RESEARCH

The anti-tumor activity of the 7A7 antibody, specific to murine EGFR, is independent of target expression levels in immunocompetent mice

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ABSTRACT

The epidermal growth factor receptor (EGFR), a molecule that is highly expressed in epithelial tumors, provides a clinically validated target for anti-cancer drug development. Anti-EGFR passive agents have shown anti-tumor activity in pre-clinical and clinical trials; optimization of these therapies is currently under active research. So far, no clear association between EGFR expression levels in the tumors and response to EGFR targeting agents has been found. One possible reason could be the lack of appropriate pre-clinical models. Therefore we explored, for the first time, a possible correlation between EGFR expression and the sensitivity to anti-EGFR monoclonal antibody (Mab) treatment, using murine tumors in normal syngeneic hosts and a specific antibody to murine EGFR (7A7 Mab). Two murine lung tumors (TC-1 and 3LL-D122), with equivalent EGFR expression levels, showed different sensitivities to 7A7 Mab treatment although similar reductions in EGFR activation in both cell lines were observed. TC-1 cells were indifferent to complement-dependent cytotoxicity (CDC) mediated by 7A7 Mab. These findings demonstrate that the effectiveness of anti-EGFR Mabs is not exclusively dependent of the EGFR expression in the tumors, suggesting that the escape to the complement cascade could represent a mechanism of resistance to anti-EGFR Mab therapy.

Keywords: EGFR, monoclonal antibodies, predictive factor, cancer therapy, drug resistance

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RESUMEN

La actividad anti-tumoral del anticuerpo 7A7, específico por el EGFR murino, es independiente de los niveles de expresión del blanco en ratones inmunocompetentes. El receptor para el factor de crecimiento epidérmico (EGFR), una molécula altamente expresada en tumores epiteliales, provee un blanco clínicamente validado para el desarrollo de drogas anti-cáncer. Los agentes de terapia pasiva anti-EGFR han mostrado actividad anti-tumoral en los escenarios pre-clínicos y clínicos, la optimización de estas terapias se encuentra actualmente bajo activa investigación. Hasta el momento no ha sido encontrada una clara asociación entre los niveles de expresión del EGFR y la respuesta a los agentes específicos por el EGFR. Una posible razón para esta interrogante pudiera ser la carencia de modelos pre-clínicos adecuados. Con esta idea en mente nosotros exploramos, por primera vez, si podía ser demostrada una correlación entre la expresión del EGFR y la sensibilidad al tratamiento con un anticuerpo monoclonal (AcM) anti-EGFR, usando tumores murinos en hospederos singénicos normales y un anticuerpo específico por el EGFR murino (AcM 7A7). Nosotros observamos que dos tumores murinos de pulmón (TC-1 y 3LL-D122) con equivalentes niveles de expresión del EGFR, muestran diferente sensibilidad al tratamiento con el AcM 7A7 a pesar de que una reducción en la activación del EGFR por el tratamiento con este anticuerpo fue obtenida en ambas. Las células TC-1 fueron indiferentes a la citotoxicidad dependiente de complemento (CDC) mediada por el AcM 7A7. Estos hallazgos demuestran que la efectividad de los AcMs anti-EGFR no depende exclusivamente de la expresión del EGFR en los tumores, sugiriendo además que el escape a la cascada del complemento podría representar un mecanismo de resistencia a la terapia anti-EGFR basada en anticuerpos.

Palabras claves: EGFR, anticuerpos monoclonales, factor predictivo, terapia de cáncer, resistencia a drogas

*I*ntroduction

The EGFR is a 170-kDa glycoprotein composed by an extracellular ligand-binding domain, a single hydrophobic transmembrane region and an intracellular domain with intrinsic tyrosine kinase activity. A high expression of EGFR has been observed in human epithelial tumors such as lung, breast, head and neck, colon, esophagus, prostate, bladder, pancreas and ovary[1], and it has been shown to correlate with advanced tumor stage and poor clinical prognosis[2]. EGFR signaling activation leads to an increase in cellular proliferation, angiogenesis and apoptosis inhibition[3].

EGFR is an attractive target for novel anti-cancer therapy. Passive immunotherapy with anti-EGFR Mabs[4-8] and treatment with EGFR-tyrosine kinase inhibitors[9, 10] are currently undergoing clinical trials showing anti-tumor activity, but their effects are modest and only benefit a group of patients[11]. One of the main challenges in the development of anti-EGFR agents is the improvement in patient selection. The level of EGFR expression required in the tumor to obtain clinical benefits from these therapies remains unknown at present.

