ErbBs inhibition by lapatinib blocks tumor growth in an orthotopic model of human testicular germ cell tumor

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Running title: **ErbB3 overexpression in testicular germ cell tumors**

Key words: **Lapatinib, testicular germ-cell tumors, ErbBs, ErbB3.**

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NOVELTY AND IMPACT OF THE WORK

We describe for first time overexpression of ErbB3 in Germ cell testicular tumors (GCTs). Moreover our results *in vivo* using an orthotopic model explain the complete lack of effect (refractoriness) to pure ErbB1 receptor inhibitors caused by an ErbB1-independent activation of ErbB2. In contrast, the dual inhibitor of ErbB1 and ErbB2 lapatinib is effective blocking tumor growth and could be a new alternative for treatment of those patients who are refractory to cisplatin (CDDP)-based chemotherapy.

ABSTRACT

In this work we have analyzed the expression of different members of the ErbB family in human samples of germ-cell testicular tumors (GCTs). We observed expression of ErbB1 or ErbB2 in different tumor subtypes, but we also found overexpression of ErbB3 in all GCTs tested. This pattern of expression was maintained when primary tumors were orthotopically implanted in nude mice. We have chosen a choriocarcinoma model characterized by high levels of ErbB1, but also of ErbB2 and ErbB3, to assay the *in vivo* effect of ErbB inhibitors on tumoral growth. Our results showed a complete lack of effect (refractoriness) to the pure ErbB1 receptor inhibitors cetuximab and gefitinib. While these inhibitors blocked ErbB1 phosphorylation, ErbB2 phosphorylation was not affected, suggesting an ErbB1-independent activation of this receptor. In order to confirm the importance of ErbB2 activation, animals were treated with lapatinib, a dual ErbB1 and ErbB2 inhibitor. Lapatinib treatment caused a 50% inhibition in tumor growth, an effect correlated with a blockade of both ErbB1 and ErbB2 phosphorylation levels, and of downstream signalling pathways (Akt, ERKs and Stat3). ErbB2 activation could still occur due to the formation of ErbB2/ErbB3 heterodimers, and ErbB3 activation was completely inhibited by lapatinib. Our results explain why lapatinib but not anti-ErbB1 agents might be effective for treatment of GCT patients.

INTRODUCTION

The ErbB family of tyrosine kinase receptors includes ErbB1/EGFR, ErbB2/Neu/Her-2, ErbB3 and ErbB4. Ligand binding induces homo or heterodimerization between ErbB receptors, which leads to the activation of their intrinsic tyrosine kinase activity, autophosphorylation of intracellular tyrosine residues and activation of multiple signal transduction cascades [1, 2]. ErbB2 lacks ligand-binding capacity, whereas ErbB3 is intrinsically inactive or at least, weakly active as a kinase [3]. Both of them heterodimerize with other ErbB family members to activate intracellular signalling [4].

Altered ErbB signalling has been implicated in the development of different tumor types [5, 6]. Furthermore, in some studies ErbB1 or ErbB2 aberrant expression has been associated with a poor prognosis and resistance to conventional therapies [4, 7-9]. The implication of ErbBs in tumor progression has turned these receptors into some of the most studied molecular targets in clinical oncology. There are two main classes of ErbB receptor inhibitors: monoclonal antibodies (mAbs) [10, 11] and small molecule tyrosine kinase inhibitors (TKIs) [4]. Targeted therapies against ErbBs have been successfully used in the treatment of some advanced breast, colorectal and non-small-cell lung cancers. FDA-approved drugs that inhibit ErbBs include two mAbs against ErbB1 (Cetuximab and Panitumumab), two mAbs against ErbB2 (trastuzumab or herceptin and pertuzumab); and four TKIs, two that specifically inhibit ErbB1 (Erlotinib, Gefitinib) and lapatinib that inhibits ErbB1 and ErbB2 [9].

Germ cell tumors (GCTs) of the testis are the most common solid malignant neoplasm in young adult males aged 15 to 40 years old, and represent the leading cause of cancer-related mortality and morbidity in this age group [12]. Testicular GCTs are divided into seminoma and non seminomatous germ cell tumors based on histological, biological, and clinical features. Non seminomatous GCTs include one or more of the following components: teratoma, embryonal carcinoma, teratocarcinoma, yolk sac tumor and choriocarcinoma, the last one being the most aggressive component of mixed GCTs [13, 14]. ErbB1 and 2 expression seem to be restricted to non seminomatous germ cell tumors [14-16], but the exact contribution of ErbBs in the development, differentiation and progression of these tumors is not known. Our results show that most GCTs express high levels of ErbB3. This ErbB3 expression explains

tumor resistance to pure ErbB1 inhibitors, in contrast to positive effects of multiple ErbBs inhibition caused by lapatinib.

MATERIALS AND METHODS

Chemical Compounds

Cetuximab (Erbitux®) was obtained from Merck. Gefitinib (Iressa®) was kindly provided by AstraZeneca and Lapatinib (Tyverb®) was kindly provided by GlaxoSmithKline. Both were dissolved in 0,5% carboxymethylcellulose - 0,1% Tween 80 (Sigma) solution.

Orthotopic implantation of testicular tumors

Male nu/nu Swiss mice were purchased from Harlan (Spain). For our studies several orthotopic testicular germ cell tumors models (TGTs) were used, originated from tumor patients: three embryonal carcinomas, two with heterogenous tumor histological compounds, one yolk sac and two choriocarcinomas, among them the TGT38. This last model and the surgical implantation methodology was previously described in Castillo-Avila et al [17].

Treatment schedule

Treatments started when a palpable intra-abdominal mass was detected (14 days); studies were terminated when tumors in vehicle-treated animals were judged to adversely affect their well-being. Mice were sacrificed by CO_2 inhalation and the effects of the different treatments on tumor response were evaluated by determining tumor weight and volume, where $Volume=(length)(width^2/2)$.

For the first treatment schedule five mice were treated with gefitinib, administered daily orally, for five consecutive days, at doses of 100 mg/kg. Four mice were treated with 1 mg cetuximab twice a week by intraperitoneal administration. As control group four mice received daily oral vehicle solution and intraperitoneal sterile serum at the same schedule as gefitinib and cetuximab mice. Mice were treated for 11 days.

For second treatment schedule, seven mice were treated with lapatinib 100 mg/kg administered daily orally. Nine mice were treated with vehicle oral solution with the same schedule as the lapatinib group. Mice were treated for 13 days.

None of these treatments had a significant effect on the body weight of mice, and animals appeared healthy and active throughout the duration of the study

Histological study

Representative fragments of the primary and xenografted tumors were fixed in buffered formalin, dehydrated and embedded in paraffin. Tissue sections (3-4 μm) were stained with hematoxylin-eosin for morphological analysis.

Anti-ErbB1 mouse monoclonal antibody (1:50) (ZYMED laboratories, 31G7), anti-ErbB2 mouse monoclonal antibody (1:100) (Novocastra, NCL-L-CB11) and anti-ErbB3 rabbit polyclonal antibody generated by Dr.Pandiella (1:75) [18] were used for immunohistochemical characterization. Previous to primary antibody incubation, tissue sections were permeabilized with saponin, for ErbB1 staining, and with pepsin for ErbB2 staining. Slides were incubated with the secondary antibody anti-mouse (DAKO envision+ System-HRP), and the detection was done with the DAB+ Chromogen System (DAKO). The slides were then dehydrated and mounted.

OCT-frozen tissue sections from control and treated tumors were used for immunofluorescence staining carried out to detect Ki67 positive cells and apoptotic cells by TUNEL staining as described [17].

Images of sections were obtained on an Olympus-BX60 microscope. To quantifiy Ki-67 and TUNEL staining six hotspot fields in viable tissue zones at x400 magnification were captured for each tumor. Quantification of staning areas was done using ImageJ software.

Western blotting

Western blot from samples from different treated tumors were performed as described [17].

Blots were incubated with polyclonal rabbit anti-phospho tyrosine 1173 ErbB1 antibody (Cell Signaling), polyclonal rabbit anti-ErbB1 antibody (Santa Cruz), polyclonal rabbit anti-phospho ErbB2 (Neu-sc18, Santa Cruz) antibody, polyclonal rabbit anti- ErbB2 antibody (Santa Cruz), polyclonal rabbit anti-phospho tyrosine 1289 ErbB3 antibody (Cell Signaling, Beverly, MA), polyclonal

rabbit anti-ErbB3 antibody (C-17, Santa Cruz), mouse monoclonal anti-phospho-ERK antibody (Sigma), rabbit anti-Erk1/2 total [19], rabbit polyclonal anti-phospho-Akt (Ser 473) antibody (Cell Signaling), rabbit polyclonal anti-AKT (Cell Signalling), rabbit polyclonal anti-phospho-Stat3 (Tyr705) (D3A7) antibody (Cell Signaling), monoclonal mouse anti-actin antibody (Sigma Chemical) or monoclonal mouse anti-tubulin antibody (Sigma Chemical) in 1/5 blocking solution overnight at 4°C.

Densitometry quantification was carried out using the Quantitiy One (BioRad Laboratories, INC) software.

Quantitative real-time PCR

Real-time PCR of cDNA obtained from TGT38 independent tumors and human testis was done as described [17], with the exception of hErbB1 primers, where the annealing was carried out at 61ºC. Human specific primers used are ErbB1 (5'-GTCCCATAGTTGGACAGGATG and 5'-TCATCAGGGGAAATGCTCTT), ErbB2 (5'-AGGGGTCTTGATCCAGC and 5'-GGTTGGTGTCTATCAGTGTGA), ErbB3 (5'-GCTTGCTTTTCAGCCTGGCC and 5'-TTCTCAGCATCGCCGGTCAC), ErbB4 (5'-GTACAGTGCTGACCCCACCG and 5'-AGGGTTCTCCTCCACTGGATTCA) and the house keeping gene β-actin (5'GAGGCAGCCAGGGCTTA and 5'AACTAAGGTGTGCACTTTTATTCAACT).

