Thoracic Cancer

Open Access

Thoracic Cancer ISSN 1759-7706

STUDY PROTOCOL

Prospective exosome-focused translational research for afatinib study of non-small cell lung cancer patients expressing *EGFR* (EXTRA study)

Yusuke Okuma[1], Kei Morikawa[2], Hisashi Tanaka[3], Takuma Yokoyama[4], Hidetoshi Itani[5], Kazuya Horiuchi[6], Hideyuki Nakagawa[7], Nobumasa Takahashi[8], Akihiro Bessho[9], Kenzo Soejima[10], Kazuma Kishi[11], Akira Togashi[12,13], Yae Kanai[14], Koji Ueda[15], Katsuhisa Horimoto[13], Noriyuki Matsutani[16] & Nobuhiko Seki[17]

1 Departments of Thoracic Oncology and Respiratory Medicine, Tokyo Metropolitan Cancer and Infectious Diseases Center, Komagome Hospital, Tokyo, Japan
2 Department of Internal Medicine, Division of Respiratory Medicine, St. Marianna University School of Medicine, Kanagawa, Japan
3 Department of Respiratory Medicine, Hirosaki University Graduate School of Medicine, Aomori, Japan
4 Department of Respiratory Medicine, Kyorin University School of Medicine, Kyorin University Hospital, Tokyo, Japan
5 Department of Respiratory Medicine, Ise Red Cross Hospital, Mie, Japan
6 Respiratory Disease Center, Showa University Northern Yokohama Hospital, Kanagawa, Japan
7 Department of Respiratory Medicine, National Hospital Organization, Hirosaki Hospital, Aomori, Japan
8 Department of General Thoracic Surgery, Saitama Cardiovascular and Respiratory Center, Saitama, Japan
9 Department of Respiratory Medicine, Japanese Red Cross Okayama Hospital, Okayama, Japan
10 Clinical and Translational Research Center, Keio University Hospital, Tokyo, Japan
11 Department of Respiratory Medicine, Respiratory Center, Toranomon Hospital, Tokyo, Japan
12 Nippon Boehringer Ingelheim Co., Ltd., Tokyo, Japan
13 Molecular Profiling Research Center for Drug Discovery, National Institute of Advanced Industrial Science and Technology (AIST), Tokyo, Japan
14 Department of Pathology, Keio University School of Medicine, Tokyo, Japan
15 Project for Realization of Personalized Cancer Medicine, Cancer Precision Medicine Center, Japanese Foundation for Cancer Research, Tokyo, Japan
16 Department of Surgery, Teikyo University School of Medicine, Tokyo, Japan
17 Division of Medical Oncology, Department of Internal Medicine, Teikyo University School of Medicine, Tokyo, Japan

Keywords
Afatinib; epidermal growth factor receptor; exosome; OMIC; translational research.

Correspondence
Nobuhiko Seki, Division of Medical Oncology, Department of Internal Medicine, Teikyo University School of Medicine, 2-11-1, Kaga, Itabashi-ku, Tokyo 173-8606, Japan.
Tel: +81 3 3964 1211
Fax: +81 3 3964 9177
Email: nseki@med.teikyo-u.ac.jp

Received: 22 September 2018;
Accepted: 3 November 2018.

doi: 10.1111/1759-7714.12923

Thoracic Cancer **10** (2019) 395–400

Abstract

Patients with *EGFR*-mutated non-small cell lung cancer (NSCLC) exhibit resistance to EGFR-tyrosine kinase inhibitors (TKIs) within 9–14 months of therapy. Recently, *EGFR*-mutated NSCLC has demonstrated the potential for heterogeneity; therefore, the manner of clonal heterogeneity may impact the duration of progression-free and overall survival and other parameters affecting EGFR-TKI treatment efficacy. However no predictive biomarker of these favorable treatment efficacies has been identified to date. The exosome-focused translational research for afatinib (EXTRA) study aims to identify a novel predictive biomarker and a resistance marker for afatinib by analyzing data from association studies of the clinical efficacy of afatinib and four "OMICs" (genomics, proteomics, epigenomics, and metabolomics) using peripheral blood from patients treated with afatinib. This study aims to: (i) conduct comprehensive multi-OMIC analyses in a prospective clinical trial, and (ii) focus on both sera/plasma and exosome as a source for OMIC analyses to identify a novel predictor of the efficacy of a specific drug. To eliminate the carryover bias of prior treatment, systemic treatment-naïve patients were enrolled. The candidates to be screened for biomarkers comprise a discovery cohort of 60 patients and an independent validation cohort of 40 patients. The EXTRA study is the first trial to screen novel biomarkers of longer treatment efficacy of EGFR-TKIs using four-OMICs analyses, focusing on both "naked or free" molecules and "capsulated" exosomal components in serially collected peripheral blood.