A critical question is the usefulness of the current pre-clinical models to clarify this issue. The pre-clinical evaluation of anti-EGFR therapies is based on *in vitro* experiments with human tumor cell lines or *in vivo*

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experiments with xenograft models. Disagreements between the pre-clinical and clinical anti-tumor efficacy of these agents have been described[12]. It now seems unlikely that available pre-clinical models can resemble the patient's real scenario. Thus, searching for new models that would offer more effective extrapolation of the pre-clinical data to the clinical setting is desirable. A reasonable strategy is moving the pre-clinical evaluation to a complete autologous scenario *in vivo*. To test this idea, we evaluated whether the anti-tumor efficacy of anti-EGFR Mabs-based therapies depended on the expression level of EGFR using tumor-bearing immunocompetent mice treated with an antibody specific to the extracellular domain of murine EGFR (7A7 Mab)[13].

Materials and methods

Antibodies

7A7 Mab[13] was produced in our laboratory. 3B11 Mab[14] was kindly provided by Dr. A. M. Vazquez (Antibody Engineering Department, Centre of Molecular Immunology, Havana, Cuba) and was used as isotype control. Antibodies specific to phospho-EGFR (Tyr^{992}), phospho-p42/p44 MAPK (Thr^{202}/Tyr^{204}), phospho-Akt (Ser^{473}), phospho-STAT 3 (Tyr^{705}), p42/p44 MAPK, Akt and STAT 3 were obtained from Cell Signaling Technology, Inc (Bervely, MA).

Cell Lines and Cell Culture

The C57BL/6-derived cell lines, thymoma EL4 (ATCC TIB-39), lung tumor TC-1 (ATCC CRL-2785) and metastatic variant of Lewis lung carcinoma (3LL-D122)[15] were cultured in DMEM:F 12 (Life Technologies Inc., Grand Island, NY) supplemented with 10% FBS, 50 µM 2-ME, 2 mM L-glutamine, 20 U/mL penicillin, and 20 µg/mL streptomycin.

Mice

Female C57Bl/6 mice were purchased from the Centre for Laboratory Animal Production (Havana, Cuba) and maintained in the animal house of the Centre of Molecular Immunology (Havana, Cuba). All animals were of 6 to 12 weeks of age. Management and experiments were performed according to institutional guidelines.

Tumor Cells

TC-1, 3LL-D122 and EL4 primary tumors were obtained by surgery. The paracancerous normal tissue derived from surgical samples was separated. The samples were then minced and digested with a type I collagenase-DNase mixture (Sigma, St. Louis, MO) in DMEM:F 12 medium for 1 h at 37 ºC. The resulting suspension was examined, and a 40 µm mesh filtration was used to obtain a single cell suspension. The collected cells had usually more than 95% viability.

RT-PCR

Total RNA was isolated from cells (5×10^6) using TRIZOL Reagent (LifeTechnologies Inc., Grand Island, NY) according to the manufacturer's instructions. The reverse transcription and polymerase chain reaction (RT-PCR) was performed using the SUPERSCRIPTTM One-Step RT-PCR System according to the manufacturer's instructions. The primers used for EGFR and β-actin amplification were designed from the published sequences[16]. After PCR amplification, 10 µL of the RT-PCR products were separated by electrophoresis on 1.5% agarose gel and visualized with ethidium bromide.

FACS Analysis for EGFR Recognition

Cells from lines or primary tumors (2×10^5) were incubated with Mabs (1 µg/mL) followed by FITC conjugated goat anti-mouse IgG+IgM antibody (Jackson, Immunoresearch Laboratories Inc., West Grove, PA). Data were obtained with a FACScan Flow Cytometer (Beckton Dickinson, San Jose, CA) by collecting a minimum of 10 000 events that were analyzed using the WinMDI version 2.8 software. EGFR relative expression levels were calculated as described previously[17].

