been shown to arrest cell-cycle progression and lead to apoptosis [13}. Cetuximab can inhibit tumor angiogenesis, neovascularization and invasion, and sensitizes tumor cells to radiation and chemotherapy-induced growth inhibition and apoptosis *in vivo* [13}. Finally, cetuximab may induce complement-dependent cytotoxicity or cytolytic effects by immune cells via ADCC [13,32,38,39,43]. An advantage of cetuximab-mediated ADCC is that it would be independent of the EGFR mutation status [34,38] and persistently activated EGFR signaling pathways [13,40}.

The primary mode of action of cetuximab *in vivo* is difficult to determine. In a murine model, the anticancer effect of cetuximab was presumed to be mediated by NK cells [41]. Depletion of NK cells in murine osteosarcoma xenograft models or in mice with syngeneic mesenchymal stem cell–induced osteosarcoma could address whether an antitumor effect of cetuximab or murine anti-EGFR mAb relies on the presence of NK cells [44–46]. In humans, the relevance of ADCC has been suggested by the finding that FcγRIIIa/CD16 polymorphism of NK cells correlated with the survival of colorectal cancer patients [47,48], as well as with the efficacy of cetuximab-mediated ADCC by NK cells *in vitro* [34}. Interestingly, the intratumoral NK cells have recently been associated with improved survival when colorectal cancer patients had been treated with cetuximab [49]. In this study, we used a unique combination of tumor specimens, primary tumor cultures, and PBMC from osteosarcoma patients. This allowed us to establish that cetuximab treatment can improve the lysis of EGFR-expressing, autologous primary osteosarcoma cells by patient-derived NK cells via cetuximab-mediated ADCC.

Cetuximab treatment is associated with relatively mild adverse effects and has been approved for clinical usage by the FDA [37,50]. Therefore, in the treatment of osteosarcoma patients, cetuximab-mediated immunotherapy could be scheduled in the presence of endogenous or adoptively transferred NK cells. As such, cetuximab may provide an interesting treatment modality for patients with chemotherapy-resistant or metastatic $EGFR^+$ sarcomas.

ACKNOWLEDGMENTS

The authors thank Els Jol-van der Zijde, Monique Ostaijen-ten Dam, Kitty Kwappenberg, and Dani€elle de Jong for technical contributions and helpful discussions, and Nicolette Leijerzapf and Jakob Anninga for the assistance in obtaining the patient samples.

REFERENCES

1. Klein MJ, Parisien MV,Scheider-Stock R. Osteogenic Tumors. In: Fletcher, C. M. D., Unni, K. K., and Mertens, F. editors. World health classification of tumours. Pathology and genetics of tumours of soft tissue and bone. Lyon: IARC Press; 2002. p. 259-285.
2. van den Berg H, Kroon HM, Slaar A, and Hogendoorn PCW. Incidence of biopsy-proven bone tumors in children: a report based on the Dutch pathology registration "PALGA". J Pediatr Orthop 2008;28:29-35.
3. Hattinger CM, Pasello M, Ferrari S, Picci P, and Serra M. Emerging drugs for high-grade osteosarcoma. Expert Opin Emerg Drugs 2010;15:615-634.
4. Anninga JK, Gelderblom H, Fiocco M, Kroep JR, Taminiau AH, Hogendoorn PCW, et al. Chemotherapeutic adjuvant treatment for osteosarcoma: Where do we stand? Eur J Cancer 2011.
5. Marina N, Gebhardt M, Teot L, and Gorlick R. Biology and therapeutic advances for pediatric osteosarcoma. Oncologist 2004;9:422-441.
6. Buddingh EP, Schilham MW, Ruslan SE, Berghuis D, Szuhai K, Suurmond J, et al. Chemotherapy-resistant osteosarcoma is highly susceptible to IL-15-activated allogeneic and autologous NK cells. Cancer Immunol Immunother 2011.
7. Cho D, Shook DR, Shimasaki N, Chang YH, Fujisaki H, and Campana D. Cytotoxicity of activated natural killer cells against pediatric solid tumors. Clin Cancer Res 2010;16:3901-3909.
8. Verhoeven DHJ, de Hooge AS, Mooiman EC, Santos SJ, ten Dam MM, Gelderblom H, et al. NK cells recognize and lyse Ewing sarcoma cells through NKG2D and DNAM-1 receptor dependent pathways. Mol Immunol 2008;45:3917-3925.
9. Moretta L, Bottino C, Pende D, Vitale M, Mingari MC, and Moretta A. Human natural killer cells: Molecular mechanisms controlling NK cell activation and tumor cell lysis. Immunol Lett 2005;100:7-13.
10. Vivier E, Raulet DH, Moretta A, Caligiuri MA, Zitvogel L, Lanier LL, et al. Innate or adaptive immunity? The example of natural killer cells. Science 2011;331:44-49.
11. Buddingh EP, Kuijjer ML, Duim RA, Burger H, Agelopoulos K, Myklebost O, et al. Tumor-infiltrating macrophages are associated with metastasis suppression in high-grade osteosarcoma: a rationale for treatment with macrophage activating agents. Clin Cancer Res 2011;17:2110-2119.
12. Hughes DP, Thomas DG, Giordano TJ, Baker LH, and McDonagh KT. Cell surface expression of epidermal growth factor receptor and Her-2 with nuclear expression of Her-4 in primary osteosarcoma. Cancer Res 2004;64:2047-2053.
13. Vincenzi B, Schiavon G, Silletta M, Santini D, and Tonini G. The biological properties of cetuximab. Crit Rev Oncol Hematol 2008;68:93-106.
14. Szuhai K, Ijszenga M, Tanke HJ, Rosenberg C, and Hogendoorn PCW. Molecular cytogenetic characterization of four previously established and two newly established Ewing sarcoma cell lines. Cancer Genet Cytogenet 2006;166:173-179.
15. Ottaviano L, Schaefer KL, Gajewski M, Huckenbeck W, Baldus S, Rogel U, et al. Molecular characterization of commonly used cell lines for bone tumor research: a trans-European EuroBoNet effort. Genes Chromosomes Cancer 2010;49:40-51.
16. Pasello M, Michelacci F, Scionti I, Hattinger CM, Zuntini M, Caccuri AM, et al. Overcoming glutathione S-transferase P1-related cisplatin resistance in osteosarcoma. Cancer Res 2008;68:6661-6668.
17. Serra M, Scotlandi K, Manara MC, Maurici D, Lollini PL, De GC, et al. Establishment and characterization of multidrug-resistant human osteosarcoma cell lines. Anticancer Res 1993;13:323-329.
18. Serra M, Reverter-Branch, Maurici D, Benini S, Shen JN, Chano T, et al. Analysis of dihydrofolate reductase and reduced folate carrier gene status in relation to methotrexate resistance in osteosarcoma cells. Ann Oncol 2004;15:151-160.
19. Wen YH, Koeppen H, Garcia R, Chiriboga L, Tarlow BD, Peters BA, et al. Epidermal growth factor receptor in osteosarcoma: expression and mutational analysis. Hum Pathol 2007;38:1184-1191.
20. Harris M. Monoclonal antibodies as therapeutic agents for cancer. Lancet Oncol 2004;5:292-302.
21. Murdoch D and Sager J. Will targeted therapy hold its promise? An evidence-based review. Curr Opin Oncol 2008;20:104-111.
22. Bokemeyer C, Bondarenko I, Makhson A, Hartmann JT, Aparicio J, de BF, et al. Fluorouracil, leucovorin, and oxaliplatin with and without cetuximab in the first-line treatment of metastatic colorectal cancer. J Clin Oncol 2009;27:663-671.

