ErbB4 increases the proliferation potential of human lung cancer cells and its blockage can be used as a target for anti-cancer therapy

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Clinical and experimental data suggest that ErbB-4, a member of the epidermal growth factor receptor family, may have a role in cancer progression and response to treatment. We found recently, using a retrospective clinical analysis, that expression of ErbB-4 receptor is correlated with metastatic potential and patient survival in non-small-cell lung cancer (NSCLC). The purpose of this work was to correlate the expression of the ErbB-4 and lung cancer cells growth in vitro and in vivo and to determine the therapeutic potential of a monoclonal antibody to ErbB-4 against lung cancer. For this aim, we ectopically expressed ErbB-4 in a human NSCLC cell line that did not express the ErbB-4 protein. Overexpression of ErbB-4 produced a constitutively activated ErbB-4 receptor. The transfected ErbB-4 positive clones showed an increased cell proliferation in vitro and in vivo in comparison with parental ErbB-4 negative cells and with the cells transfected by neomycin-resistant gene. A monoclonal antibody to ErbB-4 showed both an inhibitory effect on growth rate and an increasing apoptotic rate in the ErbB-4 positive cells and with the cells transfected by neomycin-resistant gene. Additionally, neuregulin provides marked angiogenic response in vitro and in vivo. Further, evidence for proliferative and tumorigenic properties of ErbB-4 was provided by Gilmour et al.,19 using ribozyme technology. They observed that down-regulation of ErbB-4 in breast cancer cell lines expressing relatively high levels of ErbB-4 markedly reduces the ability of the cells to grow in an anchorage-independent assay. Furthermore, ribozyme-mediated down-regulation of ErbB-4 in these breast cancer cells inhibited tumor formation in athymic nude mice. Overexpression of ErbB-4 in other tumor types suggests a strong selective advantage conferred upon tumor cells in vivo.20,21 These data propose that ErbB-4 plays a significant role in the proliferation of cells expressing high levels of ErbB-4.

Several groups have evaluated the expression of ErbB-4 in breast cancer patients22–29 and found that ErbB-4 is expressed at moderate to low levels. Its expression correlates with a favorable prognosis and this is in contradictory to ErbB-2 and ErbB-1 that are correlated with a poor prognosis. In addition to mammary carcinoma, ErbB-4 expression has been noted in several other tumors30–37. These include carcinomas of the colon, prostate, lung, ovary, pancreas, endometrium, bronchus, cervix, stomach and thyroid. Also, soft tissue sarcomas, astrocytoma and pediatric brain tumors38–41 are reported to express ErbB-4. In ependymoma,41 ErbB-4 expression is quite high in about 75% of the cases. ErbB-2 expression was found in ~30%, while the expression of ErbB-1 or ErbB-3 is relatively low. Coexpression of ErbB-2 and ErbB-4 occurs in about 75% or more of these tumors and is correlated with a high proliferative index and poorer survival outcome. Similar findings were reported in other tumors. Nevertheless, the role of ErbB-4 in tumor progression is still undefined and further investigations are needed.

The possibility of ErbB in cancer progression has led to an extensive search for selective inhibitors. The results of a large body of preclinical studies and the clinical trials thus far conducted suggest that targeting the ErbB family could represent a contribution to cancer therapy. A variety of different approaches are currently being used for this purpose. Two therapeutic approaches have been shown to be the most promising and are currently used to inhibit ErbB in clinical settings: (i) monoclonal antibodies (MAbs) to prevent ligand binding and (ii) small molecule tyrosine kinase inhibitors (TKIs) specific to ErbB tyrosine kinases, which inhibit autophosphorylation and downstream intracellular signaling. Among the MAbs that gained wide clinical use is a MAb to ErbB-2 denoted...
trastuzumab (Herceptin). It blocks signal transduction and cell growth and combined with chemotherapy it produced a higher response rate and a longer survival.\textsuperscript{45,46}

The main purpose of the present study was to determine whether ErbB-4 has a role in proliferation of non-small-cell lung cancer (NSCLC) cells \textit{in vitro} and \textit{in vivo}, and whether ErbB-4 can be used as a target for anticancer therapy.

\textbf{Materials and methods}

\textbf{Cell lines and culture conditions}

Human large-cell cancer of lung H661 and human adenocarcinoma of lung H1299 were obtained from American Type Cell Collection (Rockville, MD). The cell lines were maintained in DMEM supplemented with 10\% heat-inactivated fetal bovine serum (FBS), antibiotics, glutamine, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate (Biological Industries, Beit HaEmeq, Israel), and grown in 5\% CO\textsubscript{2} at 37\degree C in a water-jacketed incubator with 100\% humidity. The cells were harvested by trypsin solution with 1–2 passages per week in a split ratio of 1:3–5. The 24-hr cell cultures were used in all the experiments.

