Potential benefits of bevacizumab combined with platinum-based chemotherapy in advanced non-small-cell lung cancer patients with *EGFR* mutation

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Abstract

Objectives:

Oncogenic EGFR signaling has been shown to upregulate vascular endothelial growth factor A (*VEGFA*) expression involved in tumor angiogenesis. However, the clinical benefits of bevacizumab plus cytotoxic chemotherapy for *EGFR* mutation-positive patients remain unclear. This study aimed to investigate *VEGFA* mRNA expression in patients with *EGFR* mutation, and further compare the efficacy of bevacizumab combined with platinum-based chemotherapy between *EGFR*-mutant and wild-type patients.

Methods:

Gene expression of various pro-angiogenetic factors was analyzed in non-squamous non-small-cell lung cancer (NSCLC) patients, using the Cancer Genome Atlas dataset. Additionally, clinical data of patients receiving carboplatin and pemetrexed (CPem; N=104) or bevacizumab plus CPem (BevCPem; N=55) at Nagoya University hospital were retrospectively assessed for progression-free survival (PFS) and best overall response rate (ORR).

Results:

Among various pro-angiogenetic factors, only *VEGFA* expression was significantly higher in the advanced non-squamous NSCLC patients with *EGFR* mutation compared to those with wild-type (*p-value*=0.0476). In our cohort, the PFS of the BevCPem group was significantly longer in

patients with *EGFR* mutation than in wild-type patients (10.5 vs. 6.6 months; Wilcoxon *p-value*=0.0278), while the difference in the CPem group was not significant (6.6 vs. 4.5 months; Wilcoxon *p-value*=0.1822). The ORRs in BevCPem group were 54.5% and 36.4% for *EGFR*-mutant and wild-type patients, respectively, and the ORRs in CPem group were 35.5% and 28.8 % in *EGFR*-mutant and wild-type patients, respectively.

Conclusion:

VEGFA mRNA expression was significantly increased in advanced non-squamous NSCLC harboring *EGFR* mutation, and BevCPem provided better clinical benefits to patients with *EGFR* mutation than wild-type carriers.

Keywords: *VEGFA*, Bevacizumab, platinum-based chemotherapy, *EGFR* mutation, NSCLC

Introduction

Lung cancer is one of the leading causes of cancer-related mortality worldwide, accounting for 20% of all cancer-related deaths [1]. Non-small-cell lung cancer (NSCLC) is the most prevalent form of lung cancers, and is typically diagnosed at advanced stages [2]. In the past decade, the identification of key oncogenic-driver mutations has led to the development of the molecular

targeted therapies for the patients with NSCLC. Among them, epidermal growth factor receptor (*EGFR*) mutation was initially reported in 2004, and the clinical genetic testing has been well established for predicting the efficacy of EGFR tyrosine kinase inhibitors (TKIs) therapy [3, 4]. To date, the frequency of genetic alterations in exons 18-21 of *EGFR* kinase domain are widely known to be approximately 40-50% in Asian patients and 10-15% in Caucasian patients [4, 5]. The molecular targeted therapies using EGFR-TKIs have significantly improved the clinical outcomes of the patients with NSCLC harboring *EGFR* mutations; however, almost all the patients who initially benefited from such therapies eventually acquired resistance [2, 6, 7]. The alternative therapeutic strategies after failure of EGFR-TKIs treatments are still fully dependent on cytotoxic chemotherapies.

As another attractive molecular targeted therapy agent, a recombinant humanized monoclonal antibody against vascular endothelial growth factor A (VEGFA), bevacizumab, was approved in 2006 by the Food and Drug Administration [8]. Although cancer cells and various tumor-associated stromal cells express a variety of pro-angiogenetic molecules, VEGFA has been recognized as one of the key stimulators of tumor angiogenesis [9-11]. The addition of bevacizumab to standard chemotherapy against advanced non-squamous NSCLC resulted in modest improvement in median progression free survival (PFS) (up to 1.4–4.0 months) and overall response rate (ORR) (a difference of approximately 15%); however, the combination therapies increased the risk of

infrequent serious adverse reactions, such as bleeding events and neutropenia complications [8, 12-15]. In addition, there are no valid predictive biomarkers of response to the treatment with bevacizumab to screen the patients and avoid toxicity in potential non-responders. For that reason, clinical studies and/or molecular translational researches are required to develop screening techniques for the patients, who can really benefit from the treatment with bevacizumab.

Recently, some clinical trials showed that the combination of bevacizumab and first generation EGFR-TKI, erlotinib, significantly increased PFS compared to the treatment with erlotinib alone in patients with *EGFR* mutation [16, 17]. Moreover, *in vitro* studies have demonstrated that oncogenic EGFR signaling can lead to upregulation of *VEGFA* expression that promotes tumor angiogenesis [18, 19]. These evidences suggest that VEGFA plays a crucial role in driving the growth of *EGFR* mutation-positive tumor, and bevacizumab treatment plus cytotoxic chemotherapy could lead to the increased clinical benefit for such patients. However, there have been no reports which evaluate the efficacy of the addition of bevacizumab to platinum-based chemotherapy in patients with *EGFR* mutation. Thus, we analyzed the gene expression levels of *VEGFA* and other key pro-angiogenetic molecules in advanced non-squamous NSCLC patients with *EGFR* mutation, and retrospectively reviewed our cohort to evaluate the clinical efficacy of bevacizumab treatment with carboplatin and pemetrexed (BevCPem) compared with only carboplatin and pemetrexed (CPem) treatment in these patients.

Materials and methods

Study design

This retrospective cohort study was conducted with the approval of the ethical review committee of Nagoya University Hospital and was in accordance with the guidelines of the Declaration of Helsinki [20]. We retrospectively reviewed the medical records of patients between January 2004 and June 2018. Patients enrolled for this study were selected based on the following eligibility criteria: (1) diagnosed as having stage III/IV or recurrent non-squamous NSCLC as confirmed by histological or cytological examination, (2) performance status 0–1, (3) receiving carboplatin and pemetrexed (CPem), or bevacizumab plus carboplatin and pemetrexed (BevCPem), followed by the maintenance treatments of pemetrexed or bevacizumab plus pemetrexed. The demographic and clinical information of eligible patients, including age, sex, smoking history, histological subtype, clinical stage of disease at diagnosis, performance status, treatment outcomes, and *EGFR* mutation status were retrospectively obtained from medical records. Clinical stages were assigned according to the seventh edition of the American Joint Committee on Cancer. The objective tumor responses were evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST), version 1.1.

EGFR Mutation Analysis

Target sequences in exons 18, 19, 20, and 21 were amplified by polymerase chain reaction (PCR) and the PCR products were then subjected to Sanger sequencing to determine the status of *EGFR* mutation. In the Cancer Genome Atlas (TCGA http://cancergenome.nih.gov/) dataset [21, 22], the cases with genetic alterations in exons 18-21 of EGFR kinase domain were classified as *EGFR* mutation-positive (N=62), and the cases with no such detection or silent mutations in the domains were classified as wild-type *EGFR* (N=413). The cases with no data or mutations in other lesions were excluded from further analysis.

Statistical Analysis

PFS and overall survival were estimated by the Kaplan-Meier method, and was defined as the time from the start of the chemotherapy to disease progression or death, whichever was earlier, and data were censored at the last follow-up date. Gehan-Breslow-Wilcoxon and Log-rank tests were implemented to analyze the differences in PFS between patient groups. Categorical data were compared using Fisher's exact test or Chi-square tests, and continuous variables were compared using T-Test or Mann-Whitney U test. Spearman correlation was used to assess the correlation between two variables. Statistical analyses were performed using GraphPad Prism software (Version 7.0), and the differences and correlations were considered statistically

significant at *p-value* < 0.05.

