Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor Does Not Improve Paclitaxel Effect in an Orthotopic Mouse Model of Lung Cancer

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ABSTRACT

Purpose: The purpose is to evaluate whether inhibition of epidermal growth factor receptor (EGFR) activation by PKI166, an EGFR-tyrosine kinase inhibitor, affects growth of human lung cancer implanted orthotopically into the lungs of nude mice.

Experimental Design: Lungs of mice were injected with NCI-H11549 human bronchioloalveolar cancer cells. In three experiments, groups of mice (n = 10 per group) were randomized 7 days after tumor implantation to receive one of the following treatments: i.p. paclitaxel 100 or 200 µg (4 or 8 mg/kg) once per week, oral PKI166 100 or 200 mg/kg three times per week, paclitaxel plus PKI166, or i.p. saline and oral PKI166-vehicle (control) for 5 weeks. Mice were killed 6.5 to 8 weeks after tumor implantation. The experiments were repeated with PC14PE6 human lung adenocarcinoma cells to assess effect on survival.

Results: Immunohistochemical analyses revealed the expression and phosphorylation of EGFR in the growing tumors. Treatment with PKI166 alone or in combination with paclitaxel diminished activation of EGFR on tumor cells, yet maximal therapeutic effect was observed in mice treated with paclitaxel alone. Activated mitogen-activated protein kinase and basic fibroblast growth factor expression were similar in all treatment groups. Survival in mice treated with the combination of paclitaxel and PKI166 was shorter than in those treated with paclitaxel alone.

Conclusions: Our results suggest that concurrent administration of EGFR-tyrosine kinase inhibitor and chemotherapy is equivalent and may indeed be inferior to chemotherapy alone, even if EGFR is functional and its phosphorylation effectively inhibited. Our data show that the interaction of EGFR-TKIs and chemotherapy is complex and suggest that other growth factors may activate the downstream signaling events.

INTRODUCTION

Non–small-cell lung cancer (NSCLC) is the most common form of lung cancer and the leading cause of cancer-related death in the world (1). Systemic chemotherapy is the main treatment for the majority of patients with NSCLC because most are diagnosed with advanced inoperable disease. However, recent studies have shown that modern chemotherapeutics may have reached the ceiling of their clinical efficacy, and the 5-year survival rate for NSCLC has plateaued at 14% (2). These findings show that a new approach to the therapy of lung cancer is mandatory.

Targeting the epidermal growth factor receptor (EGFR) is one appealing strategy for the treatment of lung cancer because the EGFR plays a pivotal role in tumor cell proliferation, survival, adhesion, migration, differentiation, and angiogenesis (3). Indeed, increased expression of EGFR, one of the earliest and most frequently detected abnormalities in the bronchial epithelium of heavy smokers (4), is pronounced in the majority of NSCLC cases (5, 6). However, the relationship between EGFR expression and the prognosis of NSCLC patients is still not well established, and retrospective studies of EGFR expression in stage I disease have yielded conflicting results (7). Several approaches to block EGFR-mediated signaling pathways are under evaluation, including using monoclonal antibodies (MAbs) against the ligand or receptor, ligand-toxin conjugates, antisense oligonucleotides, and receptor tyrosine kinase inhibitors (8). A recent phase II trial of monotherapy with the anti-EGFR-tyrosine kinase inhibitor gefitinib (Iressa, ZD1839, AstaZeneca Pharmaceuticals, Wilmington, De) showed tumor response rate of 10%, a stable disease of 30%, and a symptomatic response rate of 40% (9, 10) for lung cancer patients. Paet et al. (11) and Lynch et al. (12) recently studied EGFR mutation status in patients treated with gefitinib and found that somatic mutations in tyrosine kinase site characterize responders to therapy.

The clinical effects of several EGFR-tyrosine kinase inhibitors were studied intensively both in vitro and in vivo, either alone or in combination with diverse chemotherapeutics. Ciardiello et al. (13) and Sirotnak et al. (14) studied the effect...
of gefitinib in combination with different chemotherapeutics. PKI166 (Novartis, Switzerland) was studied by Bruns et al. (15) in an orthotopic model of human pancreatic cancer in nude mice and by Kim et al. (16) and Weber et al. (17) in models of human prostate cancer and renal cell cancer growing in the bones of nude mice. In these models, therapy with PKI166 was associated with decreased tumor growth and metastasis, and the anti-tumor effect was enhanced by chemotherapy. We recently developed an orthotopic lung cancer model (18). In this system, human lung cancer cells were injected into the left lung of nude mice. Tumors formed from a single focus of disease and progressed to a widespread and fatal thoracic process, characterized by diffuse dissemination of lung cancer in both lungs and metastasis to intra- and extrathoracic lymph nodes.