Western Blot Analysis

Cells (1×10^6/6 well-plate) were cultured in serum-starved conditions for 12 h. After different treatments, the cells were rinsed with ice-cold PBS and lysed using 50 mM Hepes pH 7.4, 0.15 M NaCl, 1% Triton X-100 buffer containing 1 mM EDTA, 1 mM EGTA, 2 µg/mL Leupeptin, 1% Aprotinin, 2 µg/mL Pepstatin, 1 mM PMSF and 1 mM Na3VO4. The protein concentration of the lysates was determined using a BCA protein assay kit (Pierce, Rockford, IL). Equal amounts of protein (100 µg) were separated by SDS-PAGE, transferred to polyvinylidine difluoride membranes (Gelmar, Ann Arbor, MI) followed by blocking with NEGT buffer (0.15 M NaCl, 5 mM EDTA, 50 mM Tris-HCL pH 7.5, 0.02% Tween 20 and 0.04% Gelatine) overnight at 4 ºC. Then the membranes were incubated with specific antibodies (at 1:100 – 1:5 000 dilutions), at room temperature for 2 h. After washing with NEGT buffer, the membranes were incubated with secondary antibody (anti-rabbit [1: 5000] or anti-mouse [1:25 000] antibodies conjugated with horseradish peroxidase from Cell Signaling Technology, Inc [Bervely, MA]) for 30 min at room temperature. Protein bands were visualized using a chemiluminescent detection system (Perkin Elmer Life Sciences, Foster City, CA).

Cytotoxicity Assays

Flat-bottom 12-well microculture plates (Costar, Cambridge, MA) were seeded with 5×10^5 cells/well and grown in medium containing 10% FBS for 12 h. Then Mabs (0.5, 1, 5, 10, 20 µg/mL) were added at different concentrations in medium supplemented with 0.5% FBS, in the presence or absence of the autologous complement (at 1:5 dilution). Dead cells were measured by FACS using propidium iodide (20 µg/mL).

Antibody Treatment and Tumor Challenge Assays

Mice were challenged with viable cells of TC-1 (0.5×10^5), 3LL-D122 (2×10^5) or EL4 (0.5×10^5) tumors in the right flank by subcutaneous injection. For all experimental groups, Mabs (2.8 mg/Kg) were first administered the day before tumor challenge and then every 4 days until day 19. The largest perpendicular

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diameters of the resulting solid tumors were measured with a calliper two times per week, and tumor volume was calculated using the formula: $\pi/6$ x length x width^2. For ethical reasons, animals were slaughtered when tumor volume exceeded 1.8 cm^3 or when the general condition of the animals was affected.

Statistical Analysis

Equality of variances was analyzed with Levene´s test and Kolmogorov-Smirnov test was used to analyze normal data distribution, using the SPSS version 10.0 software. Differences in 3LL-D122 tumor growth were evaluated by ANOVA and Tukey test for multiple comparisons. Data were considered significant when $P < 0.05$. All statistical analyses were two-sided, and conducted using GraphPad version 4.00 software.

Results

EGFR Expression in Lung Cell Lines

We analyzed the EGFR expression in TC-1 and 3LL-D122 cell lines. First we examined the presence of EGFR mRNA in these cells by RT-PCR. Figure 1A shows the corresponding band to cDNA of EGFR from TC-1 and 3LL-D122 cells, but not from EL4 cells used as EGFR expression negative control in all experiments. A FACS analysis using the 7A7 Mab to detect EGFR in the cell membrane confirmed that TC-1 and 3LL-D122 cells are EGFR-positive. Both cell lines showed a homogenous expression of EGFR, presenting similar expression levels (Figure 1B, Table 1).

Table 1-. Comparison of protein expression level of EGFR

	Relative EGFR expression[a]	
	Cell line	Primary tumor
TC-1	3.07	13.98
3LL-D122	2.18	15.89

[a] Protein expression levels were analyzed by FACS and calculated as the ratio between the mean fluorescence intensity of cell stained with the specific antibody and the mean fluorescence intensity of cells stained with the respective isotype-matched control antibody.

Effect of 7A7 Mab on EGFR phosphorylation

We next studied if EGFR activation could be inhibited by an anti-EGFR Mab in TC-1 and 3LL-D122 cell lines. For this, we assessed the effect of 7A7 Mab on EGFR phosphorylation in these murine cells (Figure 2). EGFR phosphorylation was induced in both cell lines by treatment with EGF while EGFR basal phosphorylation was not detected for any of them. 7A7 Mab completely inhibited EGF dependent phosphorylation of EGFR in the cell lines.