Introduction

Lung cancer is the leading cause of cancer-related death and globally 85% of all lung cancer patients are diagnosed with non-small cell lung cancer (NSCLC). Recent advances in targeted therapy for NSCLC have led to the development of precision medicine with high efficacy and decreased toxicity at reasonable cost. In the 2000s, targeted therapy was developed for EGFR. Since 2009, *EGFR* mutation discovery has increased the survival of patients treated with EGFR-targeted therapy more than three-fold compared to those treated with conventional cytotoxic chemotherapy.[1,2]

Patients with *EGFR*-mutated advanced NSCLC demonstrate 9–11 months of progression-free survival (PFS) with first-generation EGFR-tyrosine kinase inhibitors (TKIs).[3] Recently, two important trials using second-generation EGFR-TKIs reported > 12 months of PFS. It is important to note that the Kaplan–Meier curve deviates after 12 months, resulting in a 20% PFS rate after two years with second-generation EGFR-TKIs.[4,5]

Afatinib, a second-generation EGFR-TKI, targets the adenosine triphosphate-binding site of the tyrosine kinase domain of EGFR, leading to irreversible inhibition of EGFR activation. Moreover, afatinib selectively targets ERBB2, ERBB3, and ERBB4, as well as EGFR (ERBB1). Signal transduction by the ERBB family is presumed to be overexpressed in cancer cells and is related to cancer-mediated proliferation, angiogenesis, apoptosis inhibition, invasion, and metastasis. Afatinib is thought to inhibit signal transduction accompanying tyrosine kinase phosphorylation following the heterodimerization of ERBB family members (ERBB1–ERBB4), but is not functional with the homodimerization of ERBB3. Originally, second-generation EGFR-TKIs were developed to treat patients with a broad spectrum of *EGFR* gene mutations and to overcome the EGFR-TKI resistance resulting from acquired EGFR T790M mutation. However, the clinical efficacy of afatinib to overcome the resistance compared to first-generation EGFR-TKIs has proven disappointing, because the inhibitory activity of afatinib on EGFR T790M has been insufficient in a clinical setting.[6] Despite this drawback, accumulating essential scientific evidence regarding the use of first-generation EGFR-TKIs suggests that a single *EGFR* mutation is responsible for activation in NSCLC. The results of clinical trials of afatinib have gradually suggested clinical differences in each EGFR-TKI. First, the presence of exon 19 and 21 *EGFR* mutations exhibits a differential therapeutic effect when using EGFR-TKIs. The overall survival (OS) of patients with advanced *EGFR* mutations treated with second-generation afatinib was longer in two combined phase III trials.[7] Second, as previously described, in clinical trials comparing afatinib and dacomitinib, patients had similar median PFS but the two-year PFS rate was greater when using a second-generation EGFR-TKI than when using a first-generation EGFR-TKI. In addition, osimertinib, a third-generation EGFR-TKI proven in the AURA-3 study to overcome T790M with a common EGFR-TKI resistance mechanism,[8] demonstrated superior PFS compared to first-generation EGFR-TKIs in patients with previously untreated *EGFR* mutation-positive NSCLC in the FLAURA study.[9] Although OS in the FLAURA study is not yet conclusive, osimertinib is considered the standard treatment for previously untreated common *EGFR* mutation-positive NSCLC. The positioning of osimertinib is thus established but not definitive. In the GIOTAG study (NCT03370770), which used real-world data, an EGFR-TKI sequential strategy of afatinib followed by osimertinib showed 46.7 months of survival when a T790M mutation appeared.[10] Moreover, new evidence of post-osimertinib resistance has demonstrated low plausibility of EGFR-TKI rechallenge and atezolizumab in combination with carboplatin/paclitaxel/bevacizumab in subgroup analysis of *EGFR* mutation (Impower150). In the analysis, patients previously treated with osimertinib were not included, and the reproducibility of the trial is uncertain.[11] Immune checkpoint inhibitors for *EGFR* mutations have lower efficacy than those harboring driver mutations; therefore, the optimal "sequential" strategy for *EGFR* mutation-positive NSCLC, including EGFR-TKIs and immune checkpoint inhibitors, is yet to be confirmed based on biological plausibility and new biomarker exploration.