The purpose of the present study was to evaluate whether inhibition of EGFR activation by PKI166 affects tumor growth and metastasis of human lung cancer implanted orthotopically into the lungs of nude mice. We studied human bronchioalveolar cancer (NCI-H358) and lung adenocarcinoma (PC14PE6), which both express the EGFR and the activated EGFR. The results of these studies show that therapy with PKI166 either alone or in combination with paclitaxel inhibited EGFR activation for lung cancer cells growing in the lung. However, the therapeutic efficacy of the regimen of concurrent PKI166 and paclitaxel was inferior to that of paclitaxel alone. These results are similar to those of recent human clinical trials with gefitinib (19, 20), which demonstrated that gefitinib and the anti-EGFR

Western Blot Analysis of EGFR, EGFR Phosphorylation of Lung Cancer Cell Lines, and Response to Treatment with PKI166. Serum-starved NCI-H358 cells were studied for the stimulation of 40 ng/mL recombinant human EGF for 15 minutes. Serum-starved NCI-H358 cells were then treated with PKI166 (0, 0.5, 1, 2.5, and 5 μmol/L) for 60 minutes and then incubated with or without 40 ng/mL recombinant human EGF for 15 minutes, washed, scraped into PBS containing 5 mmol/L EDTA and 1 mmol/L sodium orthovanadate, and centrifuged. The pellet was resuspended in lysis buffer [20 mmol/L Tris-HCl (pH 8.0), 137 mmol/L NaCl, 10% glycerol, 2 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 20 μmol/L leupeptin, and 0.15 unit/mL aprotinin], sonicated, and centrifuged to recover insoluble protein. Immunoprecipitation was done with monoclonal antibody anti-EGFR (clone EGF-RI) as described previously (15–17). Immunoprecipitates were analyzed on 7.5% SDS-PAGE and transferred onto 0.45-μm nitrocellulose membranes. The filters were blocked with 3% BSA in TDS [20 mmol/L Tris-HCl (pH 7.5) and 150 mmol/L NaCl], incubated with either polyclonal sheep antihuman EGFR (1:1000) or monoclonal anti-phospho-tyrosine (monoclonal antibody 4G10; 1:2000) in TBTBS (0.1% Tween 20 in TBS), and incubated with horseradish peroxidase-conjugated donkey antisheep IgG (1:2000; Sigma, St. Louis, MO) or sheep antihuman IgG (1:2000), respectively, in TTBTS. Protein bands were visualized by the enhanced chemiluminescence detection system.

Preparation of Cell Suspension and Orthotopic Implantation of Tumor Cells. Cells were injected with Growth Factor Reduced Matrigel, which anchors the tumor cells, thereby preventing their diffusion into the thorax. For all experiments, a
injections of PC14PE6 adenocarcinoma cells (0.5 \times 10^6 cells in 75 \mu L in Matrigel), and three mice, who received injections of NCI-H358 bronchioloalveolar cells (1 \times 10^6 cells 75 \mu L in Matrigel), were killed, and histologic examination confirmed the lesions to be actively growing lung cancer 9 days (in case of PC14PE6 tumor cells) or 14 days (in case of NCI-H358 tumor cells) after tumor implantation. Mice were then injected orthotopically into the lung with NCI-H358 tumors (1 \times 10^6 cells in 75 \mu L in Matrigel). Seven days after implantation, the mice were randomized into the following treatment groups (n = 10): once-per-week i.p. administration of pacitaxel (100 or 200 \mu g; 4 or 8 mg/kg), three times-per-week oral administration of PKI166 (100 or 200 mg/kg), and a combination of pacitaxel and PKI166 (same dosages and frequencies of each of the drugs given alone). Control mice received the oral vehicle solution for PKI166 (DMSO/0.5% Tween 80 diluted 1:20 in HBSS) and i.p. HBSS (15–17). Three separate experiments were carried out with NCI-H358 tumors for a total of 5 weeks of therapy. The experiments were terminated when the control mice became moribund, and all mice were then simultaneously killed, autopsied, and their tumor tissues were harvested.