Differential Response of Murine Lung Tumors to 7A7 Antiboby Treatment

We used propidium iodide staining to determine the effect of 7A7 Mab on the viability of the murine tumor cell lines (Figure 3). This Mab markedly decreased the viability of 3LL-D122 cells. Curiously, 7A7 Mab

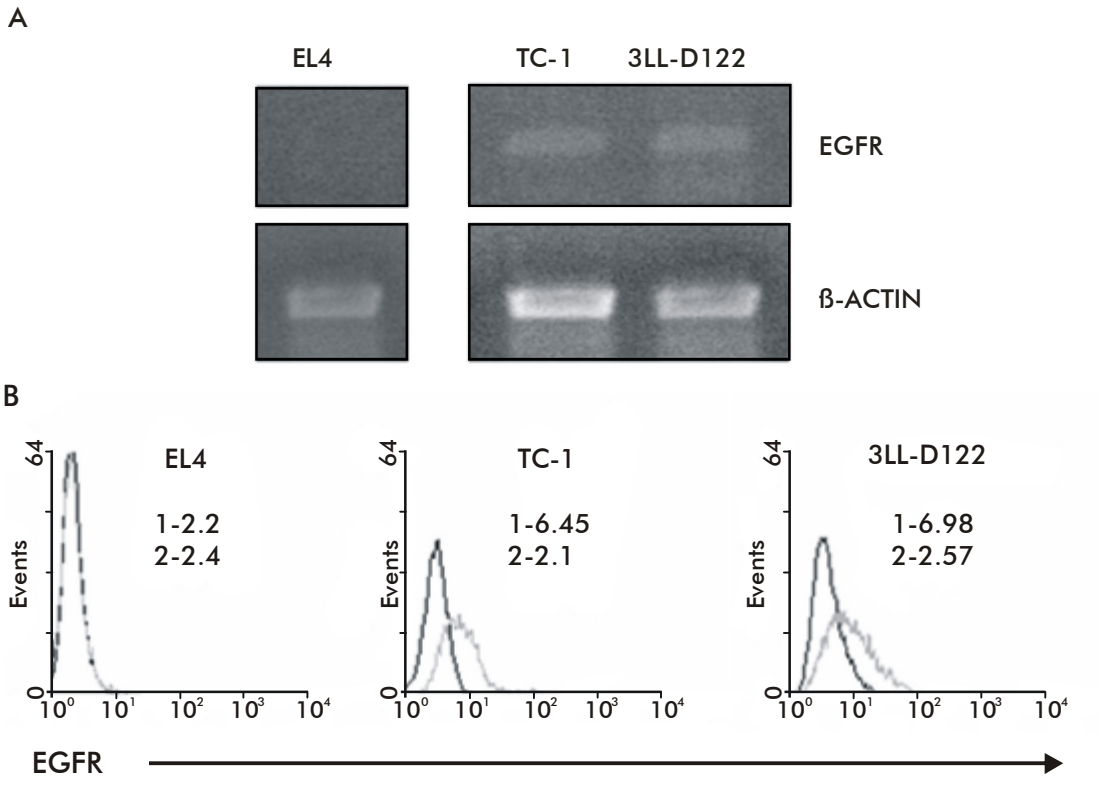

Figure 1. EGFR expression in lung cell lines. (A) Total RNA was prepared from indicated murine cells (5 x 10^6) as described in "Materials and Methods", and EGFR mRNA was determined by RT-PCR analysis. The levels of ß-actin mRNA were used as internal control. A band of EGFR was amplified. (B) Cells were stained with 1 μg/mL of 7A7 Mab (# 1, histograms with gray line) or control Mab (# 2, histograms with black line). The EGFR expression was analyzed by FACS. The numbers represent the mean fluorescence intensity from each histogram. Similar data was obtained in three separate experiments.

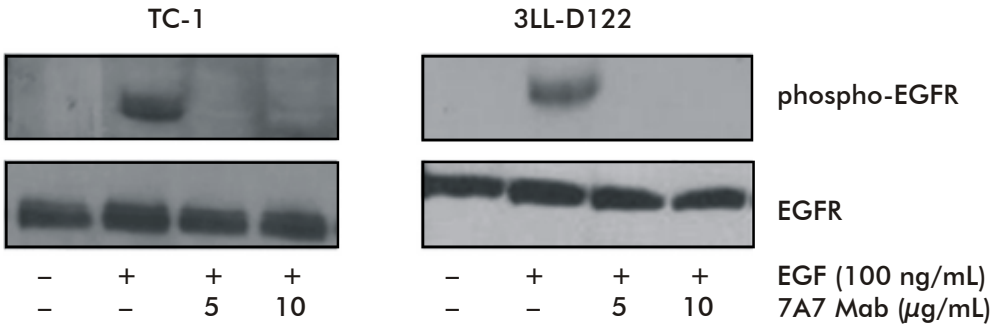

Figure 2. Inhibition of EGFR phosphorylation by 7A7 Mab in murine tumor cell lines. Serum-starved cells were treated for 1 h with the indicated concentrations of 7A7 Mab, followed by the addition of EGF. Phospho-EGFR and EGFR were determined by Western Blotting using specific antibodies. Similar results were obtained in three independent experiments.

did not have a cytotoxic effect over TC-1 cells, which express EGFR levels similar to 3LL-D122 cells. The percentages of TC-1 cells killed by the 7A7 Mab treatment were identical to those obtained for EL4 cells.