Immunoprecipitation

To immunoprecipitate ErbB1 or ErbB2 and ErbB1- or ErbB2-associated proteins, TGT38 tumors were mechanically disrupted and lysed using RIPA lysis buffer as described. Tumor lysates, with 750 μg of total protein, were first precleaned by incubation for 4h at 4ºC on the orbital shaker with 60 μl of protein A-Sepharose beads (GE, Healtcare) and 60 μl of protein G-Sepharose beads (GE, Healtcare). Beads were then discarded after centrifugation, and lysates were incubated with 1,6 μg of the rabbit polyclonal anti-ErbB1 antibody (Santa Cruz) or 1,6 μg of the rabbit polyclonal anti-ErbB2 antibody Neu (sc18): SC-284 (Santa Cruz) over-night at 4ºC on the orbital shaker; with the exception of the negative controls, that were incubated without antibody. Then lysates were

incubated for 6 h at 4ºC on the orbital shaker with 60μl of protein A-Sepharose beads (GE, Healtcare) and 60 μl of protein G-Sepharose beads (GE, Healtcare), previously cleaned with RIPA buffer. Immunoprecipitates were then collected by centrifugation, washed five times with RIPA buffer at 4ºC and denatured in SDS loading buffer for electrophoresis 2X. Then the immunoprecipitates were analysed by western blot as described previously.

Statistical analyses.

Statistical significance of differences in different parameters was determined using the Mann-Whitney U test. In all experiments, differences were considered statistically significant when $p < 0.05$.

RESULTS

Lack of effect of pure ErbB1 receptor inhibitors cetuximab and gefitinib on germ cell testicular growth

In order to assay the effect of inhibitors of ErbB1 on germ cell tumoral growth, first we evaluated the presence or absence of ErbB1 in different orthotopically-derived xenografted non seminoma testicular tumors in from different patients of nude mice (TGTs). We analyzed ErbB1 expression levels by western blot in samples from these TGTs, and were compared with ErbB expression in normal human testicle (Fig. 1A). We detected high expression of ErbB1 in choriocarcinoma and in tumors formed by heterogenous compounds, as compared to levels expressed in normal human testicle. Lower levels of ErbB1 were present in embryonal carcinoma and yolk sac tumors. We also immunodetected presence or absence of ErbB2, that was also expressed at different levels in most of our GCT samples and their orthotopic-derived models.

We chose choriocarcinoma TGT38, an orthotopic tumor that expressed high levels of ErbB1 receptor, for our *in vivo* studies. We evaluated the effect of cetuximab, a monoclonal antibody anti-ErbB1, or gefitinib, a small molecule inhibitor of the tyrosine kinase activity of ErbB1, on the TGT38 tumor growth. After tumor implantation we treated mice with these inhibitors, and at the end of the treatment tumor volume was measured. No effects on the choriocarcinoma tumoral growth were observed for any of the treatments (Fig. 1B). We confirmed the lack of effect of cetuximab or gefitinib by measuring tumoral cell proliferation (Ki67 positive cells), with similar values for controls compared with both treatments (Sup. Fig. S1A), and by measuring tumoral apoptosis (TUNEL assay) where we also obtained the same level of apoptotic cells in control tumors as compared to cetuximab- or gefitinib-treated tumors (Sup. Fig. S1B). These results indicated a lack of effect of pure ErbB1 inhibitors on choriocarcinoma germ cell testicular tumor growth in our orthotopic model.

Cetuximab and gefitinib block ErbB1 but not ErbB2 phosphorylation

Given the lack of effect of ErbB1 inhibitors on choriocarcinoma growth, we then attempted to confirm the inhibitory effect of cetuximab or gefitinib on ErbB1 tyrosine kinase activity. We measured by western blot ErbB1

phosphorylation in tyrosine 1173, one of the main tyrosines autophosphorylated when ErbB1 is active [5, 20]. Our results showed that this tyrosine was phosphorylated in control tumors while, as expected, its phosphorylation level was clearly decreased by cetuximab treatment (52% inhibition) or by gefitinib treatment (80% inhibition), in the absence of any effect on total levels of ErbB1 (Fig. 2A and Sup. Fig. S1C).

As is well known, ErbB1 receptor forms heterodimers with ErbB2, with cross phosphorylation and cross activation between receptors [4]. As our germ cell testicular TGT38 tumor model also express ErbB2 (Fig. 1A), so we studied the effect of these inhibitors on ErbB2 phosphorylation. As is shown in Fig. 2B and Sup. Fig. S1C, ErbB2 was phosphorylated in control tumors, but treatment with cetuximab or gefitinib did not significantly affect levels of phosphoErbB2. In order to evaluate whether activated ErbB2 was able to signal to downstream pathways important for cell proliferation and survival, we measured activity levels of three key signalling pathways: Akt (dowstream kinase activated by PI3K), ERKs and STAT3 [21]. Analysis by western blot of phosphorylated proteins in these three pathways revealed no detectable effect of cetuximab or gefitinib in the levels of phosphoAkt, phosphoERKs or phosphoSTAT3 (Fig. 2C, 2D and 2E and Sup. Fig. S2A). Our results indicated that ErbB2 was still active in presence of pure ErbB1 inhibitors and pathways downstream of ErbBs maintained their normal levels of activation.

Lapatinib, a dual ErbB1 and ErbB2 inhibitor, block choriocarcinoma tumoral growth

Taking into account the persistent ErbB2 activation, even in presence of cetuximab or gefitinib, we decided to assay a dual tyrosine kinase inhibitor for ErbB1 but also ErbB2, lapatinib [9]. We repeated the experiment performed with the pure ErbB1 inhibitors, this time treating mice with the dual ErbB1 and ErbB2 inhibitor. Lapatinib caused a 50% inhibition of tumoral volume growth (Fig 3A), an effect correlated to a significant block of cell proliferation (80% decrease of Ki67 positive cells) and a significant increase in apoptosis (3.5 fold) (Fig. 3B and 3C).

Then, the expression levels of activated ErbB1 and ErbB2 were analysed in control and lapatinib treated tumors by western blotting. As expected,

lapatinib caused a 95% decrease in levels of phospho-ErbB1 and an 80% decrease in levels of phospho-ErbB2 (Fig. 4A, 4B and 4C). Using the same samples, we also measured levels of activation of downstream signalling pathways Akt, ERKs and Stat3 (Fig. 4D, 4E and 4F and Sup. Fig. S2B). Lapatinib significantly inhibited levels of phosphoAkt, phosphoERKs and phosphoStat3, in contrast with the lack of effect of cetuximab and gefitinib.

ErbB3 is overexpressed in germ cell testicular tumors

As indicated by our results, ErbB2 activity was normal in the absence of ErbB1 activity (Fig. 2). As is well known, ErbB2 can also form heterodimers and become activated by other ErbB family receptors. Thus presence and activation of other ErbB receptors in these tumors could explain the ErbB2 maintained activity observed in our tumoral models treated with ErbB1-pure inhibitors. In order to confirm or discard this hypothesis, first we measured by RT-PCR mRNA levels for ErbB1, ErbB2, ErbB3 and ErbB4, comparing their levels in TGT38 tumors with levels in normal human testis. As shown in Fig. 5A, we detected overexpression of ErbB1 and ErbB2 mRNA levels in TGT38 as compared to normal testis. But more interesting, we detected an overexpression of ErbB3 mRNA levels, 16-fold higher than normal testicle levels. In contrast, ErbB4 mRNA was not expressed in our choriocarcinoma tumor model. ErbB1, ErbB2 and ErbB3 overexpression were confirmed by western blot comparing independent TGT38 tumor samples to independent human testicle samples (Fig. 5B).

Next, we analyzed ErbB3 and ErbB4 expression levels by western blot in samples from different TGTs orthotopically-derived models from different patients, and were compared with ErbBs expression in normal human testicle (Fig. 5C). We found that ErbB3 was overexpressed in all non seminoma testicular tumoral models analyzed including choriocarcinomas, yolk sac, embryonal carcinoma or tumors formed by heterogenous compounds, as compared to normal testicle. In contrast, ErbB4 was expressed at similar levels to normal testicle in the different TGTs analyzed except in choriocarcinomas, were its expression was even lower than in normal testicle (Fig. 5C).

In order to confirm the overexpression of ErbB3 in non seminoma testicular tumors from patients, we performed immunohistochemical analysis

comparing expression of the different ErbBs on different primary tumors obtained from patients with their orthotopically grown derived xenografted tumors in nude mice. As observed in Fig. 5D, all primary tumors analyzed, regardless of their distinct histological components (choriocarcinoma, embryonal carcinoma or yolk sac), expressed ErbB1, ErbB2 and ErbB3 at different levels, with ErbB1 more expressed in choriocarcinomas and ErbB2 in embryonal carcinoma tumors. When we analyzed the expression of ErbB3 we detected high levels of this receptor in all tumors analyzed. The expression pattern for the different ErbB members was maintained when primary tumors from patients were orthotopically implanted in nude mice.

ErbB3 activation depends on ErbB2 in germ cell testicular tumors

Taken account ErbB3 overexpression that we detected in GCTs, it is very likely then that ErbB3 would form heterodimers with ErbB2 in these tumor models. In order to show this was the case, we immunoprecipitated ErbB1 or ErbB2 from our samples and identified by western blot the members of the ErbB family that dimerize with these receptors (Fig. 6A and 6B). Our results indicate very low levels of ErbB2 or ErbB3 in immunoprecipitates of ErbB1. In contrast, while ErbB1 was present at very low levels in immunoprecipitated ErbB2, ErbB3 levels were high, suggesting that in our tumoral testicular model ErbB2 preferntially forms heterodimers with ErbB3.