Using similar methodologies, the therapy experiment was repeated with PC14PE6 human lung adenocarcinoma cells with overall survival as an end point. Mice were implanted with PC14PE6 cells into the lung and were randomized to the four treatment groups (n = 10) on day 7. The mice were killed and necropsied when they became moribund. Survival was evaluated by the Kaplan-Meier method (24).

Necropsy and Tissue Preparation. Mice were killed with a lethal dose of sodium pentobarbital (100 mg/kg body weight). After a laparotomy, the thoracic cavity was inspected through the diaphragm for evidence of pleural effusion. Pleural effusions were collected, and the thoracic organs were then removed en block, including all lymph nodes and tumors. After dissection and removal of the heart, the lung and tumor mass were washed in cold PBS and weighed. Other visceral organs were removed and inspected for the presence of metastases. For immunohistochemical and H&E-staining procedures, one part of the tumor was fixed in formalin and embedded in paraffin.

Immunohistochemical Determination of EGFR, Activated EGFR, bFGF, and Activated MAPK. Paraffin-embedded tissues were used to identify EGFR, activated EGFR, bFGF, and phosphorylated MAPK. Immunohistochemical procedures for EGFR, activated EGFR, and bFGF were performed as described previously (15–17, 25). Activated MAPK was determined as described by Albanell et al. (26). Briefly, tissue sections (4 to 6 \mu m) were mounted on positively charged Superfrost slides (Fisher Scientific Co., Houston, TX) and dried overnight. The sections were deparaffinized in xylene, treated with a graded series of alcohol [100, 95, and 80% etomidate/double distilled H_2O (v/v)] and then rehydrated in PBS (pH 7.5). For antigen retrieval, sections used for the activated EGFR and activated MAPK analyses were prepared in 10 mmol/L ethylene diamine tetraacetic acid buffer (pH 8) for 10 minutes in a microwave oven at 600 W. To determine EGFR, slides were prepared by pepsin digestion for 10 minutes. Before being stained for bFGF, the tissues were treated with pepsin for 20 minutes at 37°C and washed with PBS. The tissues were incubated with primary antibodies overnight at 4°C at the following dilutions: EGFR 1/200, activated EGFR 1/1000, activated MAPK 1/80, and bFGF 1/100. Control samples exposed to a secondary antibody alone showed no staining.

Quantification of Immunohistochemical Studies. To quantify the intensity of the immunohistochemical reaction, the absorbance of 100 bFGF- and activated MAPK-positive cells in 10 random 0.039-mm\(^2\) fields at \(\times 200\) of treated tumor tissues was measured with Optimas Image Analysis software (15–17, 25). The samples were not counterstained; therefore, the absorbance was attributable solely to the product of the immunohistochemical reaction. bFGF and activated MAPK cytoplasmic immunoreactivity were evaluated by computer-assisted image analysis and expressed as the ratio of tumor cell expression to normal pancreatic gland expression multiplied by 100 (27, 28).

RESULTS

EGFR Is Expressed and Activated in Human Lung Cancer Cells, and the Activation of EGFR Is Blocked by PKI166. In the first set of experiments, we determined that the NCI-H358 tumor cells express the EGFR and the activated EGFR. A Western blot analysis showed that NCI-H358 tumor cells express the EGFR protein constitutively. The activated EGFR protein was found after stimulation with the ligand EGF. Tumor cells incubated for 15 minutes in medium free of serum but containing EGF exhibited the activated EGFR (\(M_r\ 170,000\) band), as detected by antiserum on Western blot analyses of anti-EGFR immunoprecipitated cell lysates. We next determined that in vitro treatment of the cell lines with PKI166 inhibited EGF-stimulated tyrosine phosphorylation of the EGFR. Pretreatment of cells with PKI166 for 1 hour followed by a 15-minute treatment with EGF inhibited the phosphorylation in a dose-dependent manner (0 to 5 \mu mol/L). The identity of the \(M_r\ 170,000\) band was confirmed by Western blot analysis with anti-EGFR antiserum (Fig. 1). Similar results were noted with PC14PE6 cells (data not shown).