Results

VEGFA is highly expressed in advanced non-squamous NSCLC patients with *EGFR* mutation

To examine the association of *VEGFA* expression to the progression of non-squamous NSCLC in patients harboring *EGFR* mutation, we analyzed the *VEGFA* gene expression level in tumor tissues using TCGA dataset. *VEGFA* mRNA expression in *EGFR* mutation-positive patients with advanced stage non-squamous NSCLC (III + IV) was significantly higher than in patients with stage I (*p-value*= 0.0009) with *VEGFA* expression showing moderate positive correlation with stages (Spearman correlation coefficient r = 0.3901 and *p-value*= 0.0012) (Fig. 1A). Meanwhile, among individuals with wild-type *EGFR*, no significant difference in *VEGFA* expression between patients with advanced and early stages was observed (Fig. 1B). Next, we compared the expressions of 12 pro-angiogenetic genes which have been identified as key molecules regulating tumor angiogenesis, including *VEGFA* and *VEGF* receptors, between advanced stage patients with mutation and wild-type EGFR. Among them, *VEGFA* was significantly higher in patients with *EGFR* mutation compared to patients with wild-type (*p-value*= 0.0476). *Interleukin 6* (*IL-6*) mRNA expression was significantly higher in wild-type patients (Fig. 1C). The expression levels

of the other 10 pro-angiogenetic molecules and *VEGF* receptors did not show statistically significant differences between the two groups (Supplementary Fig 1). These results indicate that tumor angiogenesis in non-squamous NSCLC patients with *EGFR* mutation, compared to wild-type patients, is highly dependent on *VEGFA* expression.

The efficacy of bevacizumab plus carboplatin and pemetrexed chemotherapy in non-squamous NSCLC patients with *EGFR* mutation

To investigate the efficacy of treatment with bevacizumab in patients with *EGFR* mutation, we retrospectively reviewed our cohort to analyze the clinical data of 159 eligible patients with advanced non-squamous NSCLC. The flowchart of patient selection from our medical records is shown in Supplementary Fig. 2, and patient characteristics are summarized in Supplementary Table 1. Among the enrolled patients, the median age was 64.1 years (range 27-80 years), 64.2% were male, and 66.2% had smoking histories. The majority of patients were staged as clinical stage IV (N=96, 60.4%), and the majority histological subtype was adenocarcinoma (N=144, 90.6%). *EGFR* mutations were observed in 53 patients (33.3%). In this cohort, 55 patients were treated with BevCPem, and 104 patients with CPem as a first-line therapy without molecular-targeted agents including EGFR-TKIs. There were no significant differences in median age, gender, smoking status, stage, histological subtype, and *EGFR* mutation status between BevCPem

and CPem groups, however, the number of patients with performance status (PS) =0 was higher in BevCPem group (Supplementary Table 1 and 2). PFS in patients of the BevCPem group was significantly longer than that of the CPem group patients (Wilcoxon *p-value*=0.0028 and Log-rank *p-value*=0.0485; Fig. 2A), with median PFS being 8.4 and 5.3 months, respectively. The ORRs in the BevCPem and CPem groups were 43.6% (N=24/55) and 32.7% (N=34/104), respectively.

To evaluate the clinical benefits of the administration of bevacizumab in patients with *EGFR* mutations, we compared the efficacies of BevCPem and CPem between *EGFR*-mutant and wild-type patients. Patient characteristics of BevCPem group did not show significant differences (Table 1), however, in the CPem group, *EGFR* mutation status was significantly correlated to gender and smoking status (Table 1). The PFS of the BevCPem group was greater in patients with *EGFR* mutation than wild-type patients (10.5 vs 6.6 months; Wilcoxon *p-value* = 0.0278 and Log-rank *p- value* = 0.0730; Fig. 2B). Meanwhile, the difference in PFS between *EGFR*-mutant and wild-type patients of the CPem group was not significant (6.6 vs 4.5 months; Wilcoxon *p-value*=0.1822 and Log-rank *p-value*=0.5081; Fig. 2C). The ORRs in BevCPem group patients were 54.5% (12/22) and 36.4% (12/33) for *EGFR* mutation and wild-type patients, respectively; whereas, the ORRs in CPem group patients were 35.5% (11/31) and 28.8% (23/73) *EGFR* mutation and wild-type patients, respectively (Table 2). We further compared overall survival in

patients with stage IV between the BevCPem and CPem groups, however, the differences in both *EGFR*-mutant and wild-type patients were not statistically significant (Supplementary Fig. 3).

Discussion

Predictive biomarkers of bevacizumab treatment have been, historically, not established. High levels of *VEGFA* expression indicates a dependency of the tumor angiogenesis on *VEGFA*, however, it is known to serve as a prognostic rather than a predictive marker of bevacizumab [23, 24]. To date, despite the predominant role of VEGFA, tumor angiogenesis has been recognized as a highly complex biological process involving multiple factors [10, 11, 25]. Among the key pro-angiogenic molecules, our analysis showed only *VEGFA* expression to be significantly higher in *EGFR* mutation patients compared to wild-type patients. Furthermore, expression level of *VEGFA* mRNA showed positive correlation with advanced stages of *EGFR* mutation-positive lung cancer. These results were consistent with previous *in vitro* studies, which revealed the association of oncogenic EGFR signaling with the upregulation of *VEGFA* expression [18, 19]. On the other hand, our analysis demonstrated that *IL-6* mRNA expression was significantly increased in *EGFR* wild-type patients. Previous studies have reported that oncogenic *Ras* mutation leads to the increase of *IL-6* mRNA expression, inducing thereby its secretion [26, 27]. In addition, chronic obstructive pulmonary disease (COPD)-type airway inflammation mainly from cigarette smoke was

associated with *IL-6* upregulation, promoting the tumor microenvironment including angiogenesis [28-30]. We conducted further analysis using TCGA dataset and found that *IL-6* expression showed significantly higher in current and former smokers than in non-smokers (Supplementary Fig. 4), suggesting cigarette smoke might be one of major cause of IL-6 induction. *EGFR* mutations are well known to less association with a history of smoking and more to be mutually exclusive from oncogenic *Ras* mutation, such as *KRAS* and *NRAS*. Our findings are consistent with the previous reports and indicate that oncogenic driver mutations can be associated with discrete phenotypes of tumor angiogenesis. We did not, however, investigate the secretion levels of the pro-angiogenetic factors, which warrant further translational research for the clarification of *EGFR* mutation-positive tumors development.

This retrospective analysis showed the superiority of BevCPem over CPem in our cohort. The ORRs and median PFS in the BevCPem group were consistent with a previous single-arm phase II trial [31], and the efficacy of bevacizumab with CPem was similar to bevacizumab plus other platinum-double chemotherapies [8, 12-14]. Positive *EGFR* mutation status could be a weak predictive marker for the response to front-line platinum-based chemotherapy [32, 33] and, consistent to this, our study showed that the ORRs and median PFS of the CPem group were slightly better in *EGFR*-mutant patients as opposed to wild-type carriers. On the other hand, in the BevCPem group, median PFS in *EGFR* mutation patients was significantly longer, demonstrated by the Wilcoxon

test, and the ORR was approximately 18% higher compared with those of wild-type patients. These results indicated that bevacizumab combined with platinum-based chemotherapy could lead to greater benefits in *EGFR* mutation patients. Our study did not show the difference in overall survival in *EGFR* mutation patients to be statistically significant between the BevCPem and CPem groups. This could be due to the sample size and the length of survival of patients with *EGFR* mutation. In addition, other limitations, such as retrospectively reviewed medical records collected from a single center, may be influential factors. To investigate survival benefit of the addition of bevacizumab to platinum-based chemotherapy for the patients with *EGFR* mutation, further prospective studies are needed.