Finally, we decided to analize the activation status of ErbB3 in TGT38 tumors after different treatments. First we examined phosphoErbB3 levels in control tumors and tumors treated with lapatinib, gefitinib or cetuximab. Our results indicated a complete block of ErbB3 activation with lapatinib treatment (Fig. 6C and 6D), while in tumors treated with cetuximab or gefitinib phosphoErbB3 levels were not affected (Fig. 6E and 6F). These results suggested that in our tumoral model activation of ErbB3 depends on ErbB2 activation. In all, our results confirmed the existence of ErbB2/ErbB3 heterodimers and their capacity to signal.

DISCUSSION

In this work we have shown that pure ErbB1 inhibitors are not effective in a model of choriocarcinoma testicular tumor that overexpresses ErbB1, presumably due to maintained, ErbB1-independent ErbB2 activity. On the contrary, when activity of both receptors is blocked by the use of lapatinib, tumor growth is compromised. In the absence of ErbB1, ErbB2 can maintain its activity through the formation of active heterodimers with ErbB3.

The ErbB family of receptors not only plays a key physiological role during embryonic development and for adult tissue homeostasis, but also their normal functions are altered in many types of cancer. Thus, ErbB1 and ErbB2 receptor expression and activity are increased in different tumors, including head and neck, non small cell lung cancer, colorectal, breast and bladder [4]. For this reason, different targeted drugs have been developed against these molecules and some of them are currently used in the clinic, such as cetuximab, trastuzumab, gefitinib or erlotinib. However, it is not yet clear how co-expression of different members of the ErbB family in the same tumor might affect the response to these drugs. In our study, we have used a preclinical model of human choriocarcinoma testicular germ cell tumor orthotopically grown in nude mice which expresses high levels of ErbB1, but also ErbB2 and, as we have shown, ErbB3, to analyze its response to different ErbB-targeted drugs. When we treated this tumor with the pure ErbB1-inhibitors cetuximab or gefitinib, both failed to affect tumoral growth, even when ErbB1 activation was clearly inhibited. This was likely due to the fact that blockade of ErbB1 activation with these drugs did not significantly affect the various ErbB-dowstream signalling pathways essential for cell proliferation and/or survival, such as ERKs, Stat3 or PI3K. When we inhibited both ErbB1 and ErbB2 activity by using the dual inhibitor lapatinib, the activity of the PI3K, ERKs and Stat3 pathways was reduced and tumoral growth compromised. Re-activation of these signalling pathways is the typical mechanism to generate acquired resistance to ErbB-targeted therapies [22]. There are several mechanisms that the tumor might use to re-activate these dowstream signalling components. For example, non small cell lung cancer patients acquire resistance to gefitinib or erlotinib treatment by selection of cells containing an ErbB1 mutant (T790M) [23]; some cancer cell lines

become resistant to cetuximab by overexpression of ErbB2 or the ErbB3-ligand heregulin [24]; non-small cell lung cancer cells may become resistant to gefitinib by Met receptor amplification and consequently increased ErbB3 activation [25]; ErbB2-positive breast cancer cells become resistant to trastuzumab by overexpression of ErbB1 and its ligands (EGF, TGF-α and HB-EGF) [26]; and also ErbB2-positive breast cancer cells acquire resistance to gefitinib by restoration of ErbB3 phosphorylation [27]. Similar mechanisms could also explain initial resistance to these drugs (*de novo* resistance or refractoriness), as in head and neck cancer cells, where initial resistance to gefitinib is predicted by levels of active ErbB2 and total ErbB3 [28]; or in prostate cancer cells where pure ErbB1 inhibitors also fail to block proliferation due to ErbB3 maintained activity [29]. In our case sustained ErbB2 activity explains the refractoriness of choriocarcinoma tumors to pure ErbB1 inhibitors.

ErbB2 lacks ligand-binding capacity and requires heterodimerization with other members of the family in order to be activated. In fact, due to its extracellular domain conformation, ErbB2 is the preferred partner and common subunit for the rest of ErbB members, and the more physiological dimers formed appear to be ErbB2-containing heterodimers [4, 30]. Specific inhibitors of ErbB1 also reduce ErbB2 signalling in breast cancer cells [31-33]. However, in our model pure ErbB1 inhibitors do not affect ErbB2 phosphorylation. Thus our results indicate that ErbB2 is active in our tumoral model preferentially due to the formation of ErbB2/ErbB3 heterodimers. Moreover, we have shown that ErbB3 activation is dependent on ErbB2 activity: Block of ErbB2 and ErbB1 activation by the use of lapatinib completely inhibits ErbB3 phosphorylation, while pure ErbB1 inhibitors do not affect levels of active ErbB3. In tumors where both receptors are expressed, there appears to exist a mutual regulation. Thus, inactivation of ErbB2 leads to decreased ErbB3 activation in breast cancer cells [34, 35]. On the other hand, ErbB3 is required for ErbB2 to transform breast cancer cells [36, 37]. Moreover, in breast and bladder cancers, ErbB3 expression and activity correlate with ErbB2 expression [38-40]. In the different testicular tumor models analyzed in this work there is a high correlation between ErbB2 and ErbB3 protein expression, confirming this mutual regulation also in germ cell tumors. Our results show that non seminoma testicular tumors are characterized by high levels of ErbB3 as compared to normal testicle. Despite

the fact that ErbB3 is not overexpressed or mutated in tumors, it seems to play an important role in facilitating transformation by other oncogenes (for example ErbB2) and participates in drug resistance [41]. ErbB3 and its ligand neuregulin-1 are expressed and play a role in germinal cells during development of fetal mouse gonads, where ErbB2 is also expressed [42, 43]. Thus, overexpression of ErbB3 could be a consequence of an undifferentiated or poorly differentiated state of tumors; this possibility should be further explored in the future.

Lapatinib has been approved by FDA for the treatment of ErbB2-positive metastatic breast cancer patients after trastuzumab-progression [44] and it is under clinical trials in different solid tumors. Our results in preclinical models indicate that lapatinib is also effective in choriocarcinoma testicular tumor. Sensitivity to lapatinib could be related to ErbB3 expression and the inhibition of ErbB2/ErbB3 heterodimer formation. In fact, cells with higher levels of ErbB1, as compared to ErbB2 and ErbB3, are more sensitive to pure ErbB1 inhibitors such as gefitinib, while cells with higher levels of ErbB2 and ErbB3 are more sensitive to lapatinib [45]. Moreover, it has recently been described that certain tumor cells posses an autocrine loop: cells secrete high levels of neuregulin-1, which stimulates the formation and activity of ErbB2/ErbB3 heterodimers and downstream signalling pathways in the absence of ErbB2 amplification [46, 47]. Growth of these cells can be inhibited by lapatinib [47], as is the case with our testicular tumor models. In contrast, lapatinib has no effect on lung cancer cells that show increased ErbB3 activation mediated by Met independently of ErbB2 [25]. Finally, it is clear that other signalling pathways in addition to EGFs also play a role in testicular choriocarcinoma tumors that can explain the partial effect of lapatinib on tumor growth: c-Kit, CXCR4 or PDGFs, among others, also play a role in their physiology [17, 48] and could be responsible for tumor growth in the presence of lapatinib-mediated inhibition of EGF receptors. Combination with other drugs that block some of these other pathways might be an interesting therapeutical alternative. In fact, our results indicate a synergic effect between lapatinib and anti-angiogenic drugs (data not shown).

In all, our results indicate that lapatinib could be a therapeutic alternative for the treatment of tumors where the heterodimer ErbB2/ErbB3 plays a key role, as in testicular germ cell tumors. Our data reinforces the concept that the presence of the different members of the ErbB family can predict the

effectiveness of a drug, and the analysis of ErbB1, ErbB2 and ErbB3 expression levels in patient tumor biopsies before anti-ErbB targeted treatments should be recommended.

ACKNOWLEDGEMENTS

This study was supported by research grants from the Ministerio de Ciencia y Tecnología (SAF2007-60955 and SAF2010-20859), the Spanish Ministry of Health (RTICC RD2006-0092) and Generalitat de Catalunya (2005SGR727 and 2009SGR283) to F. Viñals, the Spanish Ministry of Health (FIS PI10-0222) to A. Villanueva; and Fundació La Marató de TV3 (051430 to FV and XGM). MJ is a recipient of a pre-doctoral fellowship from IDIBELL. WCA is a recipient of a pre-doctoral fellowship from Marató de TV3.

REFERENCES

1. Takeuchi K, Ito F. EGF receptor in relation to tumor development: molecular basis of responsiveness of cancer cells to EGFR-targeting tyrosine kinase inhibitors. Febs J 2011;277:316-26.

2. Aaronson SA. Growth factors and cancer. Science 1991;254:1146-53.

3. Shi F, Telesco SE, Liu Y, Radhakrishnan R, Lemmon MA. ErbB3/HER3 intracellular domain is competent to bind ATP and catalyze autophosphorylation. Proc Natl Acad Sci U S A 2010;107:7692-7.

4. Hynes NE, Lane HA. ERBB receptors and cancer: the complexity of targeted inhibitors. Nat Rev Cancer 2005;5:341-54.

5. Olayioye MA, Neve RM, Lane HA, Hynes NE. The ErbB signaling network: receptor heterodimerization in development and cancer. Embo J 2000;19:3159-67.

6. Petit AM, Rak J, Hung MC, Rockwell P, Goldstein N, Fendly B, Kerbel RS. Neutralizing antibodies against epidermal growth factor and ErbB-2/neu receptor tyrosine kinases down-regulate vascular endothelial growth factor production by tumor cells in vitro and in vivo: angiogenic implications for signal transduction therapy of solid tumors. Am J Pathol 1997;151:1523-30.