In 1983, exosomes were reported as granular molecules used to excrete unwanted cellular substances;[12] however, in 2008, it was revealed that exosomes deliver capsules including microRNAs and other molecules.[13] Exosomes are now regarded as a means of intercellular communication, whereas it was previously thought that intercellular communication occurred via proteins (e.g. cytokines or hormones). Exosomes consist of proteins, nucleic acids, lipids, and other cell components[14] and are secreted in various biological fluids, including blood, saliva, urine, and breast milk.[15] The function of exosomes is related to various biological processes, including antigen presentation,[16] apoptosis,[17] angiogenesis,[18] inflammation,[19] and coagulation.[20] Moreover, specific gene transduction and the exchange of proteins or lipids to target cells can induce downstream signal transduction.[13,21,22] For example, exosome-containing encapsulated nucleic acids (e.g. microRNA and messenger RNA) derived from cancer cells can promote cancer progression, influence metastatic organs,[23] and inhibit immune responses.[13,21,22] Moreover, it is suggested that exosomes are stable biomarkers because of their lipid bilayer, which protects them from enzymatic degradation.

It remains unclear which predictive factors contribute to longer survival or how resistance to afatinib is acquired. In a phase II study comprising patients with platinum-resistant metastatic urothelial cancers, afatinib was associated with better treatment efficacy in patients harboring *ERBB2* (HER2/neu) and *ERBB3* mutations compared to those expressing wild-type copies of these genes.[24] In a phase II study of patritumab (U3-1287, an anti-ERBB3 antibody) and erlotinib combination treatment, 24% of previously treated NSCLC patients harboring *EGFR* mutations demonstrated elevated levels of heregulin, a ERBB3 ligand.[25] This investigation suggested that 20–30% of patients with previously treated NSCLC harbor an *EGFR* mutation and demonstrate activated ERBB3 signaling with elevated levels of heregulin. Afatinib potentially inhibits the activated ERBB3 signaling pathway in vivo, whereas erlotinib does not. A retrospective analysis reported that among patients with an *EGFR* mutation, those who also had a *p53* mutation had shorter survival.[26]

With regard to the mechanism of acquired resistance, it remains unclear why a T790M mutation is acquired following treatment with a first-generation EGFR-TKI[27–29] or why C797S and L792F mutations are acquired following treatment with osimertinib, a third-generation EGFR-TKI.[30]

To clarify the different mechanisms underlying treatment efficacy and the development of resistance to EGFR–TKIs, a translational approach using a combination of "OMIC" analyses, including genomics, proteomics, epigenomics, and metabolomics, is required. The results of this large cohort, multi-center institutional exosome-focused translational research for afatinib (EXTRA) study could provide strategies to improve the clinical outcomes for patients with advanced NSCLC who have an *EGFR* mutation.

Methods/Design

Objectives

We intend to investigate the mechanisms underlying long-lasting treatment efficacy and acquired resistance to afatinib by evaluating free and exosome-encapsulating molecules (e.g. DNA, proteins, and metabolites) in the peripheral blood of patients with advanced or recurrent NSCLC with an *EGFR* mutation. Multi-OMIC analyses will be applied to the samples to conduct an association study of treatment efficacy.

Our primary objective is to identify a predictive biomarker and a resistant factor associated with longer OS after afatinib treatment. The secondary objectives are to elucidate relationships between the generated OMIC data and treatment response rates, disease control, PFS time, response duration, time to response, and treatment duration. We will also assess the appropriate source of OMIC analysis between the free molecules of sera/plasma and exosome-packaged molecules. This study is registered in UMIN-CTR (UMIN000024935).

Eligibility criteria

Enrollment was limited to patients: aged ≥ 20 years; with histologically or cytologically confirmed metastatic or local advanced NSCLC; with an *EGFR* mutation (common or uncommon); Eastern Cooperative Oncology Group performance status of 0 or 1; and adequate bone marrow, renal, and liver function. Patients previously treated for advanced diseases were excluded.