More recently, randomized phase III trials and meta-analyses have shown that immune checkpoint inhibitor (ICI) monotherapy in NSCLC patients has been less effective in patients, harboring *EGFR* mutations than in wild-type *EGFR* patients [34]. Meanwhile, in subgroup analysis of IMpower 150 trial, atezolizumab plus bevacizumab, carboplatin, and paclitaxel (ABCP) therapy significantly improved PFS for *EGFR* mutation NSCLC patients compared to bevacizumab with carboplatin and paclitaxel (BCP) therapy. These data suggest that the addition of bevacizumab plus ICI to chemotherapy would be beneficial for the treatment of NSCLC with *EGFR* mutations [35, 36]. The effect of cytotoxic chemotherapy with bevacizumab, resulting in the tumor mass shrinkage, can contribute to anti-tumor immune activation through the release of tumor antigens

and reduction of tumor-associated immunosuppression[37]. Our study showed that the ORRs in the BevCPem groups were considerably higher in patients with *EGFR* mutation compared to those with wild-type. Therefore, our result is consistent with the subgroup analysis of the IMpower 150 trial, which indicated that bevacizumab could enhance the therapeutic efficacy of chemotherapy with ICI in NSCLC harboring *EGFR* mutations. Our findings provide important information for applying combination therapies of bevacizumab with cytotoxic chemotherapy and ICI for patients with *EGFR* mutation.

Conclusion

Our study indicates that tumor angiogenesis in *EGFR* mutation-positive lung cancer is highly dependent on *VEGFA* expression, and the addition of bevacizumab to platinum-based chemotherapy provides greater clinical benefits for patients with *EGFR* mutation. These results are useful to screen appropriate patients for cytotoxic chemotherapy in combination with bevacizumab. The role of *EGFR* mutation as a predictive biomarker during treatment with bevacizumab requires further investigation.

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Figure legends

Fig. 1. (A) mRNA expression levels of vascular endothelial growth factor A (*VEGFA*) in non-squamous non-small-cell lung cancer (NSCLC) patients harboring epidermal growth factor receptor (*EGFR*) mutation with stage I (N=29), with stage II (N=17), with stage III (N=12), and with stage IV (N=4) (B) mRNA expression levels of *VEGFA* in NSCLC patients harboring *EGFR* wild-type with stage I (N=228), with stage II (N=96), with stage III (N=70), and with stage IV (N=19). (C) mRNA expression levels of *VEGFA* and IL-6 in advanced non-squamous NSCLC patients harboring *EGFR* mutation (N=16) and wild-type (N=89). *p-value*s were calculated by Mann-Whitney U test. Spearman correlation was used to assess the correlation between two variables.

Fig. 2. Kaplan-Meier plot of progression-free survival (PFS) in the total number of patients (A), in the patients treated with bevacizumab plus carboplatin and pemetrexed chemotherapy (BevCPem) (B), and in the patients treated with carboplatin and pemetrexed chemotherapy (CPem) (C).

Supplementary Fig. 1. mRNA expression levels of *ANGPT2*, *CXCL12*, *FGF2*, *IL-1B*, *IL-8*, *PDGFB*, *PIGF*, *VEGFB*, *VEGFC*, *VEGFD*, *VEGFR1*, and *VEGFR2* in advanced non-squamous NSCLC patients with *EGFR* mutation (N=16) and wild-type (N=89). *P-values* were calculated by Mann-Whitney U test. *ANGPT2*: angiopoietin 2, *CXCL12*: C-X-C motif chemokine ligand *12*, *FGF2*: fibroblast growth factor 2, *IL-1B*: interleukin 1 beta, *IL-8*: interleukin 8, *PDGFB*: platelet derived growth factor subunit B, *PIGF*: phosphatidylinositol glycan anchor biosynthesis class F, *VEGFB*: vascular endothelial growth factor B, *VEGFC*: vascular endothelial growth factor C, *VEGFD*: vascular endothelial growth factor D, *VEGFR1*: vascular endothelial growth factor receptor 1, and *VEGFR2*: vascular endothelial growth factor receptor 2

Supplementary Fig. 2.

Flowchart of patient selection in this cohort.

Supplementary Fig. 3.

Kaplan-Meier plot of overall survival in the patients with stage IV, treated with bevacizumab plus carboplatin and pemetrexed chemotherapy (BevCPem), and carboplatin and pemetrexed chemotherapy (CPem).

Supplementary Fig. 4.

(A) mRNA expression levels of *IL-6* in NSCLC patients, characterized as non-smokers (N=75), with current smokers (N=118), and with former smokers (N=301). (B) mRNA expression levels of *IL-6* in NSCLC patients harboring *EGFR* wild-type with non-smokers (N=41), with current smokers (N=103), and with former smokers (N=260). *p-value*s were calculated by Mann-Whitney U test.

Table 1. Clinical characteristics of 159 NSCLC patients with *EGFR* mutations or *EGFR* wild type

Characteristic	BevCPem				CPem			
	Total	***EGFR* mutation n (%)**	***EGFR* wild n (%)**	**P‡**	**Total**	***EGFR* mutation n (%)**	***EGFR* wild n (%)**	**P‡**
Total	55	22 (40.0)	33 (60.0)		104	31 (29.8)	73 (70.2)	
Median Age (Range)	62.0	62.2 (47-74)	61.9 (41-80)	0.9095	65.3	67.8 (40-76)	64.2 (27-79)	0.0768
Gender								
Male	31	9 (29.0)	22 (71.0)	0.0954	71	12 (16.9)	59 (83.1)	<0.0001
Female	24	13 (54.2)	11 (45.8)		33	19 (57.6)	14 (42.4)	
Smoking status*								
Current	12	4 (33.3)	8 (66.7)	0.5658	28	3 (10.7)	25 (89.3)	<0.0001
Former	25	9 (36.0)	16 (64.0)		37	5 (13.5)	32 (86.5)	
Never	18	9 (50.0)	9 (50.0)		36	22 (61.1)	14 (38.9)	
PS								
0	40	17 (42.5)	23 (57.5)	0.7582	57	21 (36.8)	36 (63.2)	0.0910
1	15	5 (33.3)	10 (66.7)		47	10 (21.3)	37 (78.7)	
Stage								
IIIA	2	1 (50.0)	1 (50.0)	0.4617	6	1 (16.7)	5 (83.3)	0.7177
IIIB	1	0 (0.0)	1 (100.0)		11	4 (36.4)	7 (63.6)	
IV	34	16 (47.1)	18 (52.9)		62	17 (27.4)	45 (72.6)	
Recurrence	18	5 (27.8)	13 (72.2)		25	9 (36.0)	16 (64.0)	
Subtype								
Adenocarcinoma	53	22 (41.5)	31 (58.5)	Not Available	91	31 (34.1)	60 (65.9)	0.0427
Large cell carcinoma	0	0 (0.0)	0 (0.0)		3	0 (0.0)	3 (100.0)	
NSCLC	2	0 (0.0)	2 (10.0)		10	0 (0.0)	10 (100.0)	

‡P values were calculated by T-Test, Fisher's exact test or Chi-square test.