7. Harari PM. Epidermal growth factor receptor inhibition strategies in oncology. Endocr Relat Cancer 2004;11:689-708.

8. Krause DS, Van Etten RA. Tyrosine kinases as targets for cancer therapy. N Engl J Med 2005;353:172-87.

9. Wheeler DL, Dunn EF, Harari PM. Understanding resistance to EGFR inhibitors-impact on future treatment strategies. Nat Rev Clin Oncol 2011;7:493-507.

10. Prewett M, Rockwell P, Rockwell RF, Giorgio NA, Mendelsohn J, Scher HI, Goldstein NI. The biologic effects of C225, a chimeric monoclonal antibody to the EGFR, on human prostate carcinoma. J Immunother Emphasis Tumor Immunol 1996;19:419-27.

11. Mendelsohn J. Epidermal growth factor receptor inhibition by a monoclonal antibody as anticancer therapy. Clin Cancer Res 1997;3:2703-7.

12. Winter C, Albers P. Testicular germ cell tumors: pathogenesis, diagnosis and treatment. Nat Rev Endocrinol 2011;7:43-53.

13. Ulbright TM. Germ cell tumors of the gonads: a selective review emphasizing problems in differential diagnosis, newly appreciated, and controversial issues. Mod Pathol 2005;18 Suppl 2:S61-79.

14. Moroni M, Veronese S, Schiavo R, Carminati O, Sorensen BS, Gambacorta M, Siena S. Epidermal growth factor receptor expression and activation in nonseminomatous germ cell tumors. Clin Cancer Res 2001;7:2770-5.

15. Hechelhammer L, Storkel S, Odermatt B, Heitz PU, Jochum W. Epidermal growth factor receptor is a marker for syncytiotrophoblastic cells in testicular germ cell tumors. Virchows Arch 2003;443:28-31.

16. Madani A, Kemmer K, Sweeney C, Corless C, Ulbright T, Heinrich M, Einhorn L. Expression of KIT and epidermal growth factor receptor in chemotherapy refractory non-seminomatous germ-cell tumors. Ann Oncol 2003;14:873-80.

17. Castillo-Avila W, Piulats JM, Garcia Del Muro X, Vidal A, Condom E, Casanovas O, Mora J, Germa JR, Capella G, Villanueva A, Viñals F. Sunitinib inhibits tumor growth and synergizes with cisplatin in orthotopic models of cisplatin-sensitive and cisplatin-resistant human testicular germ cell tumors. Clin Cancer Res 2009;15:3384-95.

18. Sanchez-Martin M, Pandiella A. Differential action of small molecule HER kinase inhibitors on receptor heterodimerization: therapeutic implications. Int J Cancer 2012;131:244-52.

19. McKenzie FR, Pouyssegur J. cAMP-mediated growth inhibition in fibroblasts is not mediated via mitogen-activated protein (MAP) kinase (ERK) inhibition. cAMP-dependent protein kinase induces a temporal shift in growth factor-stimulated MAP kinases. J Biol Chem 1996;271:13476-83.

20. Batzer AG, Rotin D, Urena JM, Skolnik EY, Schlessinger J. Hierarchy of binding sites for Grb2 and Shc on the epidermal growth factor receptor. Mol Cell Biol 1994;14:5192-201.

21. Song JI, Grandis JR. STAT signaling in head and neck cancer. Oncogene 2000;19:2489-95.

22. Vlacich G, Coffey RJ. Resistance to EGFR-Targeted Therapy: A Family Affair. Cancer Cell 2011;20:423-5.

23. Kobayashi S, Boggon TJ, Dayaram T, Janne PA, Kocher O, Meyerson M, Johnson BE, Eck MJ, Tenen DG, Halmos B. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. N Engl J Med 2005;352:786-92.

24. Yonesaka K, Zejnullahu K, Okamoto I, Satoh T, Cappuzzo F, Souglakos J, Ercan D, Rogers A, Roncalli M, Takeda M, Fujisaka Y, Philips J, et al. Activation of ERBB2 signaling causes resistance to the EGFR-directed therapeutic antibody cetuximab. Sci Transl Med 2011;3:99ra86.

25. Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO, Lindeman N, Gale CM, Zhao X, Christensen J, Kosaka T, Holmes AJ, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. Science 2007;316:1039-43.

26. Ritter CA, Perez-Torres M, Rinehart C, Guix M, Dugger T, Engelman JA, Arteaga CL. Human breast cancer cells selected for resistance to trastuzumab in vivo overexpress epidermal growth factor receptor and ErbB ligands and remain dependent on the ErbB receptor network. Clin Cancer Res 2007;13:4909-19.

27. Sergina NV, Rausch M, Wang D, Blair J, Hann B, Shokat KM, Moasser MM. Escape from HER-family tyrosine kinase inhibitor therapy by the kinase-inactive HER3. Nature 2007;445:437-41.

28. Erjala K, Sundvall M, Junttila TT, Zhang N, Savisalo M, Mali P, Kulmala J, Pulkkinen J, Grenman R, Elenius K. Signaling via ErbB2 and ErbB3 associates with resistance and epidermal growth factor receptor (EGFR) amplification with sensitivity to EGFR inhibitor gefitinib in head and neck squamous cell carcinoma cells. Clin Cancer Res 2006;12:4103-11.

29. Chen L, Mooso BA, Jathal MK, Madhav A, Johnson SD, van Spyk E, Mikhailova M, Zierenberg-Ripoll A, Xue L, Vinall RL, Devere White RW, Ghosh PM. Dual EGFR/HER2 Inhibition Sensitizes Prostate Cancer Cells to Androgen Withdrawal by Suppressing ErbB3. Clin Cancer Res 2011;17:6218-28.

30. Graus-Porta D, Beerli RR, Daly JM, Hynes NE. ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. Embo J 1997;16:1647-55.

31. Lenferink AE, Simpson JF, Shawver LK, Coffey RJ, Forbes JT, Arteaga CL. Blockade of the epidermal growth factor receptor tyrosine kinase suppresses tumorigenesis in MMTV/Neu + MMTV/TGF-alpha bigenic mice. Proc Natl Acad Sci U S A 2000;97:9609-14.

32. Moasser MM, Basso A, Averbuch SD, Rosen N. The tyrosine kinase inhibitor ZD1839 ("Iressa") inhibits HER2-driven signaling and suppresses the growth of HER2-overexpressing tumor cells. Cancer Res 2001;61:7184-8.

33. Moulder SL, Yakes FM, Muthuswamy SK, Bianco R, Simpson JF, Arteaga CL. Epidermal growth factor receptor (HER1) tyrosine kinase inhibitor ZD1839 (Iressa) inhibits HER2/neu (erbB2)-overexpressing breast cancer cells in vitro and in vivo. Cancer Res 2001;61:8887-95.

34. Neve RM, Sutterluty H, Pullen N, Lane HA, Daly JM, Krek W, Hynes NE. Effects of oncogenic ErbB2 on G1 cell cycle regulators in breast tumour cells. Oncogene 2000;19:1647-56.

35. Basso AD, Solit DB, Munster PN, Rosen N. Ansamycin antibiotics inhibit Akt activation and cyclin D expression in breast cancer cells that overexpress HER2. Oncogene 2002;21:1159-66.

36. Holbro T, Beerli RR, Maurer F, Koziczak M, Barbas CF, 3rd, Hynes NE. The ErbB2/ErbB3 heterodimer functions as an oncogenic unit: ErbB2 requires ErbB3 to drive breast tumor cell proliferation. Proc Natl Acad Sci U S A 2003;100:8933-8.

37. Lee-Hoeflich ST, Crocker L, Yao E, Pham T, Munroe X, Hoeflich KP, Sliwkowski MX, Stern HM. A central role for HER3 in HER2-amplified breast cancer: implications for targeted therapy. Cancer Res 2008;68:5878-87.

38. Naidu R, Yadav M, Nair S, Kutty MK. Expression of c-erbB3 protein in primary breast carcinomas. Br J Cancer 1998;78:1385-90.

39. Chow NH, Chan SH, Tzai TS, Ho CL, Liu HS. Expression profiles of ErbB family receptors and prognosis in primary transitional cell carcinoma of the urinary bladder. Clin Cancer Res 2001;7:1957-62.

40. Alimandi M, Romano A, Curia MC, Muraro R, Fedi P, Aaronson SA, Di Fiore PP, Kraus MH. Cooperative signaling of ErbB3 and ErbB2 in neoplastic transformation and human mammary carcinomas. Oncogene 1995;10:1813-21.

41. Campbell MR, Amin D, Moasser MM. HER3 comes of age: new insights into its functions and role in signaling, tumor biology, and cancer therapy. Clin Cancer Res 2010;16:1373-83.

42. Toyoda-Ohno H, Obinata M, Matsui Y. Members of the ErbB receptor tyrosine kinases are involved in germ cell development in fetal mouse gonads. Dev Biol 1999;215:399-406.

43. Zhang J, Eto K, Honmyou A, Nakao K, Kiyonari H, Abe S. Neuregulins are essential for spermatogonial proliferation and meiotic initiation in neonatal mouse testis. Development 2011;138:3159-68.

44. Arteaga CL, Sliwkowski MX, Osborne CK, Perez EA, Puglisi F, Gianni L. Treatment of HER2-positive breast cancer: current status and future perspectives. Nat Rev Clin Oncol 2011; 29:16-32.

45. Hickinson DM, Klinowska T, Speake G, Vincent J, Trigwell C, Anderton J, Beck S, Marshall G, Davenport S, Callis R, Mills E, Grosios K, et al. AZD8931, an equipotent, reversible inhibitor of signaling by epidermal growth factor receptor, ERBB2 (HER2), and ERBB3: a unique agent for simultaneous ERBB receptor blockade in cancer. Clin Cancer Res 2010;16:1159-69.