Inhibition of NSCLC Tumor Growth and Metastasis. Athymic nude mice were injected into their left lungs with NCI-H358 cells in three separate experiments. The mice were killed 6.5 to 8 weeks after tumor implantation, when the control mice became moribund. In all three experiments, oral PKI166 decreased the median lung and tumor weight of the treated mice as compared with that of the control mice but was less effective.
Concurrent EGFR-TKI and Paclitaxel

EGFR-specific bands was compared in each case with the untreated

bFGF level was elevated in PKI166 or paclitaxel-treated tumors and even higher in tumors treated with concurrent PKI166 and paclitaxel (Fig. 4).

DISCUSSION

The effects of EGFR-tyrosine kinase inhibitors in combination with chemotherapy on subcutaneous ectopic tumors have been extensively studied. Ciardiello et al. (13), with gefitinib on human cancer lines that co-expressed EGFR and its ligand transforming growth factor \( \alpha \), observed an increased antitumor effect and potentiation of cytotoxic drugs both \textit{in vitro} and \textit{in vivo} for s.c. xenografts. Sirotnak et al. (14) also showed a potentiation of cytotoxic treatment with coadministration of gefitinib for s.c. xenografts, but the effects did not correlate with the level of EGFR expression by the target tumor. However, the level of the activated EGFR, which is the presumptive actual target for gefitinib, was not tested in these studies. Furthermore, the relevance of s.c. tumor growth in animal models to that in human studies has to be carefully ascertained because experimental preclinical results frequently cannot be translated to the clinic. One reason for this is that lower doses of chemotherapy agents often used in animal models to benefit from a synergy with biological therapies (29). Another limitation of animal models is related to tumor implantation. The use of ectopic tumors does not adequately take into account the interaction between the specific organ environment and tumor cells (i.e., lung cancer cells interacting with the lung microenvironment; refs. 30–32) and may therefore alter the tumor response to therapy. Indeed, we have recently shown that paclitaxel is more effective in treating s.c. NSCLC tumors than in orthotopic ones, suggesting that the orthotopic model may be more suitable for the study of lung cancer (18).

In the present study, we examined the clinical and biological effects of targeted therapy against the EGFR alone and in combination with chemotherapy in orthotopic lung cancer models. Our data showed that concurrent therapy with an EGFR-tyrosine kinase inhibitor and paclitaxel in two human lung

**Table 1** PKI166 and paclitaxel therapy of NCI-H358 (human NSCLC) tumors

<table>
<thead>
<tr>
<th>Study</th>
<th>Incidence* (Control)</th>
<th>Median (%)</th>
<th>Incidence* (PKI166)</th>
<th>Median (%)</th>
<th>Incidence* (Paclitaxel)</th>
<th>Median (%)</th>
<th>Incidence* (PKI166 + paclitaxel)</th>
<th>Median (%)</th>
</tr>
</thead>
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<tr>
<td>Study 1</td>
<td>9/9</td>
<td>0.319</td>
<td>9/10</td>
<td>0.318</td>
<td>10/10</td>
<td>0.271</td>
<td>10/10</td>
<td>0.287</td>
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<tr>
<td>Study 2</td>
<td>9/9</td>
<td>0.345</td>
<td>9/9</td>
<td>0.217</td>
<td>9/9</td>
<td>0.215</td>
<td>9/10</td>
<td>0.226</td>
</tr>
<tr>
<td>Study 3</td>
<td>9/9</td>
<td>0.340</td>
<td>10/10</td>
<td>0.297</td>
<td>8/9</td>
<td>0.248</td>
<td>9/9</td>
<td>0.245</td>
</tr>
</tbody>
</table>

* Number of positive mice/number of treated mice.
† Percentage of control mice weight.
‡ NCI-H358 human NSCLC cells (1 \( \times \) \( 10^6 \)) were injected into the left lung of nude mice. Seven days later, groups of mice were treated with once-a-week i.p. paclitaxel [studies 1 and 2: 200 \( \mu \)g (8 mg/kg); study 3: 100 \( \mu \)g (4 mg/kg)], three times-a-week oral administration of PKI166 [studies 1 and 2: 200 mg/kg; study 3: 100 mg/kg], and combination of paclitaxel and PKI166. Control mice received oral vehicle solution for PKI166 and i.p. injections saline for a total of five weekly courses of therapy. All mice were killed on 6.5 to 8 weeks after tumor injection when control mice became moribund.

