METHODS FOR DETECTING DRUG-TOLERANT PERSISTER (DTP) CELLS IN CANCER SUBJECTS

CROSS-REFERENCE TO RELATED APPLICATION

[001] This application claims the benefit of priority of Singapore Patent Application No. 10202403467W filed 7 November 2024, the content of which being hereby incorporated by reference in its entirety for all purposes.

TECHNICAL FIELD

[002] Various embodiments relate generally to the field of oncology and molecular diagnostics. In particular, various embodiments relate to methods for detecting drug-tolerant persister (DTP) cells and intrinsically resistant cells in subjects undergoing treatment with tyrosine kinase inhibitors (TKIs). Moreover, various embodiments also relate to the use of ALPP expression as a biomarker for identifying, monitoring, and stratifying cancer cells that persist or resist TKI therapy in order to guide prognosis, treatment selection, and therapeutic intervention.

BACKGROUND

[003] Cancer is the no.2 leading cause of deaths worldwide with lung cancer being the deadliest cancer. Approximately 85% of lung cancers are classified as lung adenocarcinomas (LUAD), of which, approximately 30% and 60% harbour EGFR mutations in western and asian populations respectively. EGFR tyrosine kinase inhibitors (TKIs) are standard of care for EGFR mutated LUADs and although initially very effective with a high response rate of up to 80%, drug resistance and disease progression inevitably occur within a median period of 10-17 months. This is because, very often, a small population of cancer cells, usually undetectable by conventional means, survive the initial treatment. These cells, known as drug persisters, remain dormant initially but will eventually repopulate again when treatment pressure is lifted or when they gain further genetic or epigenetic changes to proliferate in the presence of the treatment.

[004] In contrast to the many different iterations of EGFR TKIs (4_{th} generation in the pipeline) to address resistant tumours that develop after treatment with the previous generation TKI, there is no clinically approved method to detect and target drug persisters at this early bottleneck before they adapt and become full blown resistant tumours. Furthermore, newer generations of EGFR TKIs do not work for all types of resistant tumours arising from previous generation TKIs. 3_{rd} generation Osimertinib, for example, is only effective against resistant tumours harbouring T790M+ mutations but not those without. Tumours which acquire resistance by shifting the dependence on EGFR to other mechanisms for proliferation or survival would not be sensitive to next generation TKIs. Hence, it is crucial to eliminate the drug persisters at an early stage when they are more homogeneous then at later stages when they diverge and adopt multiple different resistance mechanisms.

[005] Therefore, there is still need in the art for methods to detect drug tolerant persister cells to treatment with TKIs by identifying a suitable biomarker and target specifically enriched in drug persisters.

SUMMARY

[006] In a first aspect, there is provided a method of detecting the presence or absence of drug-tolerant persister (DTP) cells resistant to tyrosine kinase inhibitor (TKI) treatment, and/or intrinsically resistant cells to TKI treatment in a subject having cancer, comprising: detecting an expression level of ALPP in the subject, wherein the expression level of ALPP is indicative of the presence or absence of the DTP cells and/or the intrinsically resistant cells in the subject's cancer.

[007] In various embodiments, the subject has previously received TKI treatment, optionally the TKI treatment comprises treatment of the subject by administration of one or more TKIs.

[008] In various embodiments, the expression level of ALPP is detected in a sample obtained from the subject, wherein the sample is a blood or tumour sample.

[009] In various embodiments, the step of detecting the ALPP expression level comprises quantifying the amount of a gene product of ALPP.

[010] In various embodiments, the expression level of ALPP in the sample is compared with a reference ALPP expression level, optionally the reference ALPP expression level may be derived from a control or comparative sample.

[011] In various embodiments, a detected differential expression level of ALPP in the sample is indicative of the presence or absence of the DTP cells, and/or intrinsically resistant cells, in the subject.

[012] In various embodiments, an increased expression level of ALPP is indicative of the presence of the DTP cells in the sample.

[013] In various embodiments, the method further comprises distinguishing DTP cells from intrinsically resistant cells in the sample based on a detected surface expression level of ALPP, wherein a high surface expression level of ALPP ($ALPP^{high}$) is indicative of DTP cells and a low surface expression level of ALPP ($ALPP^{low}$) is indicative of intrinsically (innate) resistant cells.

[014] In various embodiments, the method further comprises distinguishing fast-cycling DTEP cells, from slow-cycling DTEP cells based on a detected surface expression level of ALPP, wherein the

surface expression level of ALPP is higher in slow-cycling DTEP cells relative to fast-cycling DTEP cells.

[015] In various embodiments, the method further comprises classifying the likelihood of the subject being responsive or non-responsive to TKI treatment and/or anti-ALPP therapies based on the expression level of ALPP and detection of DTP cells, DTEPs, and/or intrinsically resistant cells.

[016] In various embodiments, the cancer is lung cancer, and wherein the lung cancer is classified as a lung adenocarcinoma (LUAD), optionally the LUAD is an EGFR mutated LUAD.

[017] In various embodiments, the cancer is non-small cell lung cancer (NSCLC).

[018] In various embodiments, the TKI treatment comprises EGFR TKI treatment with one or more EGFR TKIs selected from 1st generation EGFR TKIs, 2nd generation EGFR TKIs, 3rd generation EGFR TKIs, and 4th Generation EGFR TKIs, preferably the TKI treatment comprises treatment with Gefitinib or Osimertinib.

[019] In various embodiments, the detecting step is performed at one or more pre-determined time points before, during, and after a period of withdrawal of the TKI treatment ("drug holiday"), and the expression level of ALPP at each pre-determined time point is compared to provide a differential expression level of ALPP, wherein a decrease in the expression level of ALPP during the period of withdrawal, followed by an increase in the expression level of ALPP after re-administration of the TKI treatment to the subject, is indicative of the presence, and quantity, of DTP cells, and optionally the absence of intrinsically resistant cells.

[020] In another aspect, there is provided a method for delaying and/or preventing the development of drug-tolerant persister (DTP) cells resistant to TKI treatment in a subject with cancer, comprising administering to the subject an anti-ALPP therapy, thereby preventing or delaying relapse and/or the emergence of acquired resistance to TKI treatment, wherein the presence or absence of drug-tolerant persister (DTP) cells resistant to TKI treatment has been detected in the subject using the method disclosed herein.

[021] In various embodiments, the anti-ALPP therapy is used as an adjuvant therapy in combination with the TKI treatment, to improve disease-free survival of the subject.

[022] In another aspect, there is provided a method of monitoring the efficacy of a TKI treatment in treating cancer in a subject, comprising:(a) administering the TKI treatment to the subject; (b) obtaining a sample from the subject; and (c) detecting the presence or absence, and optionally quantity, of responder cells and non-responder cells to TKI treatment in the sample based on detected

expression levels of ALPP of cells in the sample using the method disclosed herein, wherein the presence or absence, and optionally quantity, of responder cells and/or non-responder cells in the sample is indicative of the efficacy of the TKI treatment in treating the cancer in the subject.