\textbf{ErbB-4 gene transfection}

H1299 cells that do not express ErbB-4 were transfected with ErbB-4 pcDNA 3.1 using a calcium phosphate method. Semiconfluent cells were seeded at 5 \times 10\textsuperscript{5} cells per tissue culture plate (60 mm), and 24 hr later, they were transfected with \sim 10 \mu g of ErbB-4 pcDNA 3.1 expression vector that contained a neomycin-resistance gene. As control, the cells were transfected with a vector containing neomycin-resistance gene alone. The transfected resistant cells in both variants were selected in the medium containing 500 \mu g/ml G418. After 3–6 weeks, the colonies were expanded into confluent cultures and then transferred into tissue culture plates (35 mm). Each transfected cell clone was checked for ErbB-4 expression using immunohistochemistry, western blot and immunoprecipitation assays.

\textbf{Antibodies}

Polyclonal anti-ErbB-1, ErbB-2, ErbB-3 and ErbB-4 antibody were purchased from Santa Cruz (CA). Monoclonal antibodies against different epitopes of extracellular domain of ErbB-4 (MAb-3, clone H72.8 and MAb-1, clone H4.77.16) were purchased from Neo-Markers (Fremont, CA). Additionally, one of the monoclonal antibodies (MAb-3) was obtained from the hybridoma H72.8 developed in the laboratory of Dr. Yarden.\textsuperscript{7} This antibody was purified from the supernatant or mouse ascites using Protein-G-Sepharose (60 mm), and 24 hr later, they were transfected with ErbB-4 pcDNA 3.1 expression vector that contained a neomycin-resistance gene. As control, the cells were transfected with a vector containing neomycin-resistance gene alone. The transfected resistant cells in both variants were selected in the medium containing 500 \mu g/ml G418. After 3–6 weeks, the colonies were expanded into confluent cultures and then transferred into tissue culture plates (35 mm). Each transfected cell clone was checked for ErbB-4 expression using immunohistochemistry, western blot and immunoprecipitation assays.

\textbf{Colorimetric in vitro cytotoxicity assay (XTT)}

Cell density was evaluated by a colorimetric assay based on the conversion of tetrazolium salt XTT to orange-colored formazan compounds by means of cellular dehydrogenases. The advantage of this assay (using 96 microwell plates) is the possibility of testing numerous arms of each experiment under the same conditions. Typically, 200 l of medium with a known number of cells from exponentially growing culture were plated in 96 microwell flat-bottomed plates. Following 24 hr in culture (to attach and to resume growth), MABs were added in varying concentrations to each of the 3 replicate wells. The cell growth was evaluated by measuring the cell density during several days of incubation. The effect of MABS on cell survival was calculated by comparing the density of intact cells and the cells exposed to different concentrations of MABs for 72 hr. Cell density was determined by the XTT assay as follows. A freshly prepared mixture of tetrazolium salt XTT and an activation reagent (PMS) was added into each well. Following 2 hr of incubation at 37\degree C, the plates were placed on a mechanical plate shaker of a computerized automatic microwell plate spectrophotometer and shaken for 1 min, after which the optic density (OD) of the dye was read at 450 nm. The measurements were repeated following 4 and 6 hr of incubation. The time point of the assay with the most optimal OD readings was chosen to count the relative cell number. When more than one time point fitted these criteria, the results of the different time points were normalized and averaged.

\textbf{Immunohistochemistry}

For IHC analysis, the cells were centrifuged into a cell pellet that was embedded in paraffin for immunohistochemical staining with the ErbB-4-polyclonal (Santa Cruz, CA) or monoclonal (Neo-Markers, CA) antibodies. Antigen retrieval was performed at 95\degree C in citrate buffer pH 6.0, 6.4 M sodium citrate dehydrate and 1.6 M citric acid monohydrate for 40 min. The slides were cooled at room temperature for 20 min and washed 3 times of 3 min each with Tris buffer pH 7.6, 0.15 M sodium chloride and 0.05 M Trizma HU. They were peroxidase-blocked for 5 min and washed as above. They were then incubated for 30 min with the primary antigen, followed by the secondary antigen (visualization reagent), followed by the substrate–chromogen solution (3,3'-diaminobenzidine), and finally counter-stained with hematoxylin. Paraffin sections from skeletal muscles known to express ErbB-4 were used as a positive control. For a negative control, the primary antibodies were replaced with a nonspecific negative control antibody. Cell staining was quantified in a blind manner by 2 independent investigators (S.M. and A.S.) who graded them from 0 to 3+, according to the staining intensity and the percentage of stained cells.