The ethics committees at Teikyo University and each institution approved this study and written informed consent was obtained from each patient.

Study design and treatment plan

The EXTRA study, a prospective, single-arm, observational study, is currently underway. Serial liquid biopsies were obtained from patients with advanced or recurrent advanced NSCLC harboring *EGFR* mutations treated with afatinib as their first-line treatment (Fig 1). Afatinib was initially used at a dose of 40 mg/day; the dose was adjusted according to toxicities observed by the investigators.

The primary efficacy endpoint is to identify a novel predictive biomarker for OS after afatinib treatment via a comprehensive association study using multi-OMIC data. We obtained 33 mL of peripheral blood from registered patients before treatment (baseline); eight weeks following treatment; and at the point of disease progression, intolerable toxicity, or withdrawal of consent. All collected blood samples included 3 mL for serum isolation and six 5 mL samples for plasma isolation. We plan to enroll 60 patients in the discovery cohort and 40 in the independent validation cohort, and data for interim analysis should be available by the end of 2018.

Expected results

First, using comprehensive multi-OMIC analyses, novel markers associated with the efficacy of and resistance to afatinib treatment should be revealed. Second, recently proposed predictors for afatinib treatment, that is, ligands of ERBB family receptors and somatic gene alterations of *ERBB*s, will be evaluated for their feasibility as biomarkers. Third, a novel approach using exosomal DNA from peripheral blood to detect EGFR-TKI resistance-associated mutations should effectively assess sensitivity and specificity compared to the standard method using circulating free