46. Sheng Q, Liu X, Fleming E, Yuan K, Piao H, Chen J, Moustafa Z, Thomas RK, Greulich H, Schinzel A, Zaghlul S, Batt D, et al. An activated ErbB3/NRG1 autocrine loop supports in vivo proliferation in ovarian cancer cells. Cancer Cell 2010;17:298-310.

47. Wilson TR, Lee DY, Berry L, Shames DS, Settleman J. Neuregulin-1-mediated autocrine signaling underlies sensitivity to HER2 kinase inhibitors in a subset of human cancers. Cancer Cell 2011;20:158-72.

48. Gilbert D, Rapley E, Shipley J. Testicular germ cell tumours: predisposition genes and the male germ cell niche. Nat Rev Cancer 2011;11:278-88.

FIGURE LEGENDS

Fig. 1: Lack of effect of pure ErbB1 receptor inhibitors cetuximab and gefitinib in a model of choriocarcinoma testicular germ cell tumor.

A) Expression of ErbB1 and ErbB2 receptors and tubulin, as loading control, was analysed by western blotting of normal human testis (lane 1) and in 3 independent embryonal carcinomas (lanes 2, 3 and 4), 1 yolk sac (lane 5), 2 independent mixed heterogenous component tumors (lanes 6 and 7) and 2 independent choriocarcinoma (lanes 8 and 9) testicular tumor xenograft models.

B) Mice orthotopically implanted with the TGT38 choriocarcinoma were treated with vehicle, cetuximab or gefitinib as described in materials and methods. Final tumor volume was analysed.

Fig. 2: ErbB1 inhibitors reduce ErbB1 but not ErbB2 phosphorylation levels.

Densitometric quantification of A) phosphorylated ErbB1 relative to total ErbB1 and B) phosphorylated ErbB2 relative to total ErbB2 are shown as the mean ± SD of four control tumors, four cetuximab-treated tumors and five gefinitib-treated tumors, represented as arbitrary units relative to control group mean. $*$, $p < 0.05$.

Densitometric quantifications of C) phosphorylated AKT relative to total AKT, D) phosphorylated Erk1/2 relative to total Erk1/2 and E) phosphorylated Stat3 relative to actin are shown. Results are the mean ± SD of four control tumors, four cetuximab-treated tumors and five gefitinib-treated tumors relative to control group mean.

Fig. 3: The dual ErbB1 and ErbB2 inhibitor lapatinib inhibits tumor growth in a model of choriocarcinoma testicular germ cell tumor.

A) Mice bearing the orhotopically implanted TGT38 tumor were treated with vehicle or lapatinib, as described in materials and methods, and tumor volume was analysed.

B and C) Sections from control and treated tumors were stained for the proliferation marker Ki67 and TUNEL, an apoptotic staining assay. B) Percentage of tumor Ki67 positive cells is expressed relative to control group.

Results are the mean ± SD of four control and three lapatinib treated tumors. C) Results are expressed as the percentage of TUNEL staining-positive nuclei relative to the control group, and are the mean ± SD of three control and three lapatinib treated tumors, ∗, $p < 0.05$.

Fig. 4: Lapatinib blocks ErbB1 and ErbB2 phosphorylation and dowstream signalling pathways.

A) Expression of phosphoErbB1 (pErbB1), total ErbB1, phosphoErbB2 (pErbB2), total ErbB2 and actin were analysed by western blot in lapatinib-treated and control tumors. A representative blot showing results obtained for four independent control tumors and five independent lapatinib-treated tumors is shown.

Densitometric quantifications of B) phosphorylated ErbB1 relative to total ErbB1, C) phosphorylated ErbB2 relative to total ErbB2, D) phosphorylated AKT relative to total AKT, E) phosphorylated Erk1/2 relative to total Erk1/2, and F) phosphorylated Stat3 relative to actin are shown. Results are the mean ± SD of four control tumors and six lapatinib-treated tumors, and are represented as arbitrary units relative to the control group. ∗, $p < 0.05$; ∗∗, $p<0.01$.

Fig. 5: ErbB3 is overexpressed in patient testicular tumoral samples and in their derived orthotopic models.

A) mRNA levels of human ErbB1, ErbB2, ErbB3 and ErbB4 were analyzed by quantitative real-time PCR. Results are expressed as the mean ± SD of mRNA expression in seven TGT38 tumors relative to mRNA expression levels in four normal human testis.

B) The protein expression of ErbB1, ErbB2, ErbB3 and tubulin, as loading control, was analyzed by western blotting in three independent human testicular samples and four independent TGT38 tumor lysates.

C) Expression of ErbB3 and ErbB4 receptors and tubulin were analysed by western blotting in normal human testis (lane 1), in 3 independent embryonal carcinomas (lanes 2, 3 and 4), 1 yolk sac (lane 5), 2 independent mixed heterogenous component tumors (lanes 6 and 7) and 2 independent choriocarcinoma (lanes 8 and 9) testicular tumor xenograft models.

D) Histologic and immunohistochemical characterization of primary tumors and their respective orthotopically implanted xenografts in nude mice (TGT: TGT12 for embryonal carcinoma, TGT1 for yolk sac and TGT38 for choriocarcinoma): (A, E, I, M, Q, U) Hematoxilin-eosin staining; (B, F, J, N, R, V) ErbB1 immunostaining; (C, G, K, O, S, W) ErbB2 immunostaining; (D, H, L, P, T, X) ErbB3 immunostaining. (400x; bar: 100μm).

Fig. 6: ErbB3 forms heterodimers with ErbB2 in TGT38 tumors and ErbB3 activity is blocked by lapatinib but not by ErbB1 pure inhibitors.

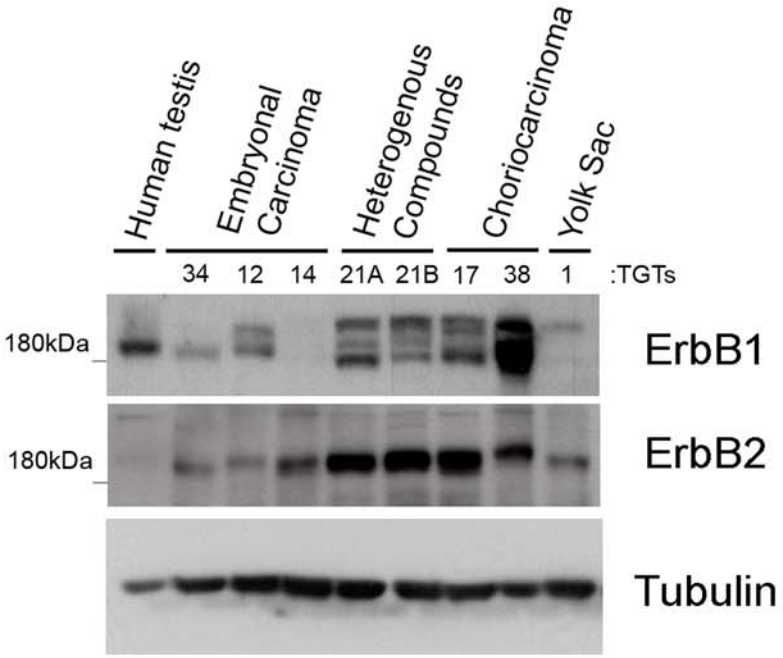
A and B) ErbB1, ErbB2 and ErbB3 detection in ErbB1 (A) and ErbB2 (B) immunoprecipitates from TGT38 tumor lysates was carried out by western blot; proteins were also analyzed in total tumor lysates (input). While low levels of ErbB3 were detected in ErbB1-immunoprecipitates, high ErbB3 levels were detected in the ErbB2-immunoprecipitates; the presence of ErbB1 or ErbB2 in the correspondent precipitates demonstrates the efficacy of the immunoprecipitation protocol.

C and D) PhosphoErbB3, total ErbB3 and actin expression were analyzed by Western blotting in independent tumors from the treatments with C) vehicle and lapatinib and D) vehicle, cetuximab and gefitib. Representative blots shows the results obtained.

E and F) Densitometric quantifications of phosphorylated ErbB3 relative to total ErbB3 in tumors from both treatments are represented as arbitrary units relative to control group. Results are the mean ± SD of E) four control tumors and six lapatinib-treated tumors and F) four control tumors, four cetuximab treated-tumors and five gefitinib-tretated tumors. ∗∗ $p<0.01$.