\textbf{Immunoprecipitation and western blot analyses}

\textbf{Cell lyses.} Cells were grown in 10-cm tissue culture dishes, washed briefly with ice-cold PBS and treated for 30 min on ice with lysis buffer containing 50 mM HEPES pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 30 mM β-glycerol phosphate, 2 mM sodium orthovanadate, 2 mM PMSF and protease inhibitors (10 μg/ml aprotinin and protein inhibitor cocktail). Cell lyses were cleared by brief centrifugation (14,000 rpm, 1 min). Cleared lyses were kept at \sim 80\degree C until use. Protein quantitation was performed using the bicinchoninic/BCA (Pierce) protein assay using bovine serum albumin as a standard.

\textbf{Immunoprecipitation.} Fifteen microliters of beads (Protein G-Sepharose, Sigma) for each sample (a “sample” is a lysate originating from one dish) were washed 3 times with HNTG buffer containing 20 mM HEPES pH 7.5, 150 mM NaCl, 0.1% Triton X-100 and 10% glycerol. Anti-ErbB-4 antibodies were coupled to the beads for 1 hr to overnight at 4\degree C while shaking. Cell lyses were added to the beads/Ab conjugates and incubated overnight at 4\degree C while shaking. Immunoprecipitates were washed 3 times with HNTG buffer, mixed with sample buffer, heated for 5 min at 95\degree C and subjected to SDS-PAGE electrophoresis (7.5%).

\textbf{Western blot.} After electrophoresis, the proteins were electrophoretically transferred to nitrocellulose membranes (1–2 hr, 4\degree C) and then saturated overnight at room temperature in a blocking solution (TBST 50 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.1% Tween 20 with the addition of 5% low fat milk). The membranes were then incubated for 1.5 hr at room temperature with the first antibody (rabbit polyclonal IgG) diluted in TBST containing 2% low fat milk. Filters were washed extensively with TBST solution and incubated for 1 hr at room temperature with conjugated goat anti rabbit IgG-HRP. Signals were detected by using the enhanced chemiluminescence method (EZ-ECL, Biological Industries, Beit Haemek, Israel).

\textbf{Flow cytometry analysis of cell cycle and apoptosis}

Cell cycle analysis was performed 2–3 times for each protocol of treatment tested. The cells were trypsinized, rinsed twice with ice-cold PBS (0.05 M, pH = 7.4) and fixed in 70% ice-cold ethanol. The fixed cells (10\textsuperscript{4} cells/ml) were stored at 4\degree C until cell cycle analysis. Before analysis, the cells were centrifuged and the pellet
was resuspended in 810 μl PBS after which RNase-A (10 mg/ml, 90 μl) was added. After 30 min of incubation at 37°C, propidium iodide (PI, 0.5 mg/ml, 90 μl) was added, and the samples were run for additional 1 hr. FACScan® (Becton Dickinson) was used for flow cytometry. The percentages of the cells in the different phases of the cell cycle were calculated using CellQuest software (Becton Dickinson).

Animals

The 4- to 6-week-old male athymic CD-1 nude mice were obtained from the Harlan Animal Production Area (Jerusalem, Israel). The animals were housed in a laminar flow cabinet under pathogen-free conditions in standard vinyl cages with air filter tops. Cages, bedding and water were autoclaved before use. The local Ethics Committee for Accreditation of Laboratory Animal Care approved all facilities in accordance with the current regulations and standards of the Israeli Ministry of Health.

Tumorigenicity assay

The harvested NSCLC tumor cells (3 × 10^6 cells in 0.2 ml PBS) were injected subcutaneously into the animal’s flank area. Each cell line was injected into 5 mice. The tumor size was measured twice weekly by digital caliper. The mean tumor size at each time point was calculated from the pooled data.

Statistics

The results for each variant in the in vitro experiments with proliferation assay were represented as an average of 2–4 experiments, and each arm was typically performed in triplicate. The mean values and standard errors were calculated for each point from the pooled normalized data.