To study if the differential response of 3LL-D122 and TC-1 cell lines to *in vitro* 7A7 Mab treatment could be reproduced *in vivo*, mice were treated with this Mab with an initial dose the day before tumor challenge and later two doses per week (Figure 4A). In agreement with *in vitro* studies, the 7A7 Mab treatment had a significant effect over the growth of 3LL-D122 tumor (Tukey test, day 17: $P < 0.01$; day 20: $P < 0.001$) whereas TC-1 tumors were as insensitive to this treatment as EL4 tumors. To determine if TC-1 and 3LL-D122 tumors have similar EGFR expression levels *in vivo*, we measured the EGFR expression in primary tumors by FACS analysis (Figure 4B, Table 1). Interestingly, TC-1 primary tumors showed EGFR expression levels similar to 3LL-D122 primary tumors, in both cases with a substantial increase in EGFR expression in comparison to cell lines. Thus, the lack of response of TC-1 tumors to the *in vivo* 7A7 Mab treatment can not be explained by the absence or a decrease in EGFR expression in primary tumors.

Resistance of TC-1 Tumor to 7A7 Antibody Treatment

The anti-tumor effect of 7A7 Mab can be ascribed to a combination of non-immunologic and immunologic mechanisms, thus we explored the possible escape routes to both mechanisms in the TC-1 tumor. The down stream effects of EGFR signaling are known to be mediated, to a large extent, by the MAPK, PI3-K and STAT 3 pathways. Hence, we investigated if some of these signaling pathways were not affected by EGFR blocking, leading therefore to the TC-1 tumor resistance to 7A7 Mab treatment. As shown in Figure 5A, a complete reduction in phospho-p42/p44 MAPK, phospho-Akt and phospho-STAT 3 levels was evident after 7A7 Mab treatment for TC-1 cells. On the other hand, the 7A7 Mab is able to induce a potent CDC over 3LL-D122 cells but this *in vitro* cytotoxic effect was not detected in TC-1 cells (Figure 5B), suggesting that the TC-1 tumor could have escape mechanisms to complement this action.

Discussion

Finding that anti-EGFR agents have clinical anti-tumor activity and a low toxicity has validated the EGFR as a target for cancer therapy. However, the use of these therapies should be optimized because their activity as single-agents is modest[18-20]; the results of phase II combination studies with conventional chemotherapy are varied[21-24]; and two phase III trials combining one of these agents with chemotherapy in NSCLC showed no improvement in response compared to chemotherapy alone[25, 26].

Challenges in the development of anti-EGFR agents include clarifying whether the EGFR over expression is a marker to obtain clinical benefit from anti-EGFR

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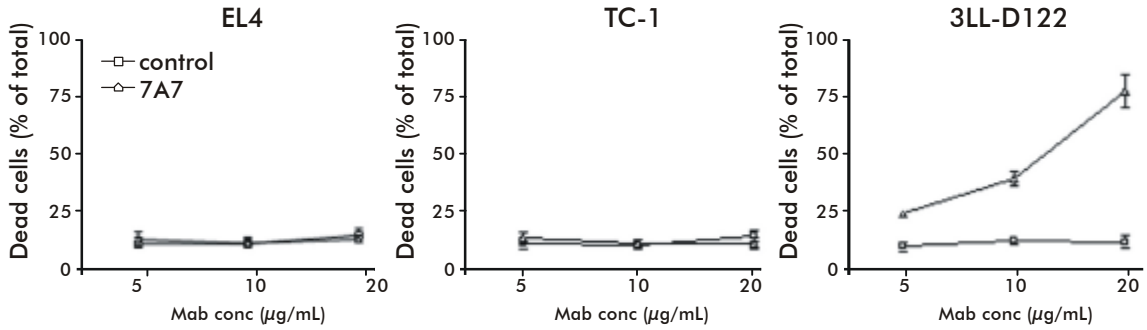

Figure 3. Response of EGFR-positive tumor cells to *in vitro* 7A7 Mab treatment. Cells were incubated with the different Mabs for 48 h and the percent of dead cells was determined by propidium iodide staining followed by FACS analysis. Each point represents the mean of triplicate wells. Error bars indicate the s.d. This experiment is representative of three independent experiments.