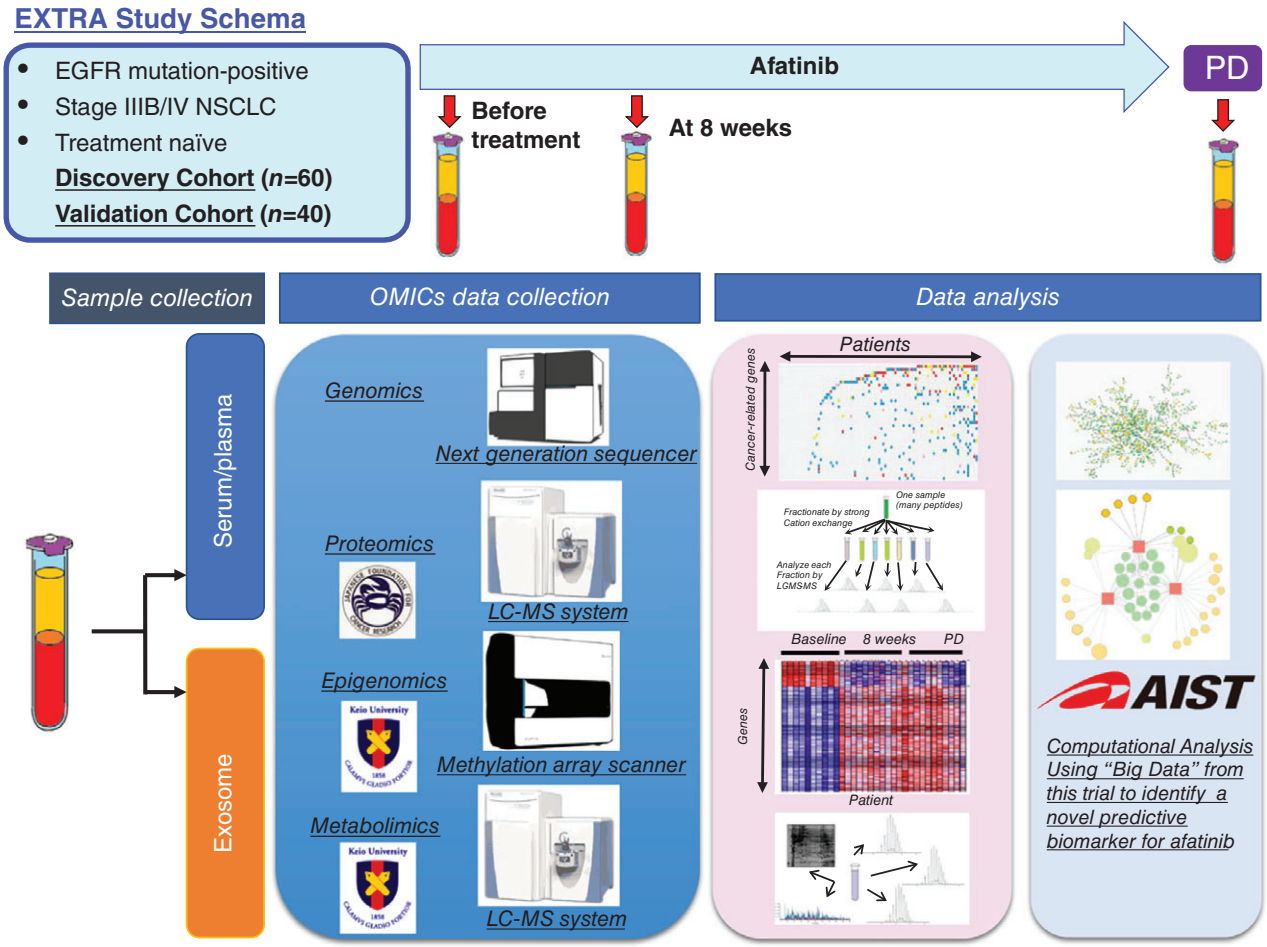

Figure 1 Study schema of the exosome-focused translational research for afatinib (EXTRA) study. LC-MS, liquid chromatography-mass spectrometry; NSCLC, non-small cell lung cancer; PD, progressive disease.

DNA. Finally, concordant results of proteomic, epigenomic, and metabolomic analyses using sera/plasma-derived and exosome-derived molecules will be obtained.

Analytical methods

Multi-OMIC analyses will be performed using both sera/plasma and exosome-capsulated molecules collected from patients treated with afatinib as follows: (i) liquid chromatography-mass spectrometry (LC/MS)-based proteomics analysis; (ii) next-generation sequencing for 508 cancer-associated genes; (iii) genome-wide DNA epigenomics analysis using a methylation array; and (iv) LC/MS or LC/MS/MS-based comprehensive metabolome analysis of metabolites. Using clinical data of efficacy and after obtaining OMIC data, statistical association analysis will be performed to identify candidates that achieve a good outcome after afatinib treatment. Additionally, using archived specimens, we will analyze drug sensitivity and resistance by evaluating their relationship to the molecular expression within cancer tissue. Alternatively, if disease progression occurs after treatment with afatinib, immunohistochemical analysis will be performed using a re-biopsied specimen.

The large amount of data obtained from four OMIC layers will be statistically analyzed to find a robust clinical predictive marker of afatinib using the following framework:

1 We will select molecular signatures and analyze biological pathways.
2 We will then select molecules from these signatures in the pathways with a significance probability in order to select functional molecules that exhibit differences between the two groups.
3 We will perform model selection based on the generalized linear mixed model,[31] where the explanatory and objective variables are the outcomes of afatinib and the selected molecules, as stated above, respectively. Biomarkers will be selected from the variables in the models.

4 If several mathematical models are selected under the criterion of model selections, we will further construct a predictor of models by boosting.[32]

The procedure will be performed for each OMIC to obtain a model for each of the four layers. The four layers of data will not be integrated to construct the model because data includes noises that are specific to each layer and because the integrated model includes variables from different layers that will not be practically used in the clinical sense. Instead, we plan to select a model from the four models in consideration of clinical usage, such as sampling burden and measuring cost.

As a coordinated multi-center investigation, the EXTRA study is designed to explore and link OMIC data generated in an academic setting with clinical data acquired at several community hospitals. It will attempt to uncover the mechanisms underlying EGFR-TKI resistance, as well as the biological mechanisms responsible for the differential response to afatinib developing in a clinical setting. We will focus on the role of exosomes and their contribution to resistance mechanisms; particularly, we will explore DNA methylation and acquired mutations other than *EGFR*. This approach will hopefully allow us to understand the mechanisms of resistance that develop in NSCLC patients and highlight potential mutations that can be targeted by novel therapeutics.

Acknowledgments

This study is sponsored by Nippon Boehringer-Ingelheim Co., Ltd. as collaborative research of the EXTRA study group. The authors would like to thank Enago (https://www.enago.jp) for English language review.