*Information was not available for 3 cases

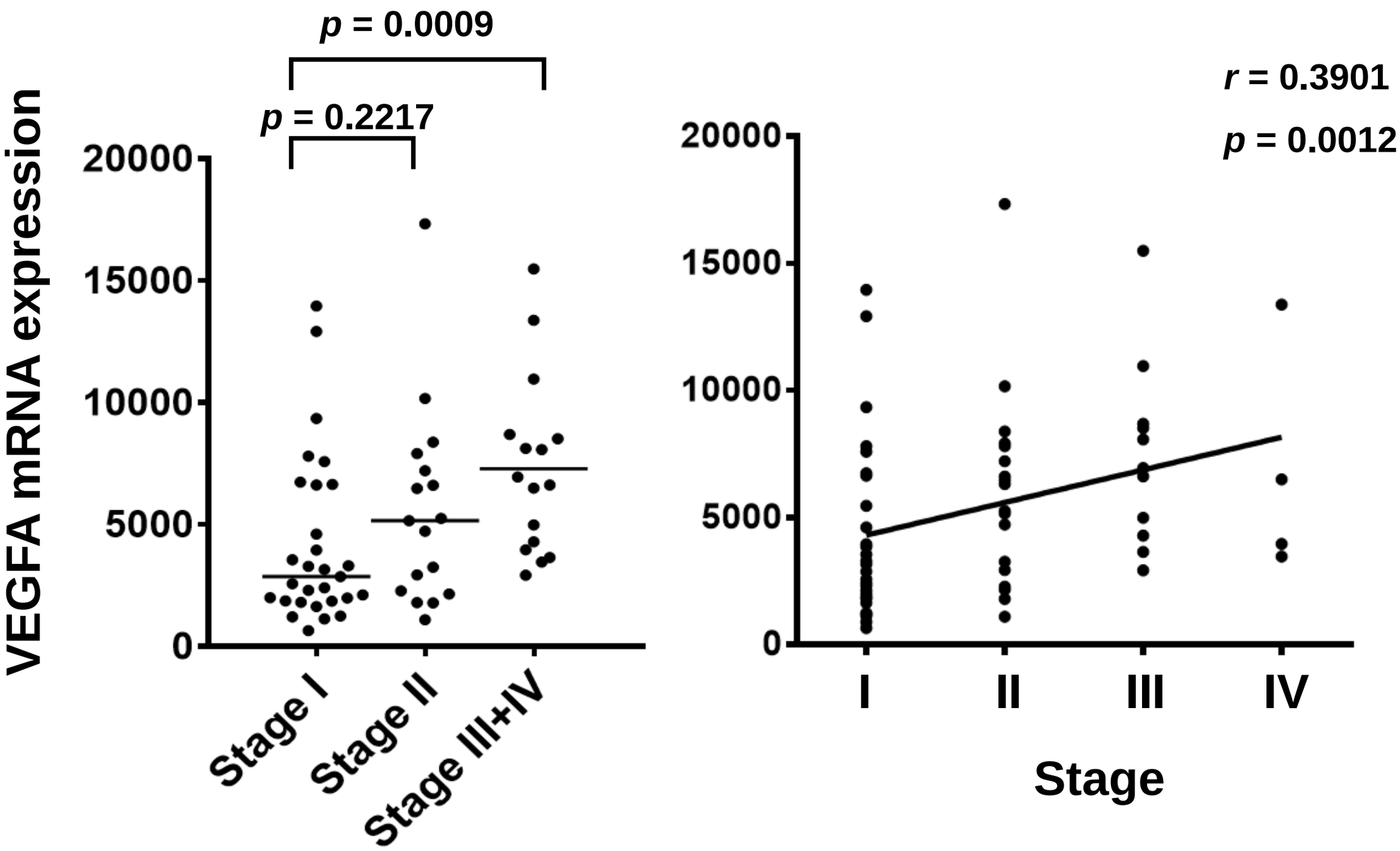
Table 2. Response to BevCPem or CPem in advanced NSCLC patients

	BevCPem				**CPem**			
	***EGFR* mutation**		***EGFR* wild**		***EGFR* mutation**		***EGFR* wild**	
	N=22	%	N=33	%	N=31	%	N=73	%
Best response								
CR	0	0.0	0	0.0	0	0.0	1	1.4
PR	12	54.5	12	36.4	11	35.5	22	27.4