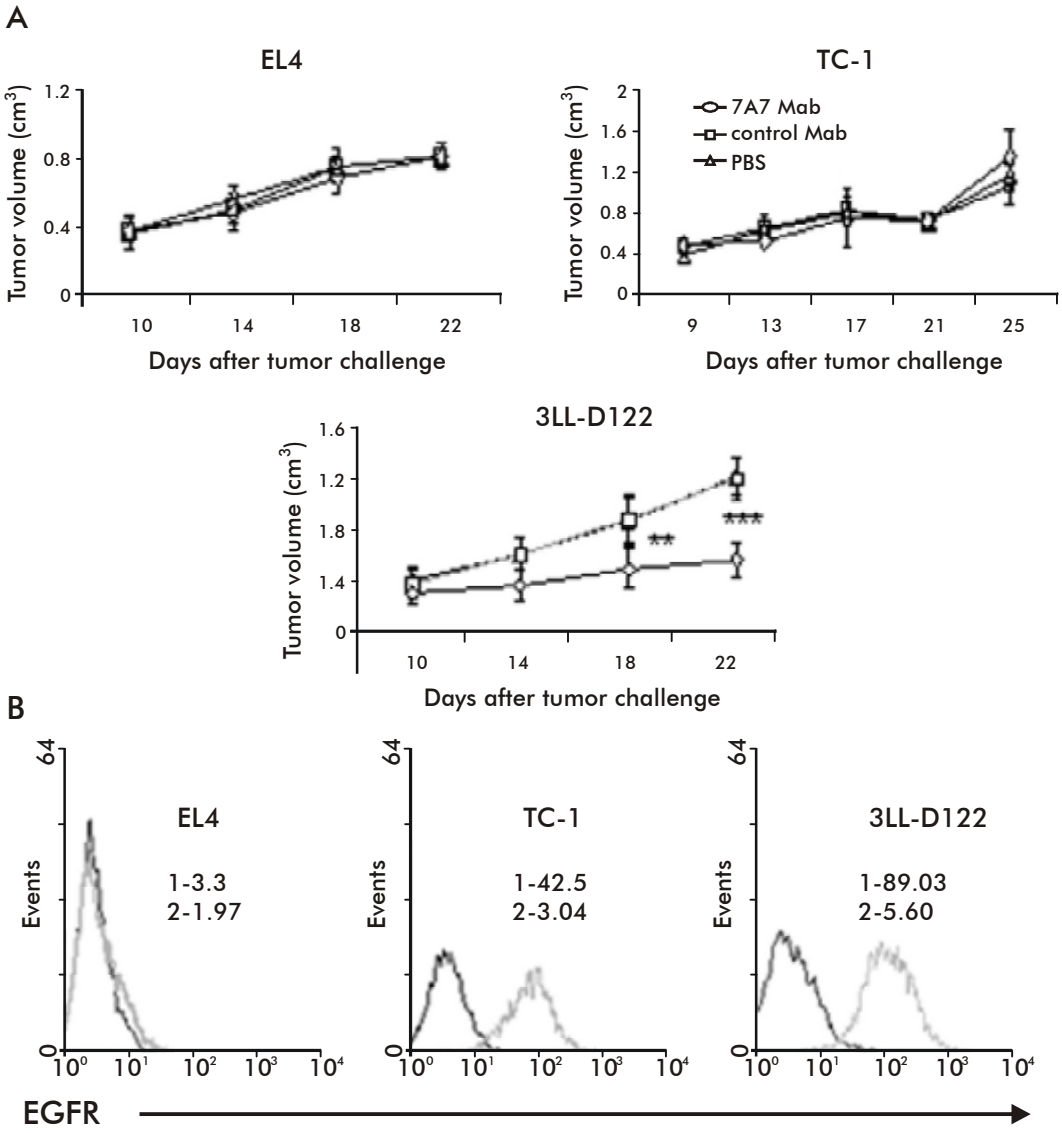

Figure 4. Sensitivity of EGFR-positive tumors to *in vivo* 7A7 Mab treatment. (A) Mice (5-8 per group) were challenged with the indicated tumor cells and treated with Mabs or PBS on the day before tumor challenge and then every 4 days until the 19. Graphs represent tumor growth in mice after tumor challenge. Each point represents the mean number of mice used. Error bars indicate the s.d. Each graph is a representative experiment of at least two experiments. For 3LL-D122 tumors, the data were analyzed according to ANOVA (**, $P < 0.01$; ***, $P < 0.001$: 7A7 Mab vs control Mab; 7A7 Mab vs PBS). (B) Cells from primary tumors were collected on day 20 after tumor challenge and stained with 1 μg/mL of 7A7 Mab (# 1, histograms with gray line) or control Mab (# 2, histograms with black line). The EGFR expression was analyzed by FACS. The numbers represent the mean fluorescence intensity from each histogram. Similar data was obtained from three different animals.