23. Borner M, Koeberle D, Von MR, Saletti P, Rauch D, Hess V, et al. Adding cetuximab to capecitabine plus oxaliplatin (XELOX) in first-line treatment of metastatic colorectal cancer: a randomized phase II trial of the Swiss Group for Clinical Cancer Research SAKK. Ann Oncol 2008;19:1288-1292.

24. Cunningham D, Humblet Y, Siena S, Khayat D, Bleiberg H, Santoro A, et al. Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. N Engl J Med 2004;351:337-345.

25. Van Cutsem E., Kohne CH, Hitre E, Zaluski J, Chang Chien CR, Makhson A, et al. Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer. N Engl J Med 2009;360:1408-1417.

26. Bonner JA, Harari PM, Giralt J, Cohen RB, Jones CU, Sur RK, et al. Radiotherapy plus cetuximab for locoregionally advanced head and neck cancer: 5-year survival data from a phase 3 randomised trial, and relation between cetuximab-induced rash and survival. Lancet Oncol 2010;11:21-28.

27. Burtness B, Goldwasser MA, Flood W, Mattar B, and Forastiere AA. Phase III randomized trial of cisplatin plus placebo compared with cisplatin plus cetuximab in metastatic/recurrent head and neck cancer: an Eastern Cooperative Oncology Group study. J Clin Oncol 2005;23:8646-8654.

28. Boulytcheva IV, Soloviev YN, Kushlinskii NE, and Mahson AN. Expression of molecular markers in the tumor and survival prognosis in osteosarcoma. Bull Exp Biol Med 2010;150:237-242.

29. Do SI, Jung WW, Kim HS, and Park YK. The expression of epidermal growth factor receptor and its downstream signaling molecules in osteosarcoma. Int J Oncol 2009;34:797-803.

30. Freeman SS, Allen SW, Ganti R, Wu J, Ma J, Su X, et al. Copy number gains in EGFR and copy number losses in PTEN are common events in osteosarcoma tumors. Cancer 2008;113:1453-1461.

31. Kersting C, Gebert C, Agelopoulos K, Schmidt H, van Diest PJ, Juergens H, et al. Epidermal growth factor receptor expression in high-grade osteosarcomas is associated with a good clinical outcome. Clin Cancer Res 2007;13:2998-3005.

32. Kurai J, Chikumi H, Hashimoto K, Yamaguchi K, Yamasaki A, Sako T, et al. Antibody-dependent cellular cytotoxicity mediated by cetuximab against lung cancer cell lines. Clin Cancer Res 2007;13:1552-1561.