SD	9	40.9	15	45.4	16	51.6	27	38.3
PD	1	4.5	6	18.2	4	12.9	23	32.9
ORRs	12	54.5	12	36.4	11	35.5	23	28.8
DCRs	21	95.5	27	81.8	27	87.1	50	67.1

CR; complete response, PR; partial response, SD; stable disease, PD; progression disease,
ORR; overall response rate, DCR; disease control rate

Figure 1

1A



1B

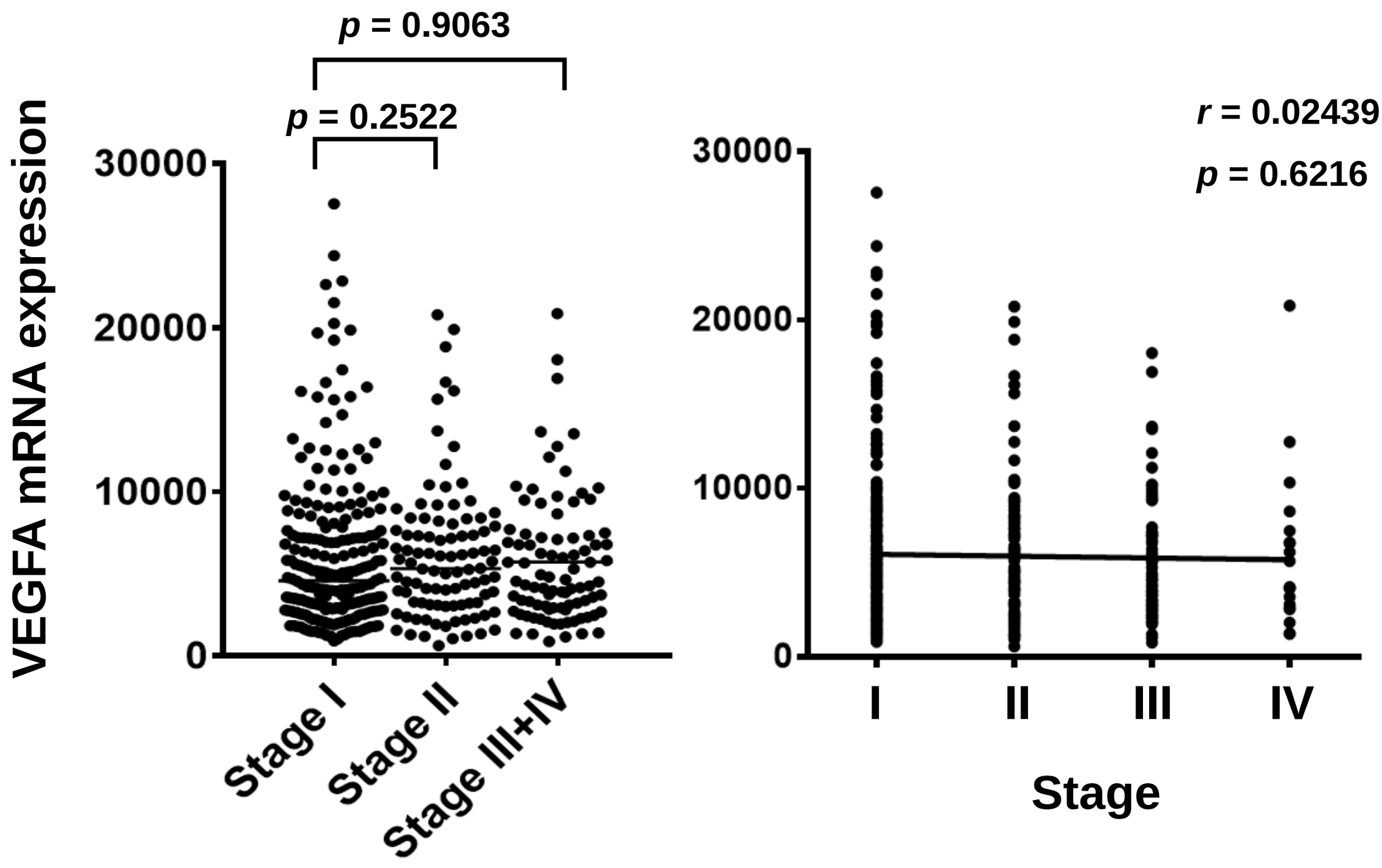
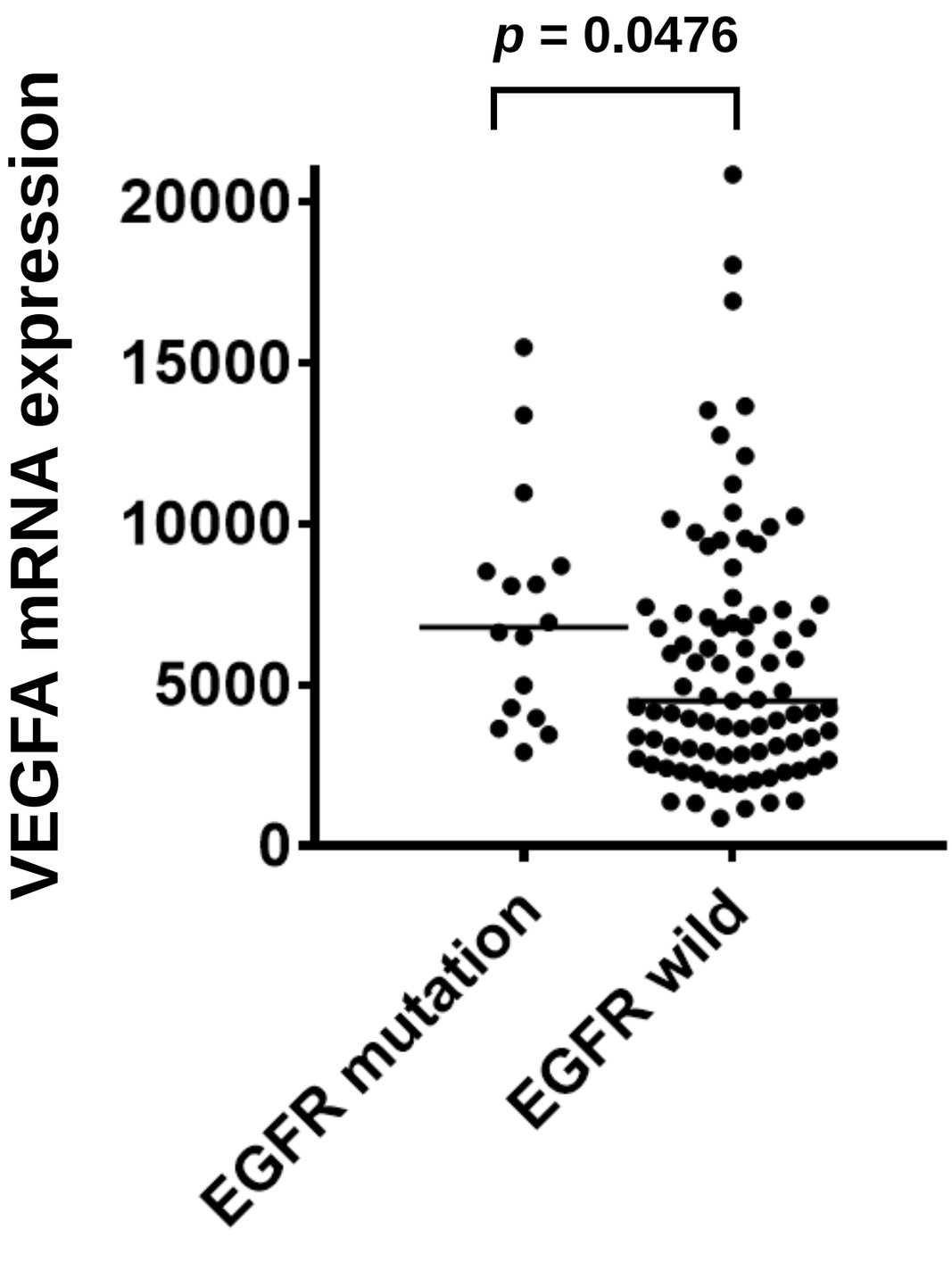