NOTE. Weight (grams) designates lung and tumor weight.

Fig. 1 Inhibition of EGF-induced autophosphorylation of EGFR: NCI-H358 NSCLC cells growing \textit{in vitro} in serum-free medium were stimulated for 15 minutes with EGF (40 ng/mL) in the presence or absence of PKI166 (0 to 5 \( \mu \)mol/L). The cells were washed and lysed, and insoluble proteins in the lysate were immunoprecipitated with an anti-EGFR monoclonal antibody, separated by 7.5% SDS-PAGE, transferred to nitrocellulose, and sequentially probed with antisera to phospho-tyrosine (\textit{anti-p-Y}) and EGFR (see Materials and Methods). The immunoreactive proteins were detected by incubating the blot with the corresponding peroxidase-conjugated IgG and visualized with the enhanced chemiluminescence system. Densitometric quantitation of the ratio between the specific organ environment and tumor cells (i.e.,

than weekly paclitaxel alone. The antitumor effects observed in the mice treated with the combination of PKI166 and paclitaxel were equivalent or inferior to those observed in the mice treated with paclitaxel alone (Table 1). Treatment with PKI166 alone or in combination with paclitaxel was well tolerated, as evidenced by maintenance of body weight (data not shown).

For the survival studies, we used the same therapeutic model with human lung adenocarcinoma cells (PC14PE6) injected into the left lungs of 10 mice in each group. The mice were killed and necropsied when they became moribund. As in the therapeutic studies in NCI-H358 cells, results of the studies in PC14PE6 cells showed that the survival of mice treated with a combination of PKI166 and paclitaxel were equivalent to or inferior to the survival of mice treated with paclitaxel alone. The survival data are presented in Fig. 2. A detailed necropsy revealed that all of the mice had thoracic tumors.

**Histology and Immunohistochemical Analysis.** The NCI-H358 tumor specimens were processed for routine histology and immunohistochemical analyses. Immunohistochemistry with specific anti-EGFR, activated EGFR, bFGF, and activated MAPK showed differences in the level of expression for the different treatment groups. The lesions did not show differences in the expression of EGFR; yet, the expression of activated EGFR was diminished in the groups treated with PKI166 alone or in combination with paclitaxel (Fig. 3). The expression of bFGF and activated MAPK was determined. Activated MAPK expression was decreased in PKI166-treated tumors, whereas bFGF level was elevated in PKI166 or paclitaxel-treated tumors and even higher in tumors treated with concurrent PKI166 and paclitaxel (Fig. 4).
cancers growing orthotopically in mice is equivalent to or inferior to therapy with paclitaxel alone, although therapy with PKI166 inhibited EGFR phosphorylation. Our studies were among the first to show the discrepancy between biological activity and clinical results in an orthotopic model of human lung cancer in nude mice. Both lung cancer models studied expressed the EGFR, the presumed target for therapy. Our data were consistent with those from the clinical trials of EGFR-tyrosine kinase inhibitors and chemotherapy for the treatment of lung cancer. Recent phase III clinical trials of gefitinib or erlotinib in combination with chemotherapy in patients with advanced NSCLC showed that concurrent gefitinib or erlotinib and chemotherapy did not improve overall survival or other efficacy as compared with chemotherapy alone (19, 20, 33, 34). The lack of improved efficacy in the combination groups was observed with different combinations of chemotherapy (carboplatin/paclitaxel or gemcitabine/cisplatin) and with two dosages of gefitinib (250 or 500 mg/day). Similar clinical results have observed for erlotinib (35).