[023] In various embodiments, the method further comprises detecting expression levels of ALPP of cells in a reference sample obtained from the subject prior to step (a), wherein the expression level of ALPP in step (c) is compared to the expression level of ALPP in the reference sample obtained prior to step (a).

[024] In various embodiments, the method further comprises selecting a cancer treatment regimen for the subject comprising a TKI treatment and/or an anti-ALPP therapy based on the presence or absence of responder and/or non-responder cells detected in the sample.

[025] In another aspect, there is provided a method for treating cancer in a subject, comprising administering to the subject an Anti-ALPP therapy after the subject has received TKI treatment, wherein the presence of responder cells resistant to TKI treatment has been detected in the subject using the method disclosed herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[026] Various embodiments will be better understood with reference to the detailed description when considered in conjunction with the non-limiting examples and the accompanying drawings.

[027] **FIG. 1A-1E** shows the development of a FT genetic reporter to isolate 'slow' DTPs vs 'fast' non-responders: **FIG. 1A** Schematic of innate and acquired resistance to drug treatment by tumour cells. Cells with innate resistance do not respond to drug from the onset. In acquired resistance, cells initially responsive to drug treatment enter a drug-tolerant persister (DTP) state and eventually regain proliferative capacity as drug-tolerant expanded persisters (DTEP). This resistance is reversible as DTPs and DTEPs are able to regain sensitivity to drug treatment after a drug holiday (Fig. 1 in **[9]**); **FIG. 1B** Schematic of the dox-inducible live cell H2B-fluorescent timer (FT) reporter. Newly synthesized FT protein emits blue fluorescence which quickly degrades into red fluorescence. The much shorter half-life of the blue protein allows the isolation of cells of differing cell cycle speed based on blue-red fluorescence ratio **[7]**; **FIG. 1C** Flow cytometry strategy to identify/isolate fast and slow cycling cells using the H2B-FT reporter in PC9 for bulk RNA-seq at various timepoints of gefitinib treatment; **FIG. 1D** Pathway enrichment analysis of differentially expressed genes (DEGs) between fast(blue) and slow(red) PC9FT cells upon Gefitinib treatment(0.2uM) for 7 days; and **FIG. 1E** Cell cycle transcriptomic profile of Fast(blue) and Slow(red) cells after 7 days of treatment in DMSO or gefitinib at 0.2uM concentration.

[028] **FIG. 2A-2G** shows ALPP is upregulated upon TKI treatment and enriched in the slow 'DTPs': **FIG. 2A** ALPP mRNA expression at various embryonic stages; **FIG. 2B** RT-qPCR for ALPP in PC9

cells across different gefitinib treatment time-course; **FIG. 2C** Western blot for ALPP and GAPDH in PC9 gefitinib treatment time-course; **FIG. 2D** Immunofluorescence (IF) staining for ALPP in PC9 cells after 3 or 7 days of gefitinib treatment; **FIG. 2E** ALPP expression from RNA-seq data of resected tumours from early-stage EGFR-mutant LUAD patients with or without neoadjuvant gefitinib treatment; **FIG. 2F** Flow cytometry of PC9 H2B-FT reporter cells co-stained for ALPP (FITC) after 7 days of gefitinib treatment; and **FIG. 2G** Western blot for ALPP in 'slow-cycling DTPs' and 'fast-cycling non-responders' after 1 month of continuous gefitinib treatment.

[029] **FIG. 3A-3C** shows ALPP is able to distinguish responders from intrinsically-resistant cells: **FIG. 3A** Flow cytometry strategy to isolate ALPPhigh and ALPPlow PC9 cells after 7 days of gefitinib treatment; **FIG. 3B** Cell viability assays of ALPPhigh and ALPPlow cells sorted after 7 days of gefitinib or Osimertinib following strategy in (FIG. 3A) and placed on drug holiday for 14 days or 1.25 months before rechallenge at various concentrations of respective drugs; and **FIG. 3C** ELISA assay for secreted ALPP in cell culture supernatant collected after 7 days of treatment with DMSO or gefitinib from treatment naïve PC9, ALPPhigh and ALPPlow cells (sorted from PC9 treated with 7 days of gefitinib and placed on 2 months drug holiday). Supernatant was diluted 2-fold for the assay except for PC9 Gef (Neat) which was used undiluted.

[030] **FIG. 4A** Schematic of the sorting strategy of ALPP high and low cells after 7 days or 21days of 0.2 μM osimertinib treatment. PC9 cells are treated for 7 days with 0.2 μM osimertinib and sorted based on ALPP expression (ALPP high / ALPP low DTPs). The cells are then either placed on drug holiday or treated with osimertinib for another 14 days and subjected to another round of sorting, once again based on ALPP levels (ALPP high / ALPP low DTEPs from the initial ALPP high DTPs, ALPP low CD-> DH from the initial ALPP low DTPs). They are then placed on drug holiday. DTP = Drug tolerant persister, DTEP = Drug tolerant expanded persister, CD->DH = Continuous drug -> Drug holiday; **FIG. 4B** Cell viability assays of ALPP high/ALPP low DTEPs and ALPP low CD->DH sorted after 21 days of osimertinib following the strategy in (FIG. 4A) and placed on drug holiday for 14 days before rechallenge at various concentrations of the same drug; and **FIG. 4C** Cell viability assays of ALPP high/ ALPP low DTEPs, ALPP low CD->DH sorted after 21 days of osimertinib, following the strategy in (FIG. 4A) and placed on drug holiday for 1 month before rechallenge at various concentrations of the same drug.

[031] **FIG. 5A-5B** shows the growth rate of PC9 DTP populations. $ALPP^{high}$ and $ALPP^{low}$ that were sorted after 7 days of osimertinib treatment at 0.2uM concentration and were cultured in drug holiday for a minimum of 14 day before investigating their growth rate in the presence of osimertinib pressure at 0.2uM concentration. Count graph (**FIG. 5A**) and bright field images (**FIG. 5B**). N=3 biological replicates, Images reflected are from 1 biological replicate in osimertinib exposure at 0.2uM.

DETAILED DESCRIPTION

[032] The following detailed description refers to, by way of illustration, specific details and embodiments in which the invention may be practiced. These embodiments are described in sufficient detail to enable those skilled in the art to practice the invention. Other embodiments may be utilized and structural and logical changes may be made without departing from the scope of the invention. Embodiments described below in context of the methods are analogously valid for the respective uses, kits, and vice versa. The various embodiments are not necessarily mutually exclusive, as some embodiments can be combined with one or more other embodiments to form new embodiments.