Fig. 1

A


Human testis
Embryonal Carcinoma
Heterogenous Compounds
Choriocarcinoma
Yolk Sac
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180kDa
ErbB1
180kDa
ErbB2
Tubulin

B

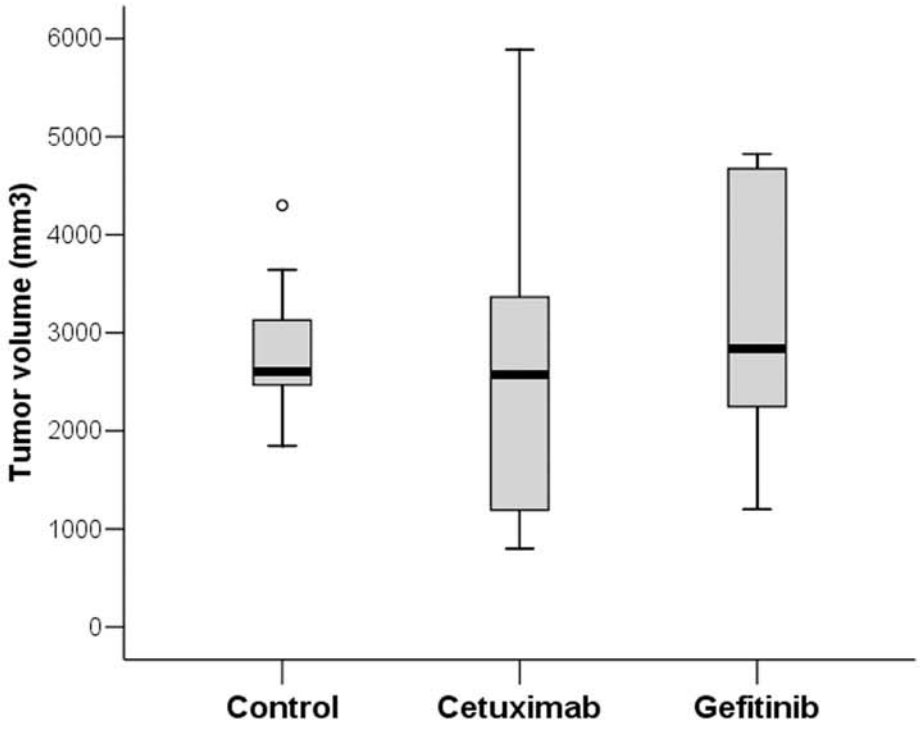
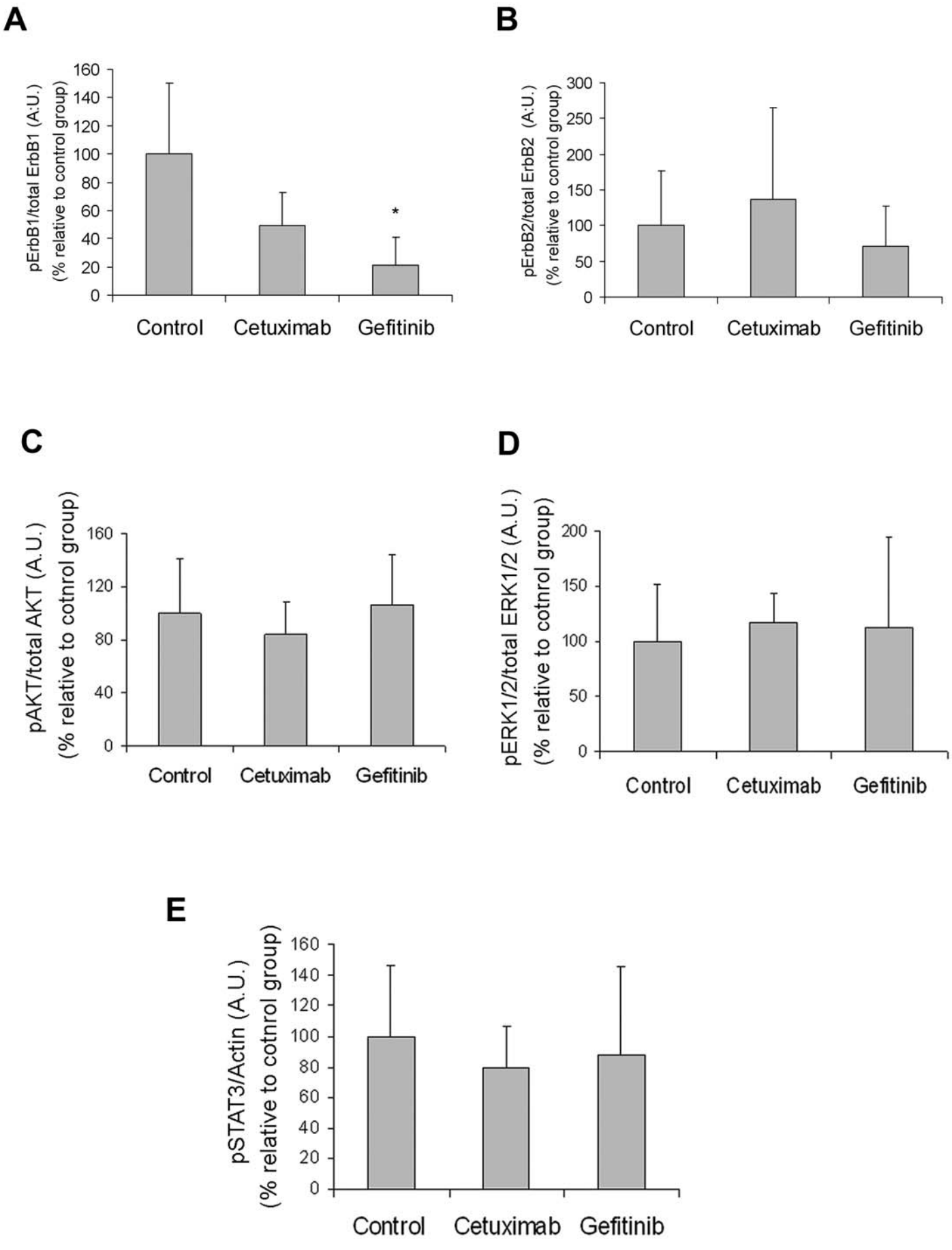

Tumor volume (mm3)
6000
5000
4000
3000
2000
1000
0
Control
Cetuximab
Gefitinib

Fig. 2


A
pErbB1/total ErbB1 (A:U.)
(% relative to control group)
160
140
120
100
80
60
40
20
0
*
Control
Cetuximab
Gefitinib
B
pErbB2/total ErbB2 (A:U.)
(% relative to control group)
300
250
200
150
100
50
0
Control
Cetuximab
Gefitinib
C
pAKT/total AKT (A.U.)
(% relative to cotnrol group)
160
120
80
40
0
Control
Cetuximab
Gefitinib
D
pERK1/2/total ERK1/2 (A.U.)
(% relative to cotnrol group)
200
150
100
50
0
Control
Cetuximab
Gefitinib
E
pSTAT3/Actin (A.U.)
(% relative to cotnrol group)
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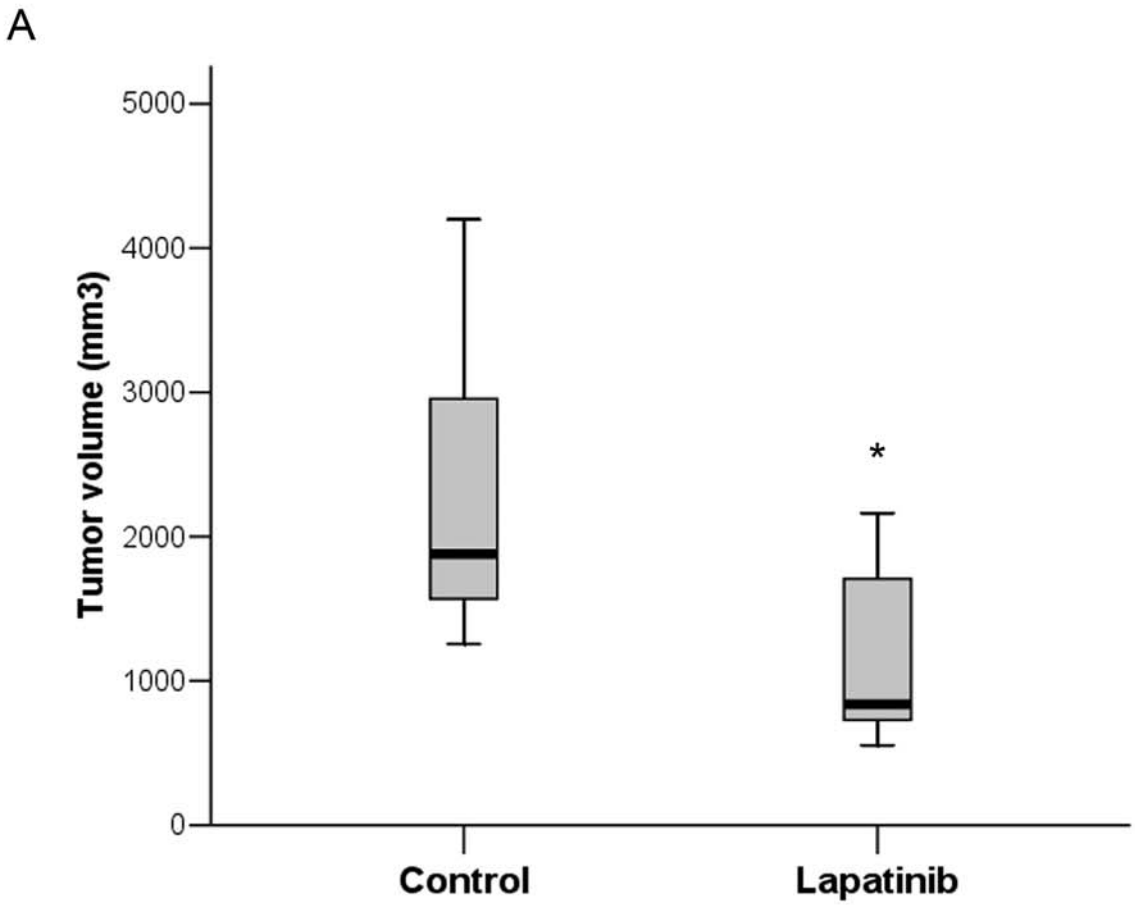
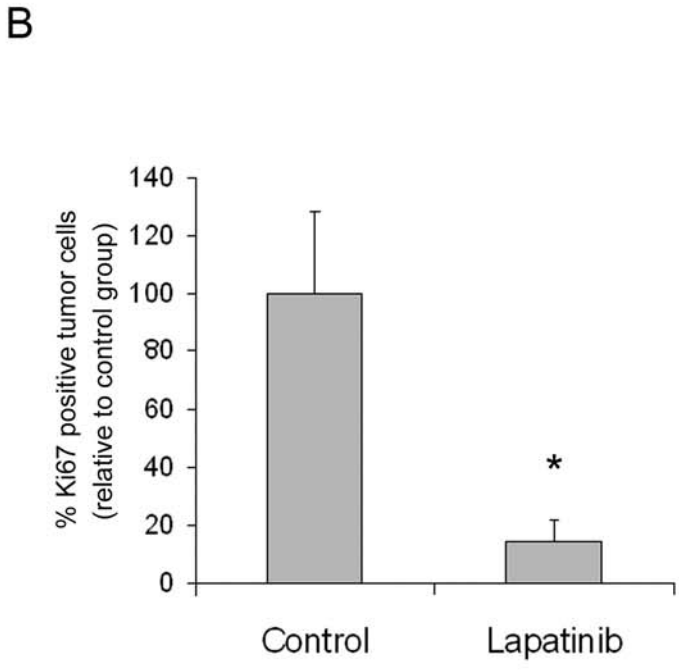
140
120
100
80
60
40
20
0
Control
Cetuximab
Gefitinib