Disclosure

YO received personal fees as honoraria from Nippon Boehringer Ingelheim Co., Ltd., AstraZeneca K.K., Chugai Pharmaceutical, and Ono Pharmaceutical. HT received personal fees as honoraria from Nippon Boehringer Ingelheim Co., Ltd. HN received research funding from AstraZeneca K.K., Pfizer Japan Inc., and Bristol–Myers Squibb Japan. AB received personal fees as honoraria from AstraZeneca K.K., Pfizer Japan Inc. KS received personal fees as honoraria from AstraZeneca K.K. and Chugai Pharmaceutical and received research funding from Chugai Pharmaceutical, Ono Pharmaceutical, and Taiho Pharmaceutical. KK received personal fees as honoraria from Nippon Boehringer Ingelheim Co., Ltd. AT is an employee of Nippon Boehringer Ingelheim Co., Ltd. and a visiting researcher of National Institute of Advanced Industrial Science and Technology (AIST). NS received personal fees as honoraria from Lily Japan, AstraZeneca K.K., Daiichi Sankyo, MSD Oncology, Chugai Pharmaceutical, and Ono Pharmaceutical, Nippon Boehringer Ingelheim Co., Ltd., and Bristol-Myers Squibb Japan and research funding from Nippon Boehringer Ingelheim Co., Ltd.

References

1 Inoue A, Kobayashi K, Usui K *et al.* First-line gefitinib for patients with advanced non-small-cell lung cancer harboring epidermal growth factor receptor mutations without indication for chemotherapy. *J Clin Oncol* 2009; **27**: 1394–400.
2 Mitsudomi T, Morita S, Yatabe Y *et al.* Gefitinib versus cisplatin plus docetaxel in patients with non-small-cell lung cancer harbouring mutations of the epidermal growth factor receptor (WJTOG3405): An open label, randomised phase 3 trial. *Lancet Oncol* 2010; **11**: 121–8.
3 Paz-Ares L, Soulieres D, Moecks J, Bara I, Mok T, Klughammer B. Pooled analysis of clinical outcome for EGFR TKI-treated patients with EGFR mutation-positive NSCLC. *J Cell Mol Med* 2014; **18**: 1519–39.
4 Park K, Tan EH, O'Byrne K *et al.* Afatinib versus gefitinib as first-line treatment of patients with EGFR mutation-positive non-small-cell lung cancer (LUX-Lung 7): A phase 2B, open-label, randomised controlled trial. *Lancet Oncol* 2016; **17**: 577–89.
5 Wu YL, Mok TS. Dacomitinib in NSCLC: A positive trial with little clinical impact - Authors' reply. *Lancet Oncol* 2018; **19**: e5.
6 Katakami N, Atagi S, Goto K *et al.* LUX-Lung 4: a phase II trial of afatinib in patients with advanced non-small-cell lung cancer who progressed during prior treatment with erlotinib, gefitinib, or both. *J Clin Oncol* 2013; **31**: 3335–41.
7 Yang JC, Wu YL, Schuler M *et al.* Afatinib versus cisplatin-based chemotherapy for EGFR mutation-positive lung adenocarcinoma (LUX-Lung 3 and LUX-Lung 6): Analysis of overall survival data from two randomised, phase 3 trials. *Lancet Oncol* 2015; **16**: 141–51.
8 Mok TS, Wu YL, Ahn MJ *et al.* Osimertinib or platinum-pemetrexed in EGFR T790M-positive lung cancer. *N Engl J Med* 2017; **376**: 629–40.
9 Soria JC, Ohe Y, Vansteenkiste J *et al.* Osimertinib in untreated EGFR-mutated advanced non-small-cell lung cancer. *N Engl J Med* 2018; **378**: 113–25.
10 Hochmair MJ, Morabito A, Hao D *et al.* Sequential treatment with afatinib and osimertinib in patients with EGFR mutation-positive non-small-cell lung cancer: An observational study. *Future Oncol* 2018; **14**: 2861–74.
11 Socinski MA, Jotte RM, Cappuzzo F *et al.* Atezolizumab for first-line treatment of metastatic nonsquamous NSCLC. *N Engl J Med* 2018; **378**: 2288–301.
12 Trams EG, Lauter CJ, Salem N Jr, Heine U. Exfoliation of membrane ecto-enzymes in the form of micro-vesicles. *Biochim Biophys Acta* 1981; **645**: 63–70.