Figure 1


1C
p = 0.0476
VEGFA mRNA expression
20000
15000
10000
5000
0
EGFR mutation
EGFR wild

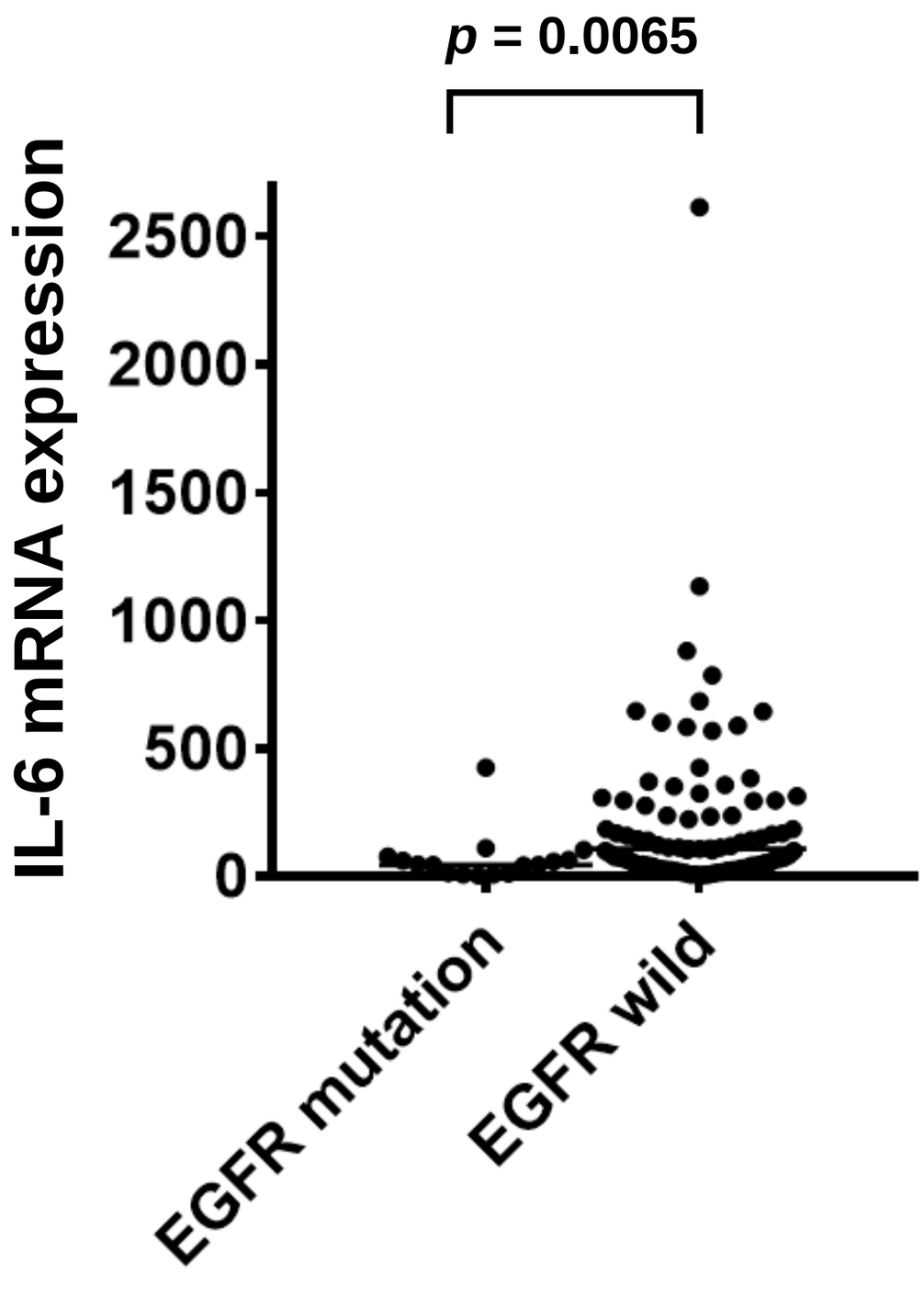
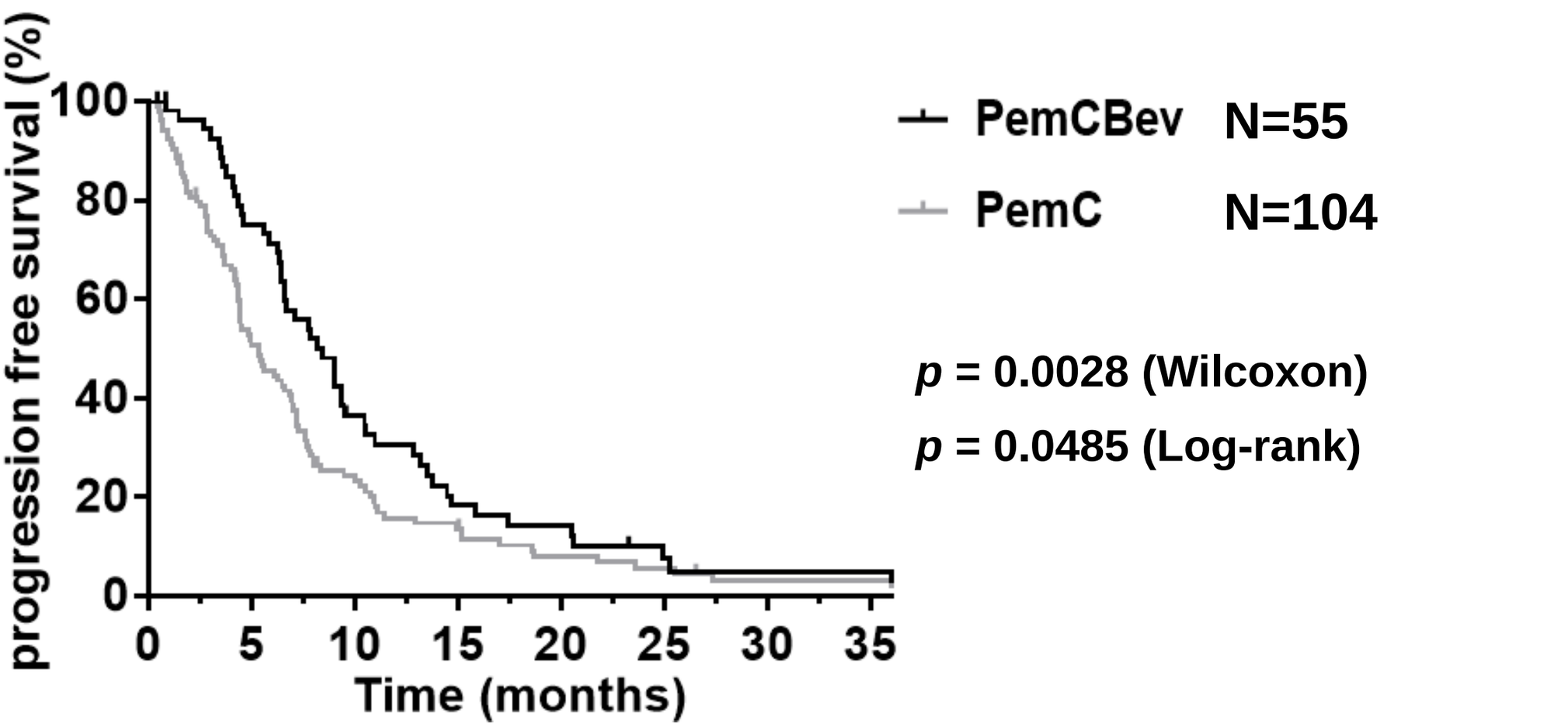
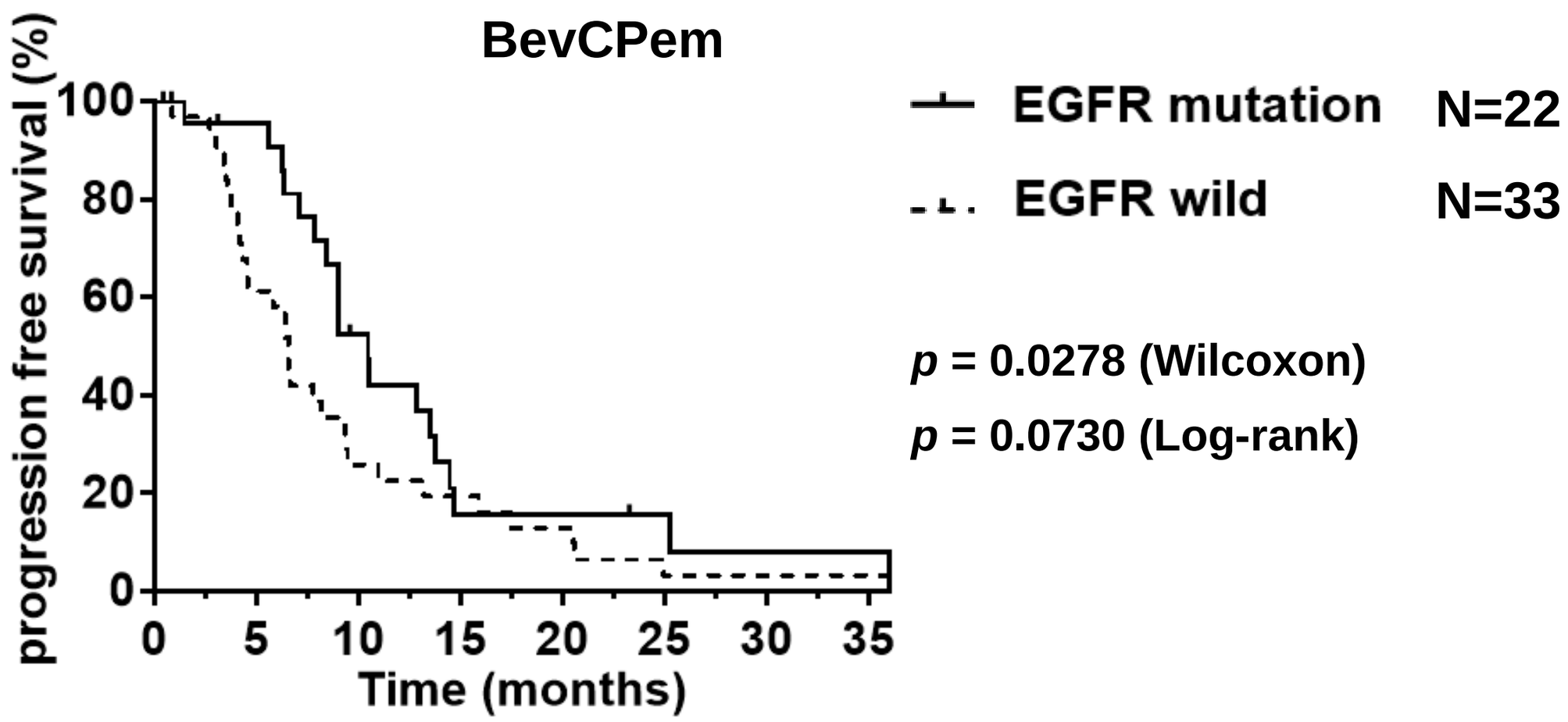

p = 0.0065
IL-6 mRNA expression
2500
2000
1500
1000
500
0
EGFR mutation
EGFR wild