Fig. 3


A
5000
4000
3000
2000
1000
0
Tumor volume (mm3)
*
Control
Lapatinib


B
140
120
100
80
60
40
20
0
% Ki67 positive tumor cells
(relative to control group)
*
Control
Lapatinib

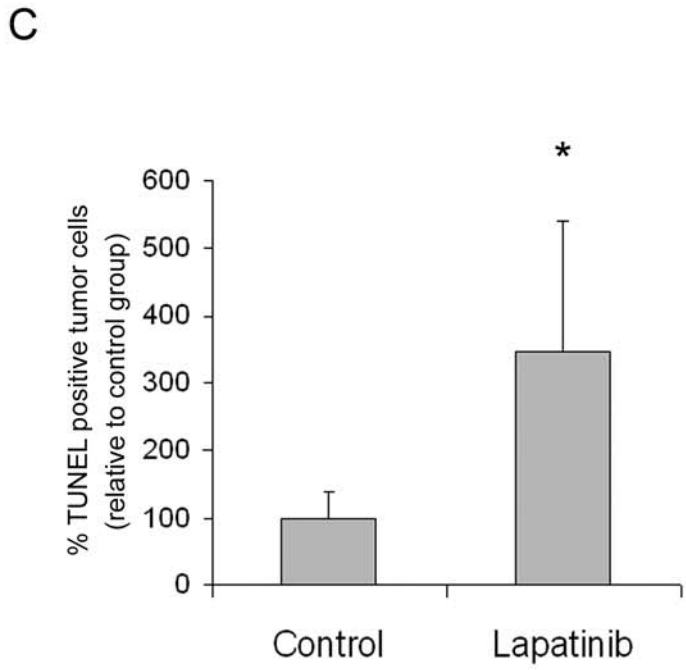

C
600
500
400
300
200
100
0
% TUNEL positive tumor cells
(relative to control group)
*
Control
Lapatinib

Fig. 4

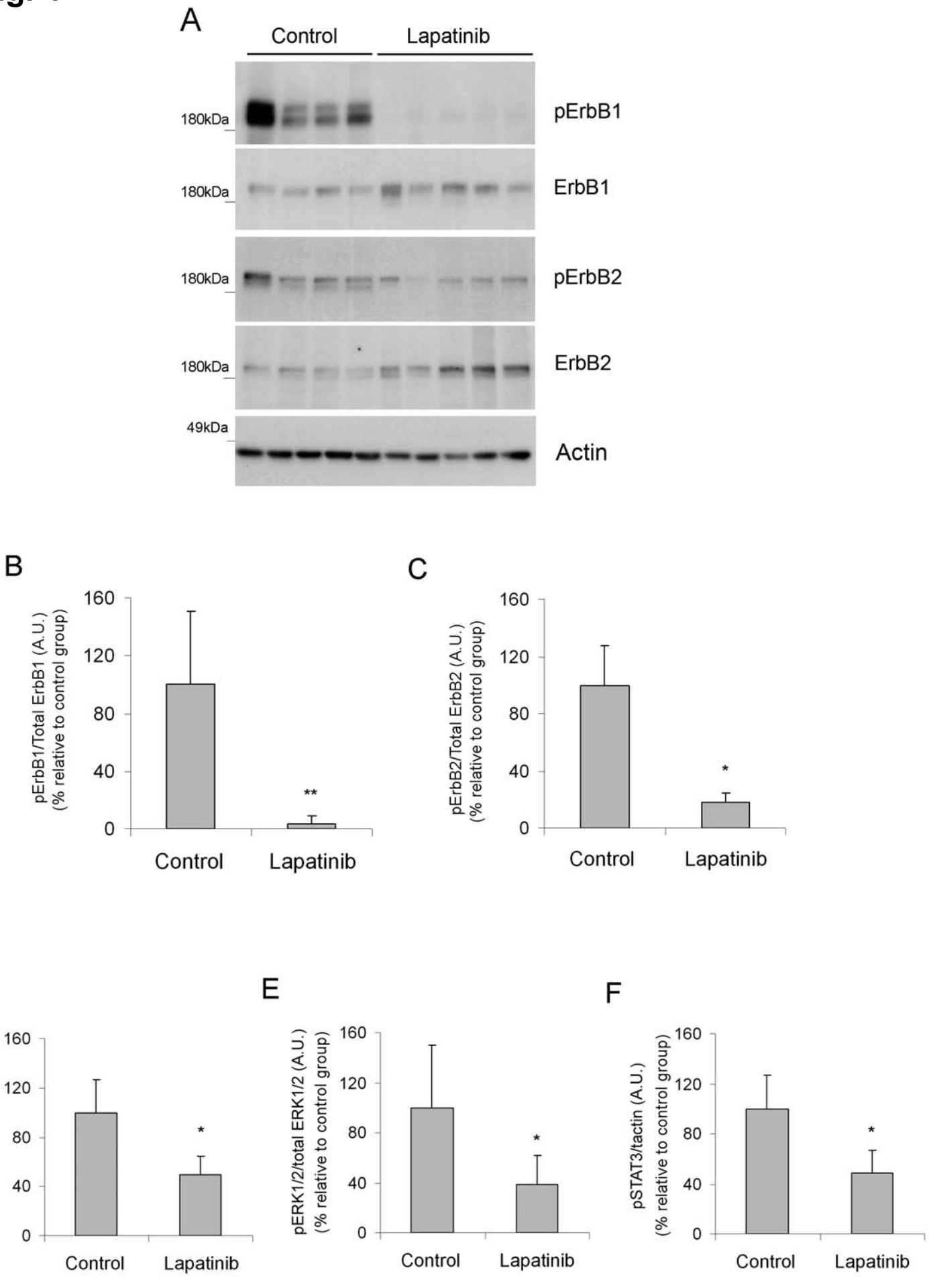
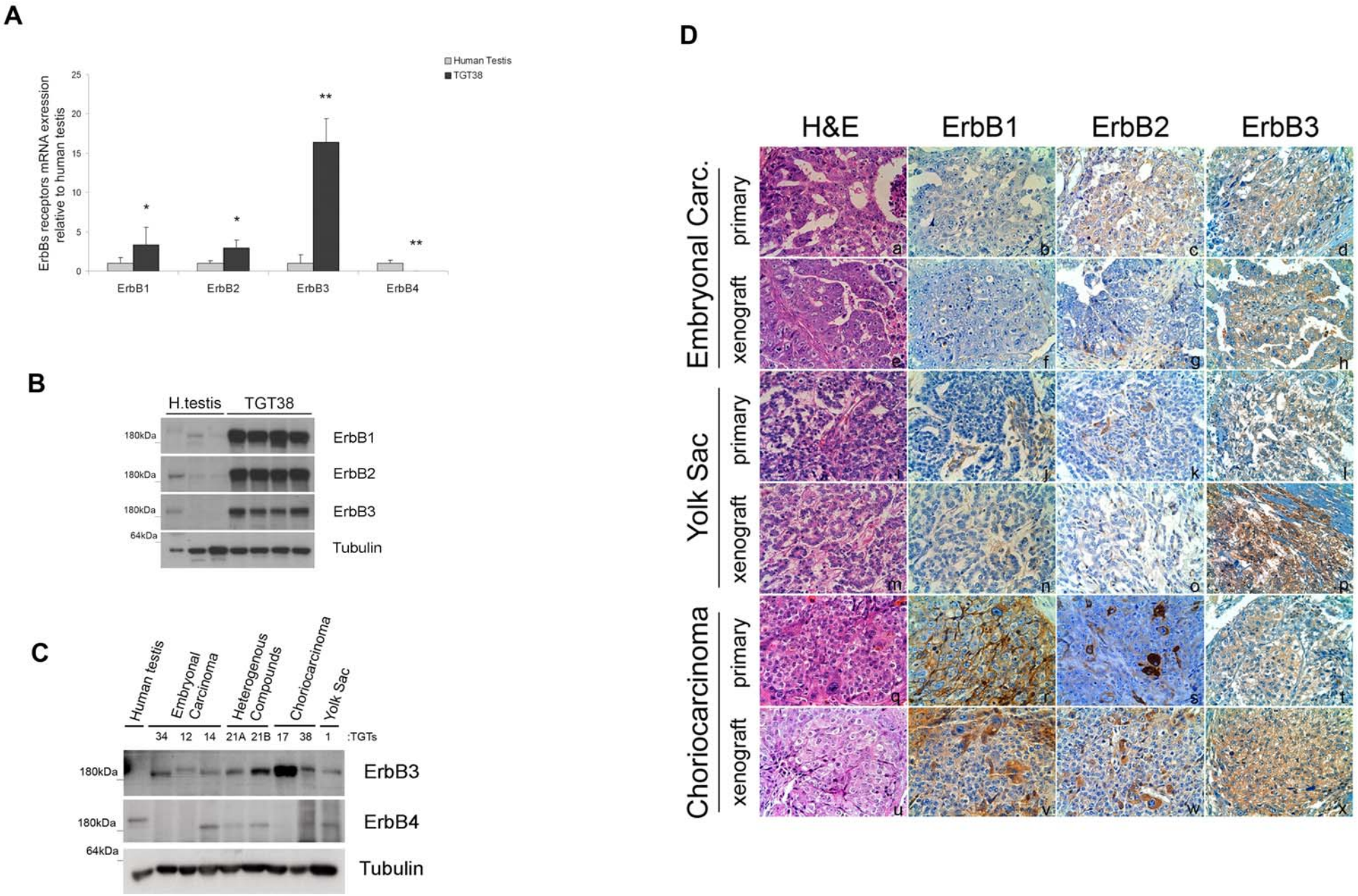