compounds. As the results of a growing number of pre-clinical studies become available, data are beginning to show the relationship between the anti-tumor efficacy of EGFR inhibition and the level of EGFR expression on tumor cells. This has been observed in tumor xenograft studies with EGF-ABX[27], where the anti-tumor effect of this Mab was only seen in tumors that express high levels of EGFR[28]. Similarly, carcinomas with low EGFR protein levels were not susceptible to EMD 72000[29] in xenotransplants derived from cancer cell lines[30]. Also, clinical data that demonstrate a correlation between gene copy number for EGFR and clinical response to cetuximab[31] and EGF-ABX in colorectal cancer have been reported[32].

Consequently, clinical trials with anti-EGFR agents are being conducted in patients with EGFR-positive tumors. However, preliminary analyses suggest that the clinical efficacy of these therapies is not related to the relative levels of EGFR expression in patients. In the phase II clinical trial of cetuximab combined with irinotecan in irinotecan-refractory colorectal cancer patients, response rates showed no correlation with EGFR expression levels[33]. Similarly, in phase II trial with NSCLC, tumor response to the tyrosine

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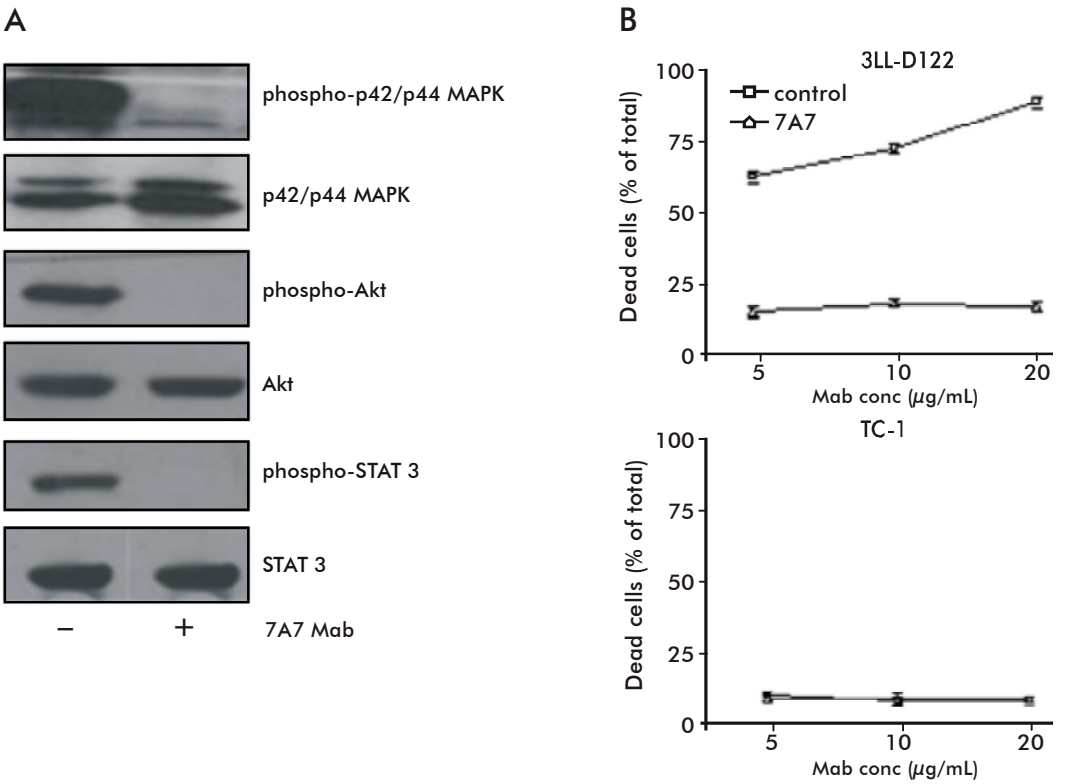