[033] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art. The singular terms "a," "an," and "the" include plural referents unless context clearly indicates otherwise. Similarly, the word "or" is intended to include "and" unless the context clearly indicates otherwise. The term "comprises" means "includes." In case of conflict, the present specification, including explanations of terms, will prevail. "About", as used herein in connection with numerical values refers to the referenced numerical value ±10% or ±5%.

[034] The present inventors discovered a biomarker in the form of a gene, placental alkaline phosphatase (ALPP), that was found to be upregulated in cancer cells that are induced to survive tyrosine kinase inhibitor (TKI) treatment. These cells are known as drug persisters, or more specifically, drug-tolerant persister (DTP) cells, and are the major cause of cancer relapse. Furthermore, it was also found that ALPP expression level can distinguish DTPs from intrinsically resistant cancer cells, providing a means to identify and isolate TKI-responsive cancer cells from nonresponsive ones. Based on these findings, ALPP may be used as a biomarker for detecting the presence or absence of drug persister cells, and optionally intrinsically resistant cancer cells, thereby enabling prediction of relapse risk and facilitating strategies to improve disease-free survival of cancer patients.

[035] The term "ALPP" refers to the gene alkaline phosphatase placental. ALPP expression is typically restricted to the placenta in healthy tissues and is also a cell surface protein, however, ALPP may also be secreted into the bloodstream via exosomes from ALPP expressing cells. The gene ALPP may also be termed as PLAP (Placental Alkaline Phosphatase), PLAP-1 (Placental Alkaline Phosphatase 1), PL (Placental Alkaline Phosphatase), PALP (Placental-type Alkaline Phosphatase), ALP (This abbreviation stands for Alkaline Phosphatase, but is often used generically; it requires context to distinguish it from other isoenzymes), Regan Isoenzyme (A designation often used in clinical settings, referring to a variant of ALPP found in certain cancers, especially germ cell tumors). ALPP may be expressed as a surface cell marker on cells. In NCBI, the human ALPP gene is assigned Gene ID 250, with the reference mRNA sequence NM_001632.5 and the corresponding protein sequence NP_001623.3. In UniProt, the ALPP is listed under accession P05187 (ALPP_HUMAN), while in Ensembl the gene is annotated as ENSG00000163283. These accession

numbers are widely used for designing probes or primers to detect ALPP mRNA expression, and for selecting assays targeting the ALPP protein in detection of its cell-surface expression or presence *in vitro, ex vivo or in vivo.*

[036] Also contemplated herein are paralogues of ALPP that may be used as biomarkers. In various embodiments, one such paralogue of ALPP may be ALPPL2 (Alkaline Phosphatase, Placental-Like 2; also termed ALPG, Nagao Isozyme), which encodes a closely related but distinct placental-type alkaline phosphatase isoenzyme to ALPP. ALPPL2 represents a distinct but paralogous gene to ALPP and shares high sequence and structural homology with ALPP. Accordingly, ALPPL2 may be used as an alternative biomarker to ALPP. In the human genome, ALPPL2 is assigned NCBI Gene ID 251 while in Ensembl the gene is annotated as ENSG00000163286.

[037] Accordingly, in one aspect, there is provided a method of detecting the presence (or absence), and optionally quantity, of drug-tolerant persister (DTP) cells resistant to TKI treatment in a subject having cancer. The method comprising the step of detecting an expression level of ALPP in the subject, wherein the expression level of ALPP is indicative of the presence (or absence), and optional quantity, of DTP cells in the subject's cancer.

[038] The term "DTP cells" refers to a subpopulation of cancer cells that can survive initial treatment with anticancer drugs, even though they are not genetically resistant to the drug. They enter a reversible, quiescent state that allows them to tolerate the drug's effects temporarily. DTP cells can re-enter a drug-sensitive state once the drug pressure is removed, distinguishing them from permanently resistant cells (i.e. intrinsically resistant cells) that are non-responsive. However, under continued drug exposure, DTP cells may evolve into drug-tolerant expanded persister (DTEP) cells. The term "drug-tolerant expanded persister (DTEP) cells" refers to a population of cancer cells that arise from drug-tolerant persister (DTP) cells under continued drug exposure. Unlike DTPs, which are quiescent, DTEPs resume proliferation in the presence of the drug and can serve as a reservoir for the eventual emergence of permanently resistant clones (acquired resistance). While DTEPs are generally less reversible than DTPs, in some instances a subset of DTEP cells may retain the capacity to regain drug sensitivity upon withdrawal of the therapeutic agent (i.e. drug holiday), reflecting heterogeneity within the expanded persister population.

[039] The term "subject", as used herein in the context of the methods, refers to a warm-blooded animal, preferably a mammal, more preferably a human. Said subject may be awaiting or receiving medical treatment for cancer, or is, or will become the subject of a medical procedure, or is being monitored for the development of cancer treatable with an TKI. Subjects include those already being afflicted by cancer as well as subjects susceptible to the progression of the cancer or for whom cancer or progression of cancer should be prevented or delayed. In various embodiments, the subject may be a human.

[040] In various embodiments, the subject may have previously received and been administered TKI treatment for the cancer.

[041] As used herein, the term "tyrosine kinase inhibitor (TKI) treatment" refers to a therapeutic regimen involving the administration of one or more tyrosine kinase inhibitors (TKIs) to a subject in need thereof. TKIs are small-molecule agents that act, directly or indirectly, to inhibit the kinase activity of receptor tyrosine kinases (RTKs) and/or non-receptor tyrosine kinases by binding to and interfering with their ATP-binding site or other functional domains. TKI treatment encompasses inhibitors that act reversibly or irreversibly, and that may selectively target a specific kinase or inhibit multiple kinases within the same or different kinase families. Examples include, but are not limited to, inhibitors of the epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2), vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR), anaplastic lymphoma kinase (ALK), c-MET, RET, BCR-ABL, JAK, and other clinically relevant tyrosine kinases. The term further encompasses all generations or classes of such inhibitors, including agents designed to overcome primary or acquired resistance mutations, and includes use as monotherapy or in combination with other TKIs or therapeutic agents. In various embodiments, the TKI treatment may be EGFR TKI treatment.

[042] In various embodiments, the TKI treatment comprises treatment of the subject by administration of one or more TKIs. In various embodiments, the one or more TKIs comprise EGFR TKIs, preferably gefitinib and/or Osimertinib.

[043] In various embodiments, the TKI treatment comprises administration of one or more TKIs to the subject according to a standard therapeutic regimen of TKIs. Typically, TKIs are administered orally once or twice daily, on a continuous dosing schedule, over a period of days, weeks, or months, depending on the specific cancer indication and clinical protocol. For example, in the treatment of EGFR-mutant non-small cell lung cancer (NSCLC), 1st, 2nd or 3rd generation EGFR inhibitors may be administered daily at fixed dosages, with initial treatment cycles often extending over 7 to 14 days of uninterrupted dosing.