13 Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol* 2007; **9**: 654–9.
14 Gutkin A, Uziel O, Beery E *et al*. Tumor cells derived exosomes contain hTERT mRNA and transform nonmalignant fibroblasts into telomerase positive cells. *Oncotarget* 2016; **7**: 59173–88.
15 Yu S, Cao H, Shen B, Feng J. Tumor-derived exosomes in cancer progression and treatment failure. *Oncotarget* 2015; **6**: 37151–68.
16 Zitvogel L, Regnault A, Lozier A *et al*. Eradication of established murine tumors using a novel cell-free vaccine: Dendritic cell-derived exosomes. *Nat Med* 1998; **4**: 594–600.
17 Thery C, Boussac M, Veron P *et al*. Proteomic analysis of dendritic cell-derived exosomes: A secreted subcellular compartment distinct from apoptotic vesicles. *J Immunol* 2001; **166**: 7309–18.
18 Skog J, Wurdinger T, van Rijn S *et al*. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat Cell Biol* 2008; **10**: 1470–6.
19 Kim SH, Lechman ER, Bianco N *et al*. Exosomes derived from IL-10-treated dendritic cells can suppress inflammation and collagen-induced arthritis. *J Immunol* 2005; **174**: 6440–8.
20 Gyorgy B, Szabo TG, Pasztoi M *et al*. Membrane vesicles, current state-of-the-art: Emerging role of extracellular vesicles. *Cell Mol Life Sci* 2011; **68**: 2667–88.
21 Pegtel DM, van de Garde MD, Middeldorp JM. Viral miRNAs exploiting the endosomal-exosomal pathway for intercellular cross-talk and immune evasion. *Biochim Biophys Acta* 2011; **1809**: 715–21.
22 Belting M, Wittrup A. Nanotubes, exosomes, and nucleic acid-binding peptides provide novel mechanisms of intercellular communication in eukaryotic cells: Implications in health and disease. *J Cell Biol* 2008; **183**: 1187–91.
23 Hoshino A, Costa-Silva B, Shen TL *et al*. Tumour exosome integrins determine organotropic metastasis. *Nature* 2015; **527**: 329–35.
24 Choudhury NJ, Campanile A, Antic T *et al*. Afatinib activity in platinum-refractory metastatic urothelial carcinoma in patients with ERBB alterations. *J Clin Oncol* 2016; **34**: 2165–71.
25 Yonesaka K, Kudo K, Nishida S *et al*. The pan-HER family tyrosine kinase inhibitor afatinib overcomes HER3 ligand heregulin-mediated resistance to EGFR inhibitors in non-small cell lung cancer. *Oncotarget* 2015; **6**: 33602–11.
26 Griesinger F, Netchaeva M, Lueers A *et al*. P53 non-disruptive mutation is a negative predictive factor for OS and PFS in EGFR M+ NSCLC treated with TKI. *J Thorac Oncol* 2017; **12** (Suppl 1): S359–60.
27 Kobayashi S, Boggon TJ, Dayaram T *et al*. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2005; **352**: 786–92.
28 Oxnard GR, Arcila ME, Sima CS *et al*. Acquired resistance to EGFR tyrosine kinase inhibitors in EGFR-mutant lung cancer: Distinct natural history of patients with tumors harboring the T790M mutation. *Clin Cancer Res* 2011; **17**: 1616–22.
29 Yu HA, Arcila ME, Rekhtman N *et al*. Analysis of tumor specimens at the time of acquired resistance to EGFR-TKI therapy in 155 patients with EGFR-mutant lung cancers. *Clin Cancer Res* 2013; **19**: 2240–7.
30 Kobayashi Y, Azuma K, Nagai H *et al*. Characterization of EGFR T790M, L792F, and C797S mutations as mechanisms of acquired resistance to Afatinib in lung cancer. *Mol Cancer Ther* 2017; **16**: 357–64.
31 Stroup WW. *Generalized Linear Mixed Models: Modern Concepts, Methods and Applications*, *Chapman & Hall/CRC Texts in Statistical Science*. CRC Press, Boca Raton, FL 2012.
32 Zhou ZH. *Ensemble Methods: Foundations and Algorithms*. Chapman and Hall/CRC, Boca Raton, FL 2012.