Figure 5. TC-1 tumor resistance to 7A7 Mab treatment. (A) Serum-starved 3LL-D122 and TC-1 cells were treated for 1 h with 7A7 Mab (5 μg/mL), followed by the addition of EGF. Phospho-p42/p44 MAPK, phospho-Akt, phospho-STAT 3, p44/p42 MAPK, Akt and STAT 3 were determined by Western Blotting using specific antibodies. Similar results were obtained in three independent experiments. (B) TC-1 cells were incubated with Mabs in the presence of autologous complement (at 1:5 dilution) for 48 h and the percentage of dead cells was determined by propidium iodide staining followed by FACS analysis. Each point represents the mean of triplicate wells. Error bars indicate the s.d. Similar results were obtained in three independent experiments.

inhibitor erlotinib was not associated with either a higher percentage of tumor cells positive for EGFR or more intensive EGFR staining[34].

A practical implication of these contradictory results can be that new models more closely resembling the clinical situation are needed to answer this question. In fact, effective and proven pre-clinical models do not exist today. A much more relevant pre-clinical strategy could be the selection of immunologically normal individuals in a complete autologous scenario. With this aim we studied EGFR expression and the sensitivity to anti-EGFR antibodies in murine tumors using the 7A7 Mab. This Mab was previously generated in our laboratory and it is able to recognize the extracellular domain of murine EGFR in tumor cells by different techniques, such as Western Blot, FACS and immunohistochemistry[13].

Our results with immunocompetent mice support that the 7A7 Mab therapy efficacy is not related to the relative levels of EGFR expression. TC-1 and 3LL-D122 tumors have equivalent EGFR expression levels and the activation of this receptor can be efficiently blocked by the 7A7 Mab in both, but only the 3LL-D122 tumor was sensitive to *in vitro* and *in vivo* treatment with this Mab. Results that demonstrate the sensitivity of the 3LL-D122 tumor to anti-EGFR therapy have been previously described by Sánchez *et al.* [35].

The ability of EGFR expression in cancer to predict response to anti-EGFR therapies is a subject of active research. Our results using a pre-clinical model closer to a clinical condition, confirm that factors other than high receptor number determine whether a particular tumor will respond to anti-EGFR agents. This pre-clinical evaluation strategy can be used to identify other markers with predictive value. Furthermore, taking in to consideration the vast complexity of the EGFR signaling network, more than searching for these known downstream potential markers, it could be necessary to analyze larger arrays of gene and protein expression levels. This approach would also require repeated tumor samples, which could be more feasible if the work is done with murine tumors rather than with patient tumor biopsies.

There are reports on human cell lines that have equivalent EGFR expression level with very different sensitivities to EGFR inhibitors[36, 37]. Hence, studies on possible mechanisms producing "innate" resistance to EGFR-targeting drugs have also been reported[38]. These include the presence of redundant tyrosine kinase receptors, increased angiogenesis, the constitutive activation of downstream mediators and mutations in the kinase domain of EGFR. Our results demonstrate that the resistance of the TC-1 tumor to 7A7 Mab was not mediated through overactivation of p42/p44 MAPK, Akt and STAT 3 proteins. However,

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alteration or overactivity in other EGFR signaling components must be present in the TC-1 tumor to explain its resistance to the *in vitro* treatment with 7A7 Mab as a single agent. Furthermore, the results described here suggest that the TC-1 tumor has escape mechanisms to CDC induced by 7A7 Mab. Complement activation is an important effector mechanism in the eradication of tumors. Human IgG1 and IgG3 are most efficient at complement activation, but not all IgG1 or IgG3 Mabs are able to activate the complement, possibly because antigen density in certain cases may be too low to support the formation of Fc dimmers. Whereas several anti-cancer Mabs activate the complement *in vitro*[39], the importance of CDC to efficacy *in vivo* has only been found in some[40]. The capacity of anti-human EGFR Mabs to induce CDC has not been explored. However, the isotype of anti-EGFR therapeutic antibodies and the over expression of EGFR in tumors could predict a potent complement activation by these Mabs. On the other hand, resistance to the complement-mediated killing by over the expression of complement restriction factors has been reported in human tumors[41, 42]. Thus, the expression of complement restriction factors can represent another mechanism by which tumor cells can become resistant to anti-EGFR Mab therapy. Additional studies to validate the complement restriction factors as a component of the resistance to 7A7 Mab treatment are required to support these findings. Moreover, it would be necessary to verify the relevance of this mechanism in patients resistant to anti-EGFR Mab therapy.

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