[044] In various embodiments, the subject may not have previously received TKI treatment, yet ALPP high cancer cells are already present within the subject's tumour. Such cell populations may arise spontaneously during tumour evolution as a result of epigenetic plasticity, stochastic entry into a quiescent state, or selective pressures unrelated to TKI exposure (e.g., hypoxia, nutrient deprivation, or prior non-TKI therapies). In this context, detection of elevated ALPP expression levels prior to initiation of TKI therapy may provide an indication that a reservoir of potential DTPs exist within the tumour. Identifying such pre-existing tolerant populations may enable early stratification of patients, prediction of response to TKI monotherapy, and implementation of combination treatment strategies

designed to eliminate or suppress these subpopulations before they expand under continuous drug pressure. In this regard, it is possible that ALPP levels are higher in patients who have not previously received TKI treatments. For example, patients who smoke have been reported to have higher baseline ALPP.

[045] In various embodiments, the cancer that the subject has may be selected from adrenal cancer, acute lymphoblastic leukaemia, acute myelogenous leukaemia, astrocytoma, basal cell carcinoma, bile duct cancer, bladder cancer, bone cancer, breast cancer, brain cancer, carcinoma, cardiac tumour, cervical cancer, childhood cancers, chronic lymphocytic leukaemia, chronic myelogenous leukaemia, colorectal cancer, embryonal tumour, epithelial cancer, oesophageal cancer, gastrointestinal cancer, germ cell tumour, gallbladder cancer, gastric cancer, glioma, head and neck cancer, haematological malignancy, Hodgkin's lymphoma, non- Hodgkin's lymphoma, intestinal cancer, intraocular melanoma, kidney cancer, laryngeal cancer, leukaemia, lung cancer, liver cancer, malignant peripheral nerve sheath tumour, melanoma, mesothelioma, nasopharyngeal carcinoma, neuroblastoma, neurofibroma, oral cancer, non-small cell lung cancer, osteosarcoma, ovarian cancer, pituitary tumour, prostate cancer, pancreatic cancer, retinoblastoma, rhabdomyosarcoma, sarcoma, small cell lung cancer, testicular cancer, throat cancer, thyroid cancer, transitional cell carcinoma, urogenital cancer, urothelial carcinoma, uterine cancer, vaginal cancer, or Wilms' tumour.

[046] In various embodiments, the cancer is lung cancer, and wherein the lung cancer is non-small cell lung cancer (NSCLC).

[047] In various embodiments, the cancer may be lung cancer. In various embodiments, the lung cancer may be classified as a lung adenocarcinoma (LUAD). In various embodiments, the LUAD may be an EGFR mutated LUAD.

[048] The term "EGFR mutated LUAD" refers to Lung Adenocarcinoma (LUAD) that harbours mutations in the Epidermal Growth Factor Receptor (EGFR) gene. These mutations are important because they lead to continuous activation of the EGFR pathway, driving cancer progression and making the tumour susceptible to targeted EGFR tyrosine kinase inhibitors (EGFR TKIs). Common EGFR mutations in LUAD include but are not limited to: Exon 19 Deletions (e.g. small deletions within exon 19 of the EGFR gene, typically involving the amino acids from positions 746 to 750 (e.g., E746-A750del); L858R Point Mutation (leucine (L) is replaced by arginine (R) at position 858); Exon 20 Insertions (e.g. Insertions or duplications in exon 20, such as the common D770_N771insNPG mutation); T790M Mutation (threonine (T) is replaced by methionine (M) at position 790); G719X Mutations (Substitutions at codon 719 in exon 18, such as G719A, G719S, and G719C); S768I Mutation (serine (S) is replaced by isoleucine (I) at position 768); and L861Q Mutation (leucine (L) is replaced by glutamine (Q) at position 861).

[049] In various embodiments, the method may be carried out *in vitro*, *ex vivo* or *in vivo*. It will be appreciated that ALPP can be detected intracellularly, at the cell surface, or as a shed/secreted protein in extracellular fluids, and such detection may be carried out in a biological sample, *in vitro* or *ex vivo* or within a living subject using appropriate detection methods.

[050] In various embodiments, the method may be an *in vitro* method and the expression level of ALPP is detected in a sample that has been obtained from the subject.

[051] The term "sample," as used herein, refers to a composition that is obtained or derived from the subject that contains a cellular and/or other molecular entity that is to be characterized and/or identified, for example, based on physical, biochemical, chemical, and/or physiological characteristics. For example, the phrase "disease sample" and variations thereof (i.e. cancer or tumour sample) refers to any sample obtained from a subject of interest that would be expected or is known to contain the cellular and/or molecular entity that is to be characterized. Samples include, but are not limited to, tissue samples, primary or cultured cells or cell lines, cell supernatants, cell lysates, platelets, serum, plasma, vitreous fluid, lymph fluid, synovial fluid, follicular fluid, seminal fluid, amniotic fluid, milk, whole blood, blood-derived cells, urine, cerebro-spinal fluid, saliva, sputum, tears, perspiration, mucus, tumour lysates, and tissue culture medium, tissue extracts such as homogenized tissue, tumour tissue, cellular extracts, and combinations thereof.

[052] In various embodiments, the sample may be a blood or plasma sample. Detection of ALPP in such liquid biopsy samples may provide an indirect yet informative measure of ALPP expression within the subject's cancer. Without wishing to be bound by theory, it is believed that DTP cells within the tumour express ALPP at elevated levels, and that ALPP protein and/or RNA can be secreted, shed, or released into the circulation. Thus, the presence and relative abundance of ALPP in blood or plasma reflect the presence, and optionally the quantity, of DTP cells in the underlying cancer. Measurement of circulating ALPP in the subject's blood can be achieved using immunoassays (e.g., ELISA, immunoblotting, or flow cytometry of extracellular vesicles), nucleic acid–based assays (e.g., RT-qPCR, digital droplet PCR, or RNA sequencing), or mass spectrometry.

[053] In various embodiments, the sample is a tumour sample, preferably the tumour is a lung tumour. In various embodiments, the sample is obtained or derived from a lung tumour, preferably a non-small cell lung cancer (NSCLC) tumour, such as a lung adenocarcinoma (LUAD) tumour or a lung squamous cell carcinoma tumour. In various embodiments, the NSCLC tumour is a lung adenocarcinoma tumour, preferably an EGFR-mutant, KRAS-mutant, or ALK-rearranged lung adenocarcinoma, or a lung squamous cell carcinoma tumour. In various embodiments, the lung adenocarcinoma may be classified according to histological subtype, including acinar, lepidic, papillary, micropapillary, or solid LUAD, or may be an unclassified NSCLC tumour.