Results

ErbB1-4 expression in NSCLC cell lines

To evaluate in detail, a possible role of ErbB-4 in cell behavior, the H1299 cell line that did not show ErbB-4 expression by IHC, IP and Western analysis was transfected either by the ErbB-4 gene in combination with the neomycin-resistance gene or by the neomycin-resistance gene alone. Two clones (c-16 and c-22), which showed stable transfection and high expression of ErbB-4 receptor revealed by IP (Fig. 1), were used in subsequent experiments. All the clones transfected with the neomycin-resistance gene alone were ErbB-4 negative (Fig. 1). Immunohistochemical staining for ErbB-4 confirmed the results of biochemical analysis (not shown). Western blot analysis for ErbB-1 and ErbB-3 showed approximately same expression in all studied cells; however, ErbB-2 was expressed in c-16 only (Fig. 1)

FIGURE 1 – Expression of ErbB oncproteins in the H1299 parental cell line and its clones transfected with ErbB-4 gene (immunoprecipitation and western blot analysis). Large cell carcinoma H661 cell line with high expression of ErbB-4 was used as positive control. C-neo cells contained the vehicle only, whereas c16 and c22 cells contained plasmid with ErbB-4 gene. After immunoprecipitation with monoclonal ErbB-4 antibody, the cell lysates were processed by Western analysis and probed by polyclonal antibody.

Effect of ErbB-4 expression on NSCLC cell growth in vitro

The growth rate of H1299 cells (parental line), two ErbB-4 positive clones (c16 and c22) and a neomycin-resistant clone (c-neo) was tested in vitro. The cells were seeded in 96 microwell plates, and the numbers of living cells were determined by XTT assay during 4 days of cultivation. Both ErbB-4 positive clones demonstrated a greater growth in comparison with parental cells (par - parental line of H1299 cells) and c-neo clone (the clone of H1299 cells transfected with neomycin resistance gene only).

Effect of anti-ErbB-4 monoclonal antibodies on cell growth in vitro

To test whether the ErbB-4 receptor might mediate growth function, the blocking effect of ErbB-4 monoclonal antibody (MAB-3) on H-661 (human large-cell carcinoma) and H1299 cell lines (including c16, c22 and c-neo clones) was evaluated. The incubation of ErbB-4 positive H661 cells with MAB-3 resulted in dose-dependent inhibition of cell survival: 30 μg/ml MAB-3 decreased cell density up to ~50% relative to untreated cells (Fig. 4). Similar findings were found by using MAB-3 on ErbB-4-expressing c16 and c22 clones of the H1299 cell line, whereas H1299 parental cells and its neomycin-resistant clone (H1299-neo) were unaffected (Fig 4).

To investigate a possible mechanism of the inhibitory effect of the blocking antibody on proliferation of ErbB-4 expressing cells, two mouse monoclonal antibodies—MAB-1 and MAB-3 were used. Both antibodies are directed to epitopes that map in the extracellular domain of human ErbB-4. MAB-1 does not interfere with the receptor-heregulin binding but stimulates tyrosine kinase activity and internalization of ErbB-4. In opposite, MAB-3 strongly inhibits the bind-
ing of heregulin to ErbB-4. It has no effect on tyrosine kinase activity or internalization of the receptor. As was demonstrated before, the proliferation of H661 cells was inhibited by MAb-3 (Fig. 4) but not by MAb-1 (data not shown). These results indicate that the growth-inhibitory effect produced by MAb-3 in NSCLC cells was mediated by the blocking of heregulin binding to ErbB-4.

**Induction of apoptosis by anti-ErbB-4 monoclonal antibodies in NSCLC cells**

The distribution of the cells in the cell cycle and the extent of apoptosis were assessed by flow cytometry, following 72 hr incubation of the cells with and without MAb-3 (30 μg/ml). In contrast to the H1299 parental cell line and c-neo clone, the incubation of ErbB-4-expressive clones (c16 and c22) with MAb-3 significantly increased the percentage of cells with sub-diploid DNA content indicating the development of apoptosis (Fig. 5). In the same time, the other phases of the cell cycle were almost not affected by MAb-3 in all cells tested (data not shown).

**Discussion**

ErbB-4 has a significant role in the growth and survival of different human tumors. We had earlier studied the correlation between expression of ErbB-4 and prognosis in human lung cancer. Using immunohistochemistry, we analyzed ErbB-4 expression in 80 patients with NSCLC who underwent surgery. The cumulative survival rate for patients with ErbB-4 protein overexpression was 16–22 months compared to 33–40 months in patients without ErbB-4 over-expression (p < 0.002). Strong immunostaining was found in 78.1% of the tumors metastatic to lymph nodes, whereas weak staining was detected in 80% of nonmetastatic tumors, indicating a significant correlation between lymph node involvement and ErbB-4 over-expression (p < 0.001). In another clinical study, which considered response and survival of lung cancer patients following gemcitabine and cis-platin chemotherapy, ErbB-4 was shown to have a possible role in the treatment outcome. These findings suggested that ErbB-4 protein over-expression in NSCLC tumors may be a useful biological marker for indicating a tendency to early lymph node metastasis, a poor clinical outcome and decreased response to chemo- and radiotherapy. Therefore, down-regulation of ErbB-4 signaling might provide an effective target for molecular cancer therapy.