Figure 2

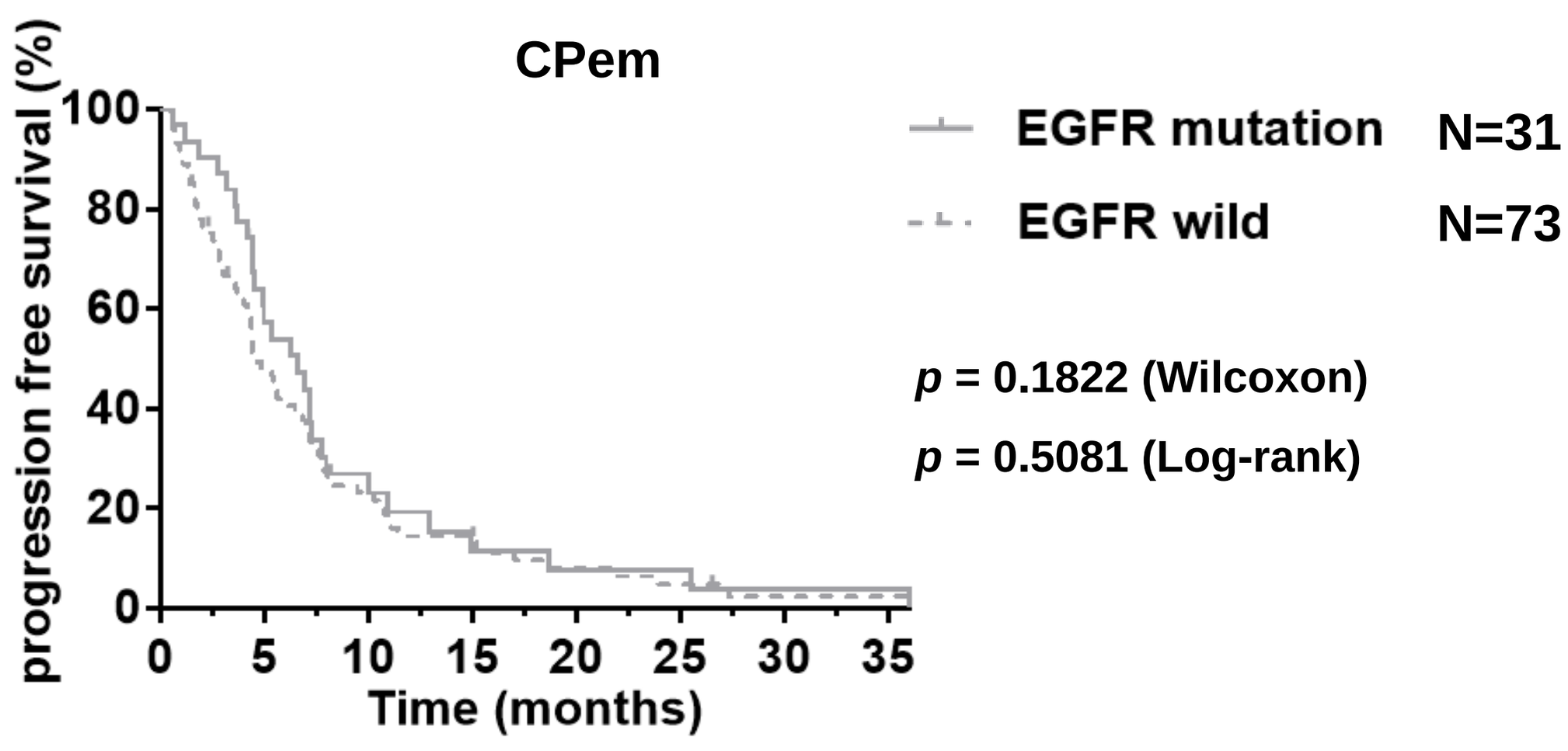
2A



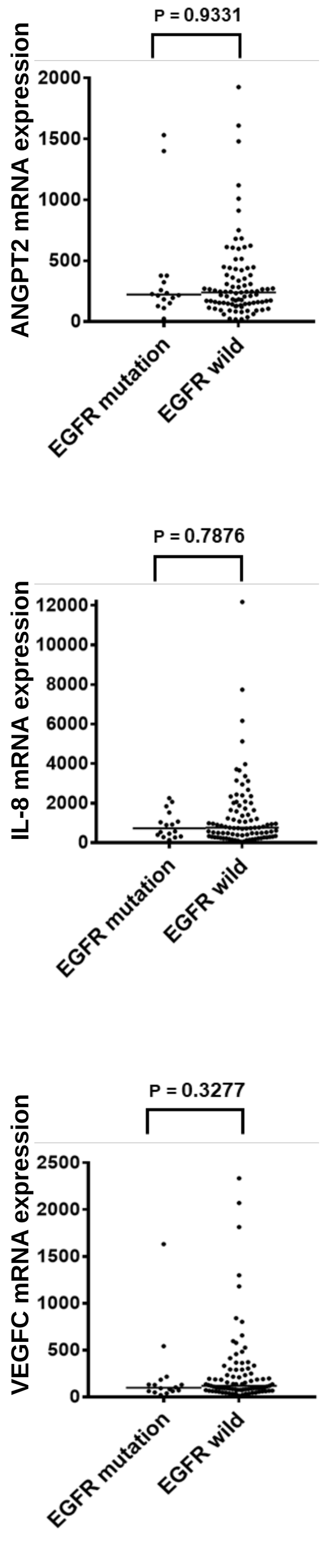
2B

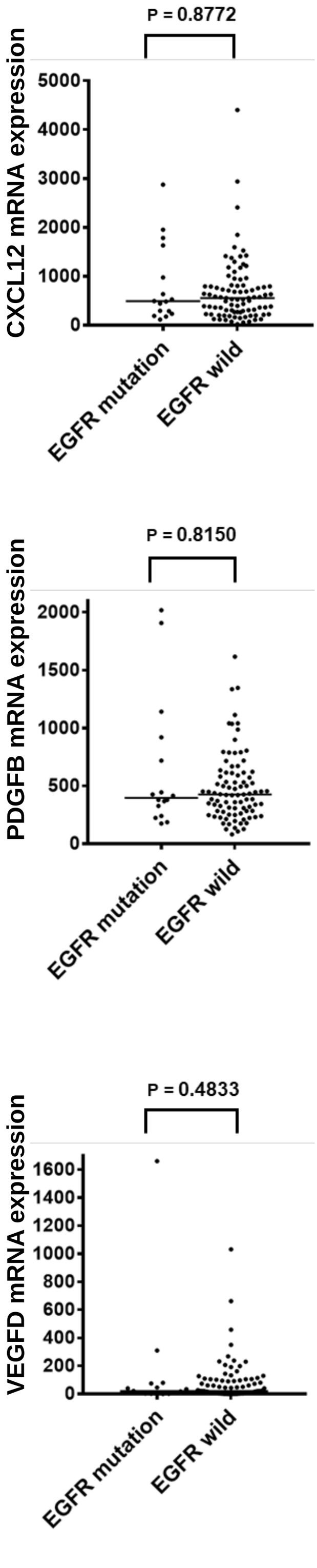


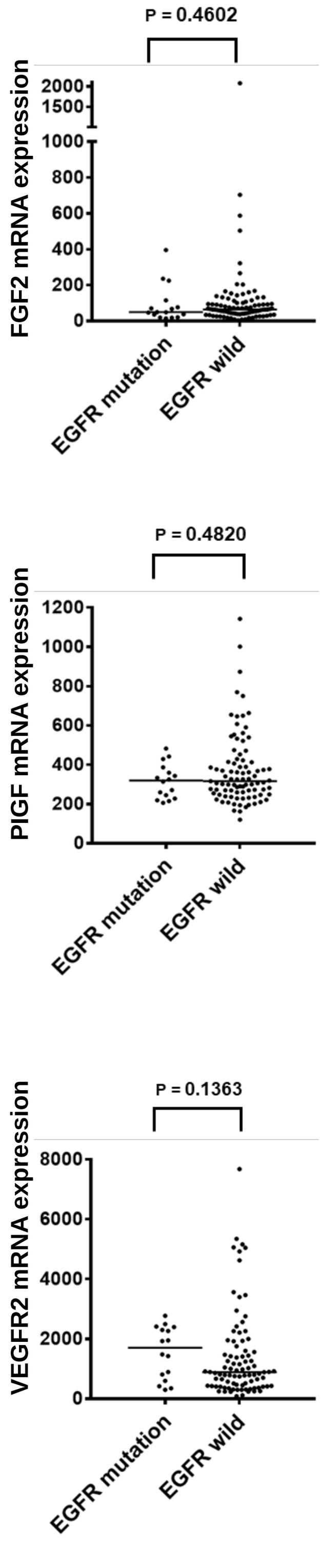
2C