[054] In various embodiments, the sample is a lung tumour sample, such as a non-small cell lung cancer (NSCLC) sample, comprising one or more cell types including but not limited to malignant epithelial tumour cells, infiltrating immune cells, stromal cells, fibroblasts, endothelial cells, or combinations thereof in an admixture of cells. In various embodiments, the lung tumour sample is a sample comprising malignant epithelial cells, such as lung adenocarcinoma (LUAD) or squamous cell carcinoma cells. In various embodiments, the lung tumour sample comprises fibroblast cells, including cancer-associated fibroblasts. In various embodiments, the lung tumour sample comprises stromal cells (e.g., endothelial cells, fibroblasts, or macrophages) admixed with malignant epithelial cells within the epithelial compartment.

[055] The term "tissue sample" or "cell sample" as used herein, refers to a collection of similar cells obtained from a tissue of a subject or individual. The source of the tissue or cell sample may be solid tissue as from a fresh, frozen and/or preserved organ, tissue sample, biopsy, and/or aspirate; blood or any blood constituents such as plasma; bodily fluids such as cerebral spinal fluid, amniotic fluid, peritoneal fluid, or interstitial fluid; cells from any time in gestation or development of the subject. The tissue sample may also be primary or cultured cells or cell lines. Optionally, the tissue or cell sample is obtained from a disease tissue/organ. For instance, a "tumour sample" is a tissue sample obtained from a tumour (e.g., a brain or nasopharyngeal tumour) or other cancerous tissue. The tissue sample may contain a mixed population of cell types (e.g., tumour cells and non-tumour cells, cancerous cells and non-cancerous cells). In particular, the tumour sample may be obtained from one or more compartments within the tumour. For example, the tumour sample may comprise or consist of an epithelial compartment that contains epithelial cells.

[056] In various embodiments, the method may be an *ex vivo* method and the expression level of ALPP is detected in a sample that has been obtained from the subject.

[057] In various embodiments, the sample may comprise primary cells, circulating tumour cells, or tissue biopsies isolated from the subject and subsequently cultured, enriched, or otherwise manipulated *ex vivo* to permit detection of ALPP expression. In various embodiments, *ex vivo* expansion of patient-derived cells or organoids may be performed prior to measurement of ALPP expression, thereby enabling characterization of tumor-derived cells outside of the body while preserving relevant cellular context.

[058] In various embodiments, the method may be an *in vivo* method and the expression level of ALPP is detected using agents specifically targeting and binding to ALPP, such as small-molecule probes, peptides, or ligand-based conjugates, that are administered to the subject for imaging such as CT or MRI imaging.

[059] In various embodiments, the step of detecting the ALPP expression level may comprise quantifying the amount of a gene product of ALPP in the sample or or within a living subject.

[060] In various embodiments, the gene product may include a protein or RNA transcript encoded by the ALPP gene, or a fragment of the protein or RNA transcript. In various embodiments, the detection of the ALPP expression may comprise obtaining the quantitative gene expression level of ALPP. In various embodiments, the gene product may include the ALPP protein expressed on the surface of a cancer cell, and the detection of the ALPP expression may comprise measuring the level of ALPP protein present on the cell surface.

[061] The term "expression level", as used herein generally refers to the amount of a gene or its product in a sample or within a living subject. "Expression" generally refers to the process by which information (e.g., gene-encoded and/or epigenetic information) is converted into the structures present and operating in the cell. Therefore, as used herein, "expression" may refer to transcription into a polynucleotide, translation into a polypeptide, or polynucleotide and/or polypeptide modifications (e.g., posttranslational modification of a polypeptide). Fragments of the transcribed polynucleotide, the translated polypeptide, or polynucleotide and/or polypeptide modifications (e.g., posttranslational modification of a polypeptide) shall also be regarded as expressed, whether they originate from a transcript generated by alternative splicing or a degraded transcript, or from a posttranslational processing of the polypeptide, e.g., by proteolysis. "Expressed genes" include those that are transcribed into a polynucleotide as mRNA and then translated into a polypeptide, and also those that are transcribed into RNA but not translated into a polypeptide (for example, transfer and ribosomal RNAs). The "amount" or "level" of a gene is a detectable level in a sample or within a subject. These levels may be measured by methods known to one skilled in the art and disclosed herein.

[062] In various embodiments, the ALPP expression level may be detected and measured in a sample by protein expression analysis (i.e., Western immunoblotting, ELISA, immunofluorescence, immunohistochemistry (IHC), flow cytometry) or mRNA expression analysis (i.e., qRT-PCR, RNA-seq).

[063] In various embodiments, the ALPP expression level may be detected and measured in a sample or within a subject using antibody-based conjugates or other molecular probes specific for ALPP that are linked to imaging agents (e.g., contrast agents, nanoparticles, radioactive tracers, fluorescent dyes) and detecting the signal by imaging modalities, or optical imaging.

[064] The term "antibody-based conjugates" refers to antibody conjugate used for *in vitro* or *in vivo* imaging as a molecular complex comprising of a monoclonal antibody that is chemically linked (conjugated) to an imaging agent such as a contrast agent, nanoparticle, radioactive tracer, or

fluorescent dye, which permits visualization of the bound antibody conjugates within the subject using one or more imaging modalities. The antibody is designed to specifically bind to ALPP expressed in the tissues or cells of interest within a sample or living subject. Once the antibody conjugate binds to its target, the attached imaging agent allows visualization of the location and concentration of the protein using various imaging modalities such as flow-cytometry, Magnetic Resonance Imaging (MRI), positron emission tomography (PET), single-photon emission computed tomography (SPECT), or fluorescently labelled conjugates may be detected using in vivo optical imaging. In various embodiments, the "antibody-based conjugates" may be an antibody-nanoparticle conjugate, wherein a gold nanoparticles may be suitable for imaging via computed tomography (CT) imaging or iron oxide nanoparticles may be suitable for imaging via MRI. By comparing the imaging signal intensity and distribution in tissues of interest relative to a reference or control, the relative expression level of ALPP may be quantified *in vivo*, thereby enabling non-invasive detection, monitoring, and longitudinal assessment of ALPP expression in the subject.

[065] In various embodiments, the amount of a gene product of ALPP may refer to the absolute amount, relative amount, or concentration of the gene product of ALPP.

[066] The term "absolute amount", as used herein, may refer to a direct quantitative measurement of ALPP nucleic acid or protein, for example expressed as copy number, mass (e.g., nanograms of mRNA), or molar quantity (e.g., femtomoles of protein) obtained from a sample or in vivo measurement. The term "relative amount" refers to a comparative measurement of ALPP expression levels, such as normalized to a reference gene or protein (e.g., housekeeping genes for mRNA, or loading controls for protein), or relative to a baseline or control sample (e.g., comparing ALPP levels in tumour tissue versus adjacent normal tissue, or in post-treatment versus pre-treatment samples). The term "concentration" refers to the amount of ALPP gene product per unit volume or per unit number of cells, for example nanograms of ALPP protein per millilitre of serum, or ALPP mRNA copies per cell equivalent. These quantifications may be obtained by methods including, but not limited to, nucleic acid amplification assays (qRT-PCR, digital PCR, RNA-seq), protein-based assays (ELISA, Western blotting, flow cytometry, immunohistochemistry), or imaging-based quantification when antibody-conjugates or molecular probes are employed in vivo.