We demonstrated that introducing ErbB-4 gene in the ErbB-4 negative cell line resulted in the stimulation of proliferation of NSCLC cells in vitro, and in increasing the growth of human NSCLC cells implanted subcutaneously into athymic nude mice. In addition, specific monoclonal antibody to ErbB-4 (MAb-3) inhibited the cell growth and enhanced induction of apoptosis in endogenously ErbB-4 expressed NSCLC cell line as well as in H1299 transfected clones that demonstrated ErbB-4 expression. The same antibodies were noneffective in the H1299 parental cell line or in their clones not expressed ErbB-4. A number of mechanisms can be proposed as characterizing the effect of the ErbB-4 antibody on the growth and apoptotic rate of H1299 cells. The direct mechanism is most probably the inhibition of ErbB-4 tyrosine kinase activation. The outcome of this blockade is reflected in the disruption of a number of processes regulated by the ErbB-4 that include: (i) regulation of cell cycle progression, (ii) cell survival pathways, (iii) tumor cell invasion and (iv) angiogenesis. Blockade of ErbB-4 signaling using antibodies against ErbB-4 have been shown to inhibit receptor autophosphorylation in vitro.
Numerous articles have reported the influence of neuregulin/heregulin on the growth of cell lines that endogenously express ErbB-4. Transfection of ErbB-4 into 3T3 cell lines that do not normally express ErbB receptors has established that growth factor activation of ErbB-4 in the absence of other ErbB receptors provokes a significant increase in cell proliferation. Also, this study demonstrated that activation of ErbB-4 by neuregulin stimulated cell proliferation and chemotaxis.

Other mechanisms of action of anti-ErbB-4 monoclonal antibody used in our experiments as well antibodies to other ErbB receptors may be suggested:

- **Block of NRG binding to ErbB-4.** The first step in signaling cascade from ErbB-4 is binding of the specific ligand to extracellular domain of the receptor. To test this interaction, we examined the effect of two mouse monoclonal antibodies—MAb-1 and MAB-3—on proliferation of different lung cancer cell lines. Both MABs interact with the extracellular domain of human ErbB-4 but to different epitopes. In addition, MAB-3 strongly inhibits the binding of heregulin to ErbB-4 while Mab-1 does not interfere with the receptor-heregulin binding. In our experiments, MAB-1 did not inhibit the proliferation of ErbB-4 positive NSCLC cells, whereas MAB-3 demonstrated an inhibitory effect. These results indicate that the growth-inhibitory effect of MAB-3 in lung cancer cells was associated with the blocking of heregulin binding to ErbB-4.

- **Disturbances of ErbB-4 heterodimerization with other EGFR members.** Some investigators have evaluated ErbB-4 growth-stimulating capacity by transfection of the receptors into the hematopoietic cell lines 32D (myeloid) or BaF3 (lymphoblastoid) that do not exogenously express any ErbB family members. It is agreed that by itself ErbB-4 has a weak capacity to support cell survival in the presence of its cognate ligand. However, when coexpressed with ErbB-2, most studies show that ErbB-4 more effectively mediates cell survival in a manner that is dependent on the presence of an ErbB-4 ligand. The picture that emerges is that ErbB-4 can mediate a proliferative signal, but its potential to do so is significantly enhanced by the presence of ErbB-2. Several studies have reported cooperative effects of MAB combinations. Combining MABs that engage distinct epitopes significantly accelerates receptor degradation. In addition, MAB combinations are more effective than single MABs in inhibiting ErbB signaling in vitro and tumorigenicity in animals.

- **Influence on cell cycle and apoptotic death.** The antitumor activity of ErbB-4 can be attributed to its influence on cell cycle and apoptosis. In our experiments, we did not find the cell cycle deceleration but MAB-3 induced apoptosis.

In conclusion, our study demonstrates the proliferative and the tumorigenic potential of ErbB-4 gene as well as antitumor activity of anti-ErbB-4 MAB in *in vitro* NSCLC model. This treatment was markedly effective against an established NSCLC cell line that expressed the ErbB-4. The ability of ErbB-4 monoclonal therapy to inhibit the growth of lung cancer cell lines in *vitro* suggests that the ErbB-4 blockage by ErbB-4 MAB has potential therapeutic strategy for anticancer therapy.

### References