33. Levy EM, Sycz G, Arriaga JM, Barrio MM, von Euw EM, Morales SB, et al. Cetuximab-mediated cellular cytotoxicity is inhibited by HLA-E membrane expression in colon cancer cells. Innate Immun 2009;15:91-100.

34. Lopez-Albaitero A, Lee SC, Morgan S, Grandis JR, Gooding WE, Ferrone S, et al. Role of polymorphic Fc gamma receptor IIIa and EGFR expression level in cetuximab mediated, NK cell dependent in vitro cytotoxicity of head and neck squamous cell carcinoma cells. Cancer Immunol Immunother 2009;58:1853-1864.

35. Patel D, Guo X, Ng S, Melchior M, Balderes P, Burtrum D, et al. IgG isotype, glycosylation, and EGFR expression determine the induction of antibody-dependent cellular cytotoxicity in vitro by cetuximab. Hum Antibodies 2010;19:89-99.

36. Luo FR, Yang Z, Dong H, Camuso A, McGlinchey K, Fager K, et al. Correlation of pharmacokinetics with the antitumor activity of Cetuximab in nude mice bearing the GEO human colon carcinoma xenograft. Cancer Chemother Pharmacol 2005;56:455-464.

37. Tan AR, Moore DF, Hidalgo M, Doroshow JH, Poplin EA, Goodin S, et al. Pharmacokinetics of cetuximab after administration of escalating single dosing and weekly fixed dosing in patients with solid tumors. Clin Cancer Res 2006;12:6517-6522.

38. Kimura H, Sakai K, Arao T, Shimoyama T, Tamura T, and Nishio K. Antibody-dependent cellular cytotoxicity of cetuximab against tumor cells with wild-type or mutant epidermal growth factor receptor. Cancer Sci 2007;98:1275-1280.

39. Kawaguchi Y, Kono K, Mimura K, Sugai H, Akaike H, and Fujii H. Cetuximab induce antibody-dependent cellular cytotoxicity against EGFR-expressing esophageal squamous cell carcinoma. Int J Cancer 2007;120:781-787.

40. Roberti MP, Barrio MM, Bravo AI, Rocca YS, Arriaga JM, Bianchini M, et al. IL-15 and IL-2 increase Cetuximab-mediated cellular cytotoxicity against triple negative breast cancer cell lines expressing EGFR. Breast Cancer Res Treat 2011.

41. Roda JM, Joshi T, Butchar JP, McAlees JW, Lehman A, Tridandapani S, et al. The activation of natural killer cell effector functions by cetuximab-coated, epidermal growth factor receptor positive tumor cells is enhanced by cytokines. Clin Cancer Res 2007;13:6419-6428.

42. Berger C, Krengel U, Stang E, Moreno E, and Helene M, I. Nimotuzumab and Cetuximab Block Ligand-independent EGF Receptor Signaling Efficiently at Different Concentrations. J Immunother 2011;34:550-555.

43. Dechant M, Weisner W, Berger S, Peipp M, Beyer T, Schneider-Merck T, et al. Complement-dependent tumor cell lysis triggered by combinations of epidermal growth factor receptor antibodies. Cancer Res 2008;68:4998-5003.

44. Fan TM. Animal models of osteosarcoma. Expert Rev Anticancer Ther 2010;10:1327-1338.

45. Garrido G, Sanchez B, Rodriguez HM, Lorenzano P, Alonso D, and Fernandez LE. 7A7 MAb: a new tool for the pre-clinical evaluation of EGFR-based therapies. Hybrid Hybridomics 2004;23:168-175.

46. Mohseny AB, Szuhai K, Romeo S, Buddingh EP, Briaire-de B, I, de JD, et al. Osteosarcoma originates from mesenchymal stem cells in consequence of

aneuploidization and genomic loss of Cdkn2. J Pathol 2009;219:294-305.

47. Bibeau F, Lopez-Crapez E, Di FF, Thezenas S, Ychou M, Blanchard F, et al. Impact of Fc{gamma} RIIa-Fc{gamma}RIIIa polymorphisms and KRAS mutations on the clinical outcome of patients with metastatic colorectal cancer treated with cetuximab plus irinotecan. J Clin Oncol 2009;27:1122-1129.
48. Zhang W, Azuma M, Lurje G, Gordon MA, Yang D, Pohl A, et al. Molecular predictors of combination targeted therapies (cetuximab, bevacizumab) in irinotecan-refractory colorectal cancer (BOND-2 study). Anticancer Res 2010;30:4209-4217.
49. Marechal R, De SJ, Nagy N, Demetter P, Lemmers A, Deviere J, et al. Putative contribution of CD56 positive cells in cetuximab treatment efficacy in first-line metastatic colorectal cancer patients. BMC Cancer 2010;10:340.
50. Fakih M and Vincent M. Adverse events associated with anti-EGFR therapies for the treatment of metastatic colorectal cancer. Curr Oncol 2010;17 Suppl 1:S18-S30