[067] In various embodiments, the ALPP expression level may be detected using an ELISA-based assay, for example by measuring ALPP in a cell culture supernatant obtained from tumour cell cultures, or in a blood sample obtained from a subject. In various embodiments, the ALPP expression level may be detected using an ELISA-based blood test and a blood sample obtained from the subject.

[068] In various embodiments, the ALPP expression level may be detected and quantified based on the surface expression of ALPP on cells. Surface expression may be measured using binding agents, such as antibodies or antibody-based conjugates specific for ALPP, that selectively recognize

and bind to ALPP epitopes presented on the plasma membrane of tumour cells. Detection, and optional quantification, may then be performed using immunoassay-based methods, including flow cytometry, immunofluorescence microscopy, immunohistochemistry, or live-cell staining protocols, which allow direct measurement of ALPP molecules expressed on the cell surface. In various embodiments, cancer cell surface expression level of ALPP may be detected in blood or tumour cell samples. Fluorescently labelled antibodies or antibody conjugates that bind to extracellular epitopes of ALPP may be employed, enabling quantification of ALPP-positive cells or comparative measurement of ALPP expression intensity per cell. Flow cytometry analysis may be used to distinguish cell subpopulations, for example drug-tolerant persister (DTP) cells characterized by higher ALPP surface expression, from intrinsically resistant or non-responder cells characterized by low or absent ALPP surface expression (i.e. ALPP negative cells).

[069] In various embodiments, surface expression of ALPP may be quantified as an absolute value, such as the mean fluorescence intensity (MFI) per cell, or as a relative measure, such as the percentage of ALPP-positive cells in a tumour cell population. Such surface expression analyses may be performed on tumour-derived samples (e.g., primary tumour tissue, pleural effusion cells, or circulating tumour cells (CTCs) in blood samples), or on cultured tumour cell lines and patient-derived organoids.

[070] In various embodiments, surface ALPP expression detection may be combined with multiplexed staining to simultaneously quantify additional biomarkers of tumour heterogeneity or therapeutic response, such as markers of proliferation, apoptosis, or stemness. Such multiplex analyses may enable phenotypic characterization of tumour subpopulations and may provide diagnostic and prognostic insights.

[071] In various embodiments, the expression level of ALPP may be compared with a reference ALPP expression level. In various embodiments, the reference ALPP expression level may be derived from a control or comparative sample.

[072] The term "reference expression level" refers to baseline or control data against which results of the method (i.e. detected expression level of ALPP) are compared. These controls and reference data account for variables like the vehicle (e.g., DMSO) or untreated state to ensure that any changes in the experimental group are due to the cell type and/or TKI treatment, not the control condition. These controls and reference data may serve as a standard for normal or known levels of ALPP, allowing for the assessment of deviations or changes or similarities that may indicate the presence/absence of DTP cells in the subject. The reference expression level may be derived from a reference sample or control sample obtained from the subject or an individual that is not the subject. The term "reference sample," "reference cell," "reference tissue," "control sample," "control cell," or "control tissue," as used herein, refers to a sample, cell, tissue, standard, or level that is used for

comparison purposes. A reference sample, reference cell, reference tissue, control sample, control cell, control tissue, may be obtained from a healthy and/or non-diseased individual, or a non-healthy and/or diseased individual, or a cell line such as a cancer cell line (e.g. PC9 LUAD cell line). In various embodiments, the "reference expression level" may be obtained from a control sample that has not been treated or exposed to any experimental conditions, treatments, or interventions, such as drugs, chemicals, or other stimuli, and in particular has not been treated with a TKI to obtain a baseline level of ALPP in determining if the ALPP expression level has been increased or decreased relative to.

[073] In various embodiments, the reference ALPP expression level may be derived from the same subject prior to treatment with one or more TKIs or other cancer treatments (i.e. treatment naïve). In various embodiments, the reference ALPP expression level may be derived from the same subject during, and/or after treatment with one or more TKIs.

[074] In various embodiments, the reference ALPP expression level may be derived from a cell line that is treatment naïve, for example, the reference ALPP expression level for all the subsequent populations that are derived from it (i.e. DTP, DTEP, intrinsically resistant cell etc.) may be derived from treatment naïve PC9 cells.

[075] In various embodiments, the reference ALPP expression level may be derived from non-diseased healthy individuals. In various embodiments, the reference ALPP expression level may be derived from (i) the same subject prior to treatment with one or more TKIs or other cancer treatments, and (ii) non-diseased healthy individuals. In such embodiments, the subject sample may be a blood plasma sample.

[076] In various embodiments, the method comprises obtaining a reference (baseline) sample from the subject prior to commencement of therapy (e.g., TKI therapy), and subsequently obtaining one or more follow-up samples after therapy initiation. The measured expression level of ALPP in the follow-up sample may be compared to the baseline reference sample.

[077] In various embodiments, samples may be obtained from the subject at a baseline, and then at one or more predefined time-points during TKI therapy (for example, Day 7, Day 14, Day 28). The trend in ALPP expression levels (for example a rising ALPP despite ongoing TKI therapy) may be used as a prognostic indicator of imminent therapy-resistance or relapse. This allows dynamic therapeutic decision making (for example, switching to combination therapy at the first indication of DTP expansion). Accordingly, the method may comprise serial monitoring of ALPP expression in the subject over the course of TKI therapy.

[078] In various embodiments, based on the comparison step, a detected differential expression level of ALPP may be indicative of the presence or absence of DTP cells, and optionally intrinsically resistant cells, in the subject's cancer.

[079] The term "differential expression level" refers to a significant variation in the expression levels of a gene product, relative to a reference expression level or threshold value (cut-off). Differentially expressed may be defined as being upregulated (higher/increased expression) or downregulated (lower/decreased expression) relative to the reference/control expression level. Statistically significant differences may be determined by applying statistical tests well-known to those skilled in the art. The differential expression level may include "aberrant expression level" which refers to any deviation from the normal, physiological levels of expression of the gene (ALPP) within a biological system. A suitable fold change, log2 fold change (Log2FC) value or TPM value may indicate a differential expression level.

[080] The term "increased gene expression level", may refer to a higher or increased expression level of a gene above or greater than a reference gene expression level and may be characterised as being $ALPP^{high}$. The increased or higher gene expression level of ALPP may be above a threshold value. In contrast, the term "decreased expression level" may refer to a lower or decreased expression level of ALPP below or less than a reference expression level and may be characterised as being $ALPP^{low}$. The decreased or lower gene expression level of ALPP may be below a threshold value. A threshold value or level may be associated with a statistic, whereby a threshold value or level is a value or level above or below which the difference in the measured detectable signal is assigned significance (i.e. via a statistical method), for example a p-value <0.05 may be considered as statistically significant.