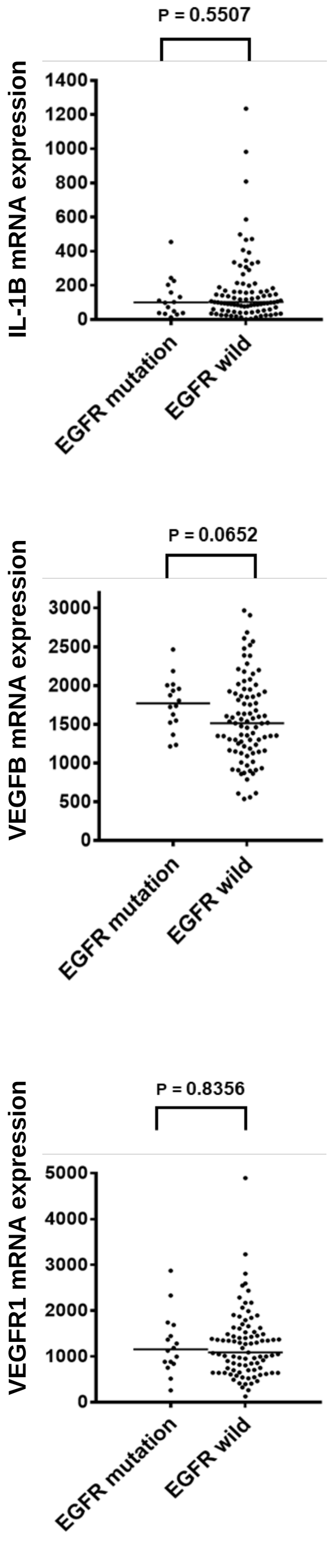


Supplementary Figure 1

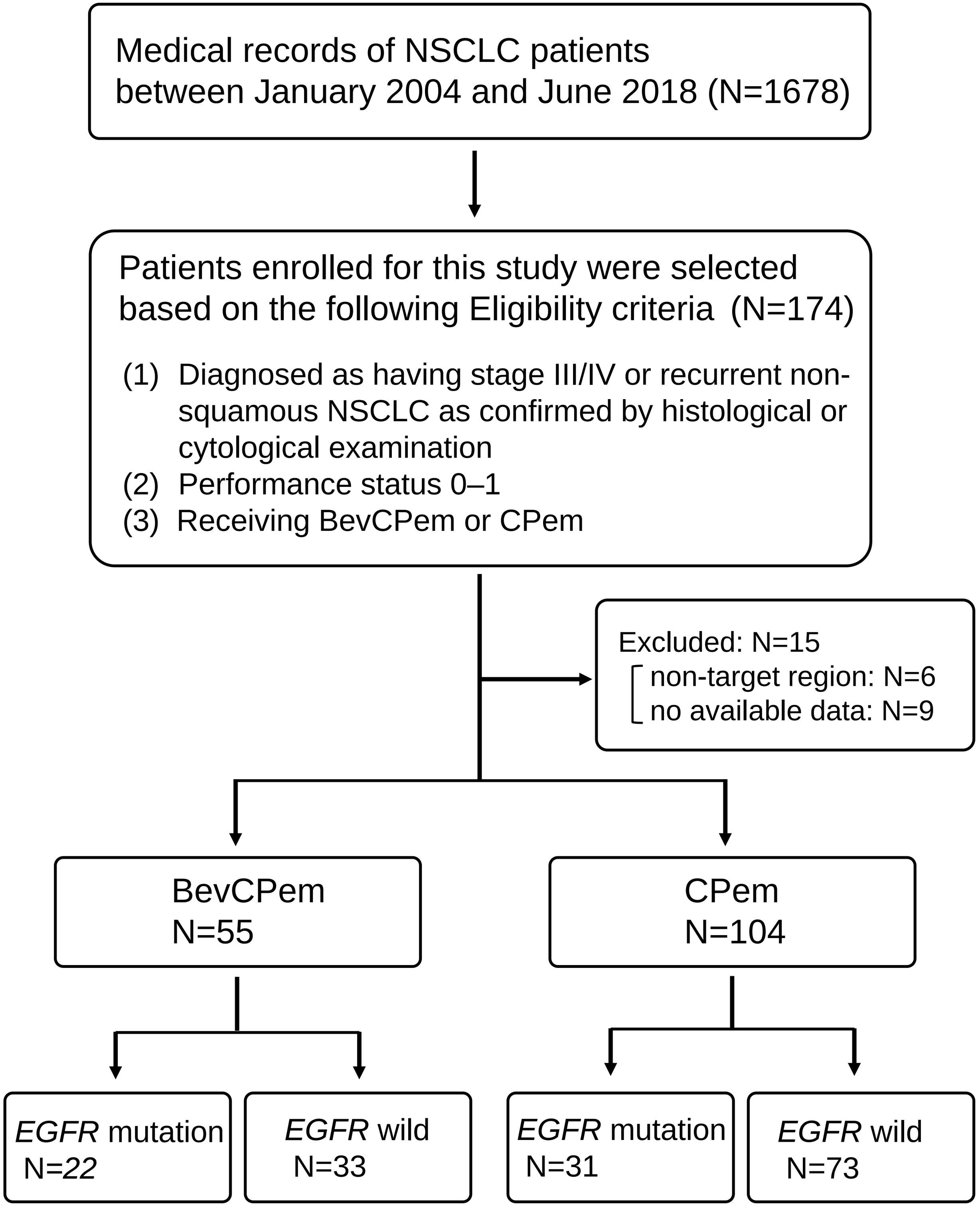




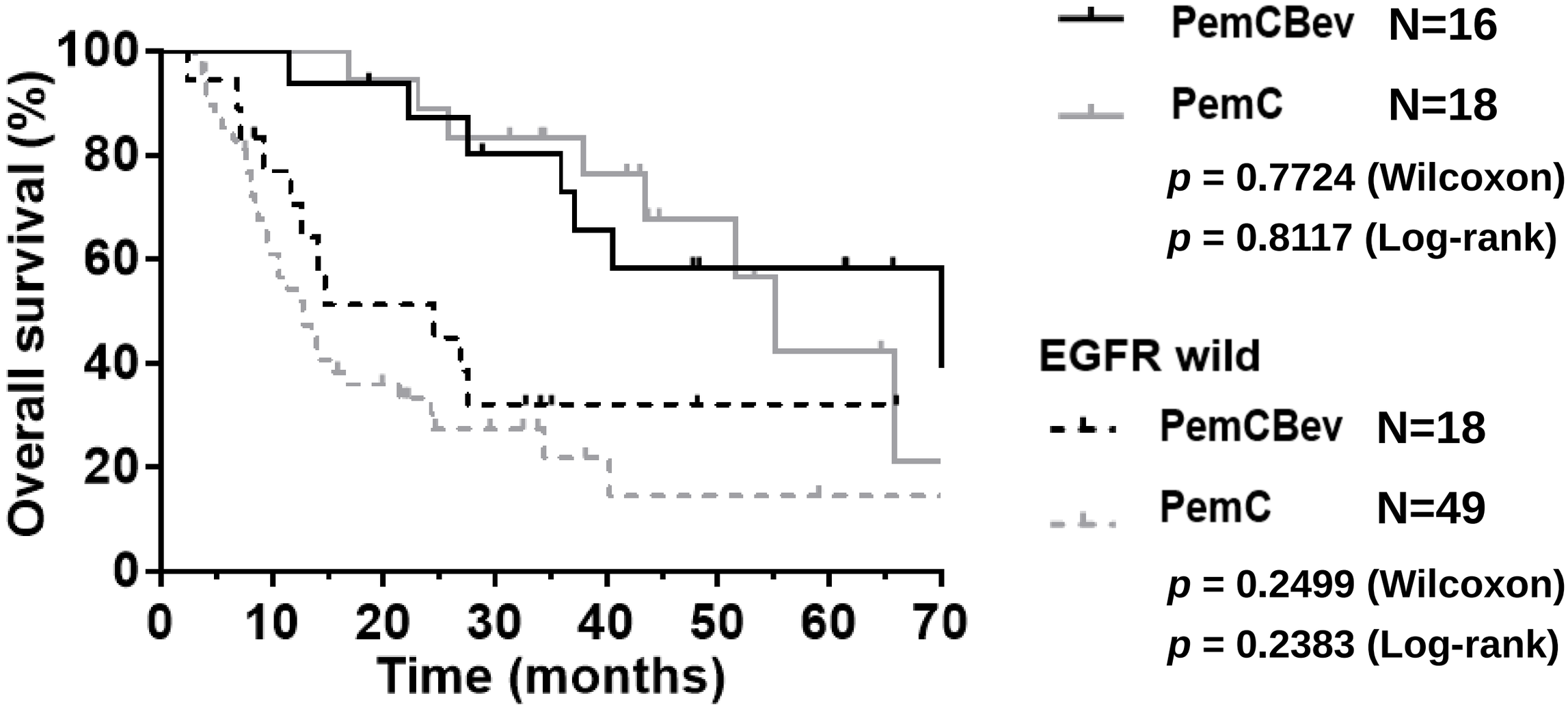




Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4