A
Control
Lapatinib
180kDa
pErbB1
180kDa
ErbB1
180kDa
pErbB2
180kDa
ErbB2
49kDa
Actin
B
pErbB1/Total ErbB1 (A.U.)
(% relative to control group)
160
120
80
40
0
**
Control
Lapatinib
C
pErbB2/Total ErbB2 (A.U.)
(% relative to control group)
160
120
80
40
0
*
Control
Lapatinib
D
pAKT/total AKT (A.U.)
(% relative to control group)
160
120
80
40
0
*
Control
Lapatinib
E
pERK1/2/total ERK1/2 (A.U.)
(% relative to control group)
160
120
80
40
0
*
Control
Lapatinib
F
pSTAT3/tactin (A.U.)
(% relative to control group)
160
120
80
40
0
*
Control
Lapatinib

Fig 5


A
Human Testis
TGT38
ErbBs receptors mRNA exression relative to human testis
25
20
15
10
5
0
*
*
**
**
ErbB1
ErbB2
ErbB3
ErbB4
B
H.testis
TGT38
180kDa
180kDa
180kDa
64kDa
ErbB1
ErbB2
ErbB3
Tubulin
C
Human testis
Embryonal Carcinoma
Heterogenous Compounds
Choriocarcinoma
Yolk Sac
34
12
14
21A
21B
17
38
1
:TGTs
180kDa
180kDa
64kDa
ErbB3
ErbB4
Tubulin
D
H&E
ErbB1
ErbB2
ErbB3
Embryonal Carc.
Yolk Sac
Choriocarcinoma
primary
xenograft
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
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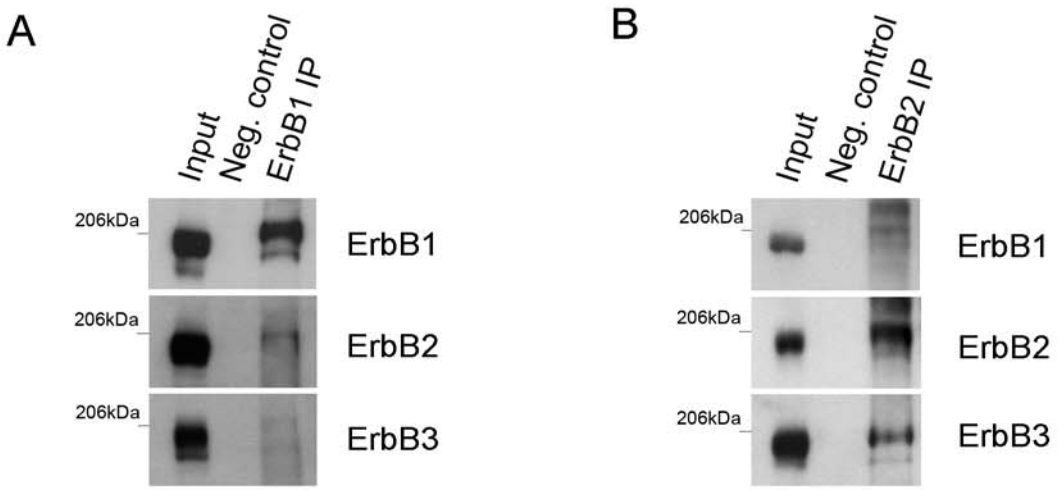
q
r
s
t
u
v
w
x

Fig. 6


A
Input
Neg. control
ErbB1 IP
206kDa
ErbB1
206kDa
ErbB2
206kDa
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ErbB2 IP
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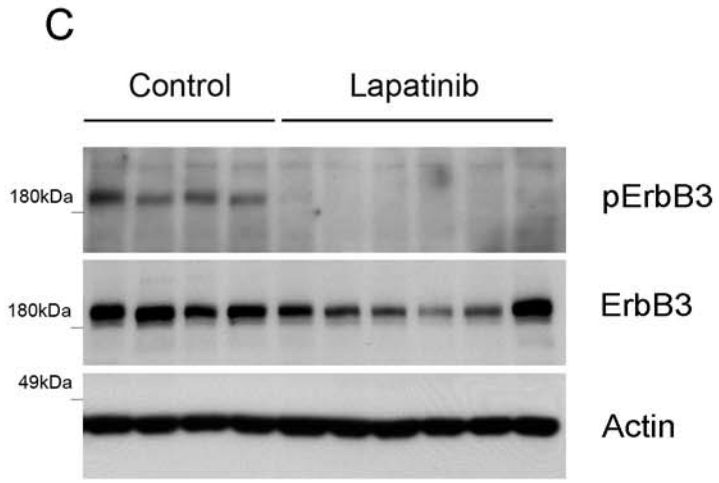
ErbB1
206kDa
ErbB2
206kDa
ErbB3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Lapatinib
180kDa
pErbB3
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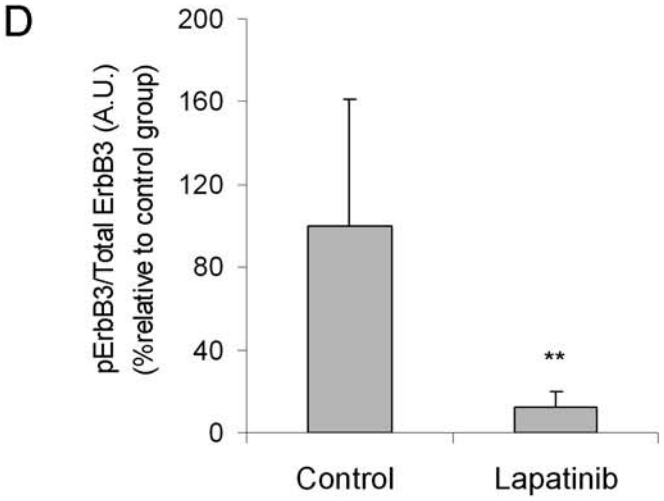
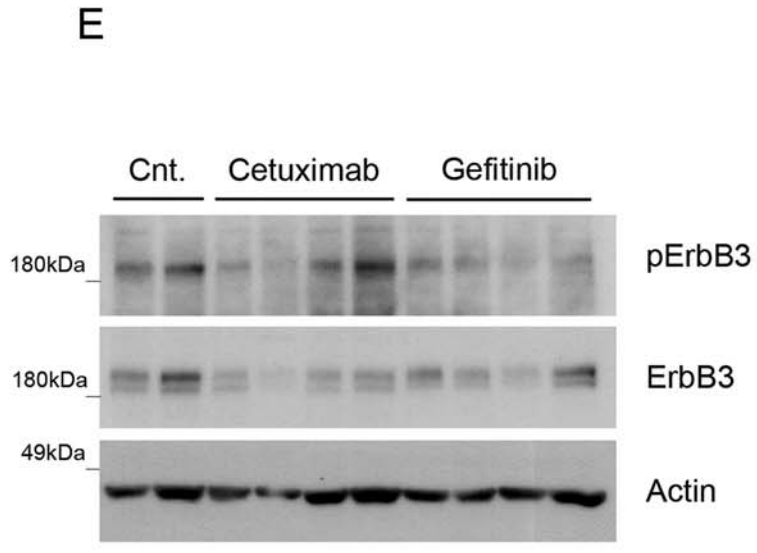
ErbB3
49kDa
Actin


D
pErbB3/Total ErbB3 (A.U.)
(%relative to control group)
200
160
120
80
40
0
**
Control
Lapatinib


E
Cnt.
Cetuximab
Gefitinib
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pErbB3
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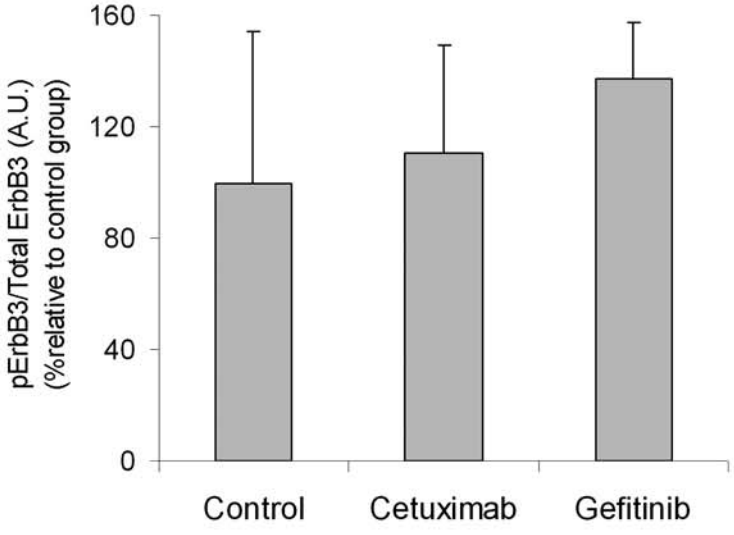
ErbB3
49kDa
Actin


F
pErbB3/Total ErbB3 (A.U.)
(%relative to control group)
160
120
80
40
0
Control
Cetuximab
Gefitinib