[081] In various embodiments, the expression level of the ALPP in the subject may be upregulated (i.e., increased) or downregulated (i.e. decreased) by at least 2 fold, at least 3 fold, at least 4 fold, at least 5 fold, at least 6 fold, at least 7 fold, at least 8 fold, at least 9 fold, at least 10 fold, at least 15 fold, at least 20 fold, at least 25 fold, at least 30 fold, at least 35 fold, at least 40 fold, at least 45 fold, at least 50 fold, at least 60 fold, at least 70 fold, at least 80 fold, at least 90 fold, at least 100 fold, at least 125 fold, at least 150 fold, at least 175 fold, at least 200 fold, at least 250 fold. In various embodiments, the fold difference may be determined and based upon surface expression of ALPP using FACS. In various embodiments, a fold change (i.e. relative increase to the reference value) of at least 10 may be termed as $ALPP^{high}$, wherein $ALPP^{low}$ may be deemed as minimal or lack of any fold change (e.g. a fold change less than 2) of ALPP expression or a decrease of ALPP expression by at least 2 fold.

[082] In various embodiments, the expression level of the ALPP in the subject may be measured to have a TPM value of 100 or greater, 200 or greater, 300 or greater, preferably 1000 or greater,

more preferably in a range of about 300 to 2000, indicating a high or upregulation (i.e., increased). In various embodiments, a TPM value of 300 or greater may be deemed as $ALPP^{high}$. In various embodiments, the expression level of the ALPP in the subject may be measured to have a TPM value of 99 or lower, 90 or lower, 80 or lower, indicating a low or downregulation (i.e., decreased). In various embodiments, a TPM value of 80 or lower may be deemed as $ALPP^{low}$.

[083] In various embodiments, the expression level of the ALPP in the subject may be upregulated (i.e., increased) or, downregulated (i.e. decreased) by a log2(fold-change) that is equal to or greater than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, preferably equal to or greater than 7. The Log2FC value for up-regulation of ALPP may be at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, or at least 7. Similarly, the Log2FC value for downregulation of ALPP may be less than 0, at least -1, at least -2, at least -3, at least -4, at least -5, at least -6, or at least -7. In various embodiments, a Log2FC value of at least 7 may be deemed as $ALPP^{high}$, and thus a Log2FC value of less than 7 may be deemed as $ALPP^{low}$.

[084] In various embodiments, detection of surface ALPP expression level of cells in a sample may enable the identification and quantification of ALPP-positive and ALPP-negative cells within the sample. Based on the signal intensity relative to a defined threshold, individual cells may be classified as ALPP-positive or ALPP-negative. The proportion or percentage of ALPP-positive cells within the total cell population may then be determined, providing a quantitative measure of the biomarker distribution in the sample. In various embodiments, the absolute number or relative percentage of ALPP-positive cells in a sample may serve as a diagnostic or prognostic indicator of DTP cell burden in the subject's cancer, and may be used to monitor treatment response, predict relapse risk, or stratify patients for additional therapeutic interventions.

[085] In various embodiments, surface ALPP expression levels of cells may be detected and quantified using fluorescence-activated cell sorting (FACS), with fluorescence intensity measured in one or more detection channels appropriate for the fluorophore employed. Suitable fluorophores may include, without limitation, fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), PerCP, or other spectrally distinct fluorescent labels. In various embodiments, the fluorophore is FITC and the detection channel may be FITC-A channel. Fluorescence intensity may be expressed in logarithmic arbitrary units (10^0–10^6). In various embodiments, a threshold of approximately 10^3 units and lower may be used to determine ALPP-low or ALPP-negative cells. Cells with fluorescence intensity greater than 10^4 may be considered as being $ALPP^{high}$. The proportion of $ALPP^{high}$ cells within a tumour cell population may serve as a diagnostic or prognostic measure.

[086] In various embodiments, an increased expression level of ALPP, relative to a reference expression level and/or threshold value, is indicative of the presence of DTP cells in the subject's cancer, and more particularly in the sample. Conversely, a decreased expression level of ALPP,

relative to a reference expression level and/or threshold value, is indicative of the absence or decrease of DTP cells in the subject's cancer, and more particularly in the sample.

[087] In various embodiments, the step of detecting the expression level of ALPP in the sample may comprise differential gene expression analysis using methods readily known to those skilled in the art.

[088] In various embodiments, the differential gene expression analysis may comprise normalization, statistical testing, and interpretation of results. In various embodiments, the differential gene expression analysis may comprise one or more differential gene expression methods, whereby the output of differential gene expression methods is used to indicate the presence/absence, and optional quantity, of DTP cells in the subject.

[089] In various embodiments, expression of ALPP can be correlated with cell cycle speed under TKI treatment. The step of detecting cell cycle speed may comprise the use of a reporter detectable by flow cytometry (i.e. Fluorescence-Activated Cell Sorting (FACS)). In various embodiments, the reporter may be a fluorescent timer (FT) genetic reporter. In various embodiments, the fluorescent timer (FT) genetic reporter may be a genetically encoded H2B-fluorescent timer (FT) reporter.

[090] In various embodiments, the step of detecting the expression level of ALPP could be possible by employing a fluorescent reporter construct under the control of an ALPP regulatory sequence.

[091] As mentioned above, the present inventors also found that ALPP expression level can be used to distinguish DTPs from intrinsically resistant cancer cells, providing a means to identify and isolate TKI-responsive cancer cells (responders) from nonresponsive ones (non-responders) via cell sorting. This may allow for subjects to be classified as responders versus non-responders.

[092] The term "intrinsically resistant cells" refers to pre-existing resistant cells in tumors prior to therapy that already pre-existed in the bulk cell population, whereas DTPs have the ability to undergo reversible resistance upon a drug holiday. Thus, intrinsically resistant cells are cancer cells that are naturally resistant to a particular drug from the outset of treatment. This resistance is not acquired or adaptive but is due to the inherent (innate) properties of the cells, and may be referred to as non-responder cells. In various embodiments, the intrinsically resistant cells referred to herein are cells that are intrinsically resistant to TKI treatment.

[093] Accordingly, in another aspect, there is provided a method of detecting the presence (or absence), of intrinsically resistant cells to TKI treatment in a subject having cancer, comprising: detecting an expression level of ALPP in the subject, wherein the expression level of ALPP is

indicative of the presence or absence, and optional quantity, of the intrinsically resistant cells in the subject.

[094] In various embodiments, the intrinsically resistant cells may be characterised as cells that fail to exhibit any ALPP expression or exhibit an expression level of ALPP below a reference expression level or threshold (e.g. $ALPP^{low}$), upon TKI treatment.