Two recent studies suggested that response to mono-therapy with gefitinib was associated with EGFR mutations. In these important reports, the authors suggested that patients whose tumors express EGFR mutations were likely to respond to gefitinib, whereas patients whose tumors did not express the mutations did not show response (11, 12). Our cell lines did not express EGFR mutations (data not shown). We propose that other mechanisms may be involved in response or resistance to EGFR-tyrosine kinase inhibitors. In our orthotopic lung model, the expression of bFGF by lung tumors was increased in all of the treatment groups with a trend of higher level in tumors treated with the combination of PKI166 and paclitaxel. Furthermore, the expression and activation of MAPK were lower in tumors treated with PKI166, as shown in prior studies with gefitinib (26). MAPK can be activated by bFGF (36), and our data suggested that the effects of EGFR blockade could possibly be offset by downstream signaling events mediated by bFGF and other growth factors, thereby promoting tumor cell survival. Combination therapy of bFGF signaling inhibitors, e.g., IFNs (37) and anti-EGFR agents, may be warranted. Furthermore, when taken together with the clinical trials, our data suggested that the sequencing of conventional therapy and biological therapy with EGFR-tyrosine kinase inhibitors has to be considered and that concurrent therapy may be detrimental. In this regard, Magne et al. (38) recently found differential effect of gefitinib in different combinations with chemotherapy and radiotherapy.

An additional intriguing explanation for the discrepancy between the biological effect and the clinical efficacy of PKI166 was provided by recent studies in animal models that showed a significant response to the combination of PKI166 therapy with

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**Fig. 2** Therapeutic effects of EGFR blockade and paclitaxel: percentage of cumulative survival. PC14PE6 adenocarcinoma cells were implanted in the lungs of nude mice. The mice were randomized into the above four treatment groups (n = 10) and were killed when moribund. Survival analysis was computed by the Kaplan-Meier method. *P = 0.1; **P = 0.004; ***P = 0.001. Compared to the control, PKI166 alone improved survival by 2.5 days, combination PKI166 and paclitaxel by 4.5 days, and paclitaxel alone by 13.8 days.

**Fig. 3** Representative panels of immunohistochemical determination of EGFR and activated EGFR in lung cancer tumors (×200 magnification): human NCI-H358 tumors from the lung of nude mice were harvested and processed for histology and immunohistochemical analyses 6.5 to 8 weeks after treatment with once-per-week i.p. paclitaxel (100 to 200 μg; 4 to 8 mg/kg), three times-per-week oral PKI166 (100 to 200 mg/kg), combination of paclitaxel and PKI166, or control. Tissue sections were stained for expression of tyrosine-phosphorylated, activated EGFR, and total EGFR as described previously (15–17). Tumors from all treatment groups stained positive for EGFR. Only tumors from control mice or mice treated with paclitaxel alone stained positive for activated EGFR.
chemotherapy (15–17) in an orthotopic model of pancreatic cancer and an orthotopic model of prostate carcinoma metastasis to the bone. Interestingly, in these studies, both the tumor cells and the tumor-associated blood vessels expressed the target receptor, and the tumor cells expressed the ligand transforming growth factor α (25). In contrast, in our studies of lung cancer, the NCI-H358 and the PC14PE6 tumors did not express transforming growth factor α, and the tumor endothelial cells did not express EGFR (data not shown).

In summary, our data suggest that in lung cancer, EGFR signaling may have an important role, yet it is insufficient to control tumor growth. The combination of several-targeted molecular therapies with conventional chemotherapy may be the basis of future therapy for lung cancer. Future directions for the study of EGFR inhibition in lung cancer should focus on the following topics: identifying the actual target for EGFR-tyrosine kinase inhibitors; identifying the best tumor biological profile that predicts response to therapy with EGFR inhibition, including the role of transforming growth factor α and the expression of EGFR by tumor endothelial cells; and the sequencing of chemotherapy and targeted therapy.

REFERENCES


Fig. 4 For quantification of the immunohistochemistry reaction intensity, the absorbance of 100 phosphorylated MAPK-positive cells (A) or bFGF-positive cells (B) from each of the differently treated tumor tissues were measured in 10 random fields at ×200 magnification with the Optimas Image Analysis software (Bioscan). The cytoplasmic immunoreactivity was evaluated by computer-assisted image analysis and was expressed as a ratio of tumor cell expression to normal muscle cell expression multiplied by 100. Student’s t test was compared the results between control and each of the three different treatment groups. A, *P = 0.003; **P = 0.5; ***P < 0.00001. B, *P < 0.00001.


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