[095] Thus, the method disclosed herein is capable of distinguishing DTP cells from intrinsically resistant cell populations based on the expression levels of ALPP. By using ALPP as a selective biomarker, the method enables the identification of responder cells that survive TKI treatment in a reversible, drug-tolerant state, and to differentiate these bona fide DTPs from non-responsive cells that are intrinsically resistant to TKIs. This enables clear discrimination between transient, adaptive persistence and fixed resistance, thereby facilitating improved monitoring, stratification, and therapeutic targeting of cancer cell populations during TKI treatment.

[096] As used herein, the term "responder cells" refers to cancer cells that survive exposure to a TKI in a reversible, drug-tolerant state, also referred to as DTP cells. Responder cells are characterized by a high expression level of ALPP ($ALPP^{high}$), which distinguishes them from intrinsically resistant cells. Responder cells retain the capacity to regain drug sensitivity upon withdrawal of the TKI, thereby representing a transient and adaptive survival phenotype.

[097] As used herein, the term "non-responder cells" refers to cancer cells that exhibit innate or intrinsic resistance to TKI treatment, such that they remain viable and proliferative under drug exposure independently of adaptive persistence mechanisms. Non-responder cells are characterized by an absence or low expression level of ALPP ($ALPP^{low}$), which differentiates them from bona fide DTPs. Unlike responder cells, non-responder cells do not regain drug sensitivity upon TKI withdrawal, thereby representing a fixed resistance phenotype.

[098] In various embodiments, the method disclosed herein may further comprise the step of distinguishing DTP cells (responder cells) from intrinsically resistant cells (non-responder cells) based on the detected expression level of ALPP. In various embodiments, a high expression level of ALPP (i.e. $ALPP^{high}$) is indicative of DTP cells and a low expression level of ALPP (i.e. $ALPP^{low}$) is indicative of intrinsically resistant cells.

[099] The inventors noted that DTP cells characterised by $ALPP^{high}$ expression exhibit a reversible phenotype upon withdrawal of tyrosine kinase inhibitor (TKI) pressure ("drug holiday"). When the drug is removed, $ALPP^{high}$ DTP cells resume proliferation and their ALPP expression decreases to a baseline or near-baseline level, indicating restoration of drug sensitivity. When the same cells are

subsequently exposed again to the TKI treatment ("re-challenge"), ALPP expression is increased and the cells re-enter the drug-tolerant state.

[0100] Accordingly, in various embodiments, the method further comprises detecting ALPP expression before, during, and after a defined period of drug withdrawal, that is, the detection step may be repeated at one or more pre-determined time points based on TKI treatment and withdrawal of TKI treatment. The expression level of ALPP at each time-point may be compared with each other to detect, and quantify, any differential expression level. A decrease in ALPP expression during the drug holiday, followed by an increase in ALPP expression after re-exposure to the TKI treatment, may be indicative of the presence, and quantity, of reversible drug-tolerant persister (DTP) cells rather than intrinsically resistant cells.

[0101] This dynamic ALPP response provides a functional diagnostic marker distinguishing reversible tolerance from fixed resistance. It further enables longitudinal monitoring of persister dynamics in subjects undergoing cyclical or intermittent TKI regimens, and may guide clinical decisions on the duration or timing of drug holidays or combination therapy interventions.

[0102] In various embodiments, the detecting step may be performed before, during, and after a period of withdrawal of the tyrosine kinase inhibitor (TKI) treatment ("drug holiday"), and wherein a decrease in ALPP expression during the withdrawal period followed by an increase upon re-administration of the TKI treatment may be indicative of drug-tolerant persister (DTP) cells, and not intrinsically resistant cells. The reversibility of ALPP expression upon drug withdrawal and re-challenge may be used to detect the DTP cells and distinguish them from intrinsically resistant cells.

[0103] In various embodiments, the defined period, or drug holiday, may be at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 days, or 1 month, or 1 month and 1 week. In various embodiments, the defined period, or drug holiday, may be 7 days, or 14 days, or 1 month or 1 month and 1 week.

[0104] In various embodiments, serial measurement of ALPP expression in samples during and after TKI withdrawal may be used to monitor recovery of drug sensitivity and to determine optimal timing for re-administration of the TKI.

[0105] In various embodiments, the DTP cells and intrinsically (innate) resistant cells may be distinguished using Fluorescence-Activated Cell Sorting (FACS) based on the detected expression level of ALPP.

[0106] In various embodiments, since ALPP is expressed as a cell surface glycosyl-phosphatidylinositol (GPI)–anchored protein, the differential expression levels of ALPP ($ALPP^{high}$ vs.

ALPP^{low}) can be exploited as a basis for selective separation of cells within a heterogeneous sample. Thus, in various embodiments, the method disclosed herein not only distinguishes DTP cells from intrinsically resistant cells based on ALPP expression, but may further comprise isolating the respective cell populations for downstream analysis or therapeutic intervention. In various embodiments, the method disclosed herein may further comprise isolating the DTP cells and/or intrinsically (innate) resistant cells in a sample.

[0107] In various embodiments, the isolating step may comprise fluorescence-activated cell sorting (FACS). Cells may be incubated with a fluorescently labelled antibody that specifically binds ALPP, and sorted based on fluorescence intensity. In this manner, ALPP^{high} (responder/DTP) cells and ALPP^{low} (non-responder/intrinsically resistant) cells can be separated into distinct fractions, thereby enabling recovery of pure populations for functional assays, transcriptomic profiling, or drug-sensitivity testing. In various embodiments, ALPP^{low} cells may be distinguished from ALPP^{high} cells by defining fluorescence intensity thresholds in flow cytometry, enabling their separation and quantification as distinct subpopulations.

[0108] In various embodiments, the isolating step comprises magnetic-activated cell sorting (MACS). Cells may be incubated with ALPP-specific antibodies conjugated to magnetic beads, and application of a magnetic field allows enrichment or depletion of ALPP^{high} cells, depending on the selection strategy. This provides a scalable means to enrich DTPs from bulk tumour cell populations. In yet other embodiments, the isolating step may be carried out using microfluidic cell sorting techniques. For example, microfluidic devices functionalized with anti-ALPP ligands may capture ALPP^{high} cells from a flow stream. Captured cells may then be released by enzymatic cleavage, competitive elution, or disruption of GPI anchorage. Such approaches offer high sensitivity and minimal sample requirement, making them suitable for isolating DTP populations.

[0109] Cells isolated by any of the above methods may be used in a range of downstream applications. In various embodiments, the isolated cells are subjected to molecular profiling, such as RNA sequencing, proteomic analysis, or epigenomic mapping, to characterise DTP-specific adaptive programs. In various embodiments, the isolated cells are subjected to drug testing to identify candidate therapeutic agents that selectively eliminate DTPs while sparing normal cells. In various embodiments, functional assays may be performed, for example to measure the reversibility of drug tolerance by culturing isolated DTPs after TKI withdrawal to confirm restoration of sensitivity. In various embodiments, isolated cells may be used for biomarker validation, such as correlating ALPP expression with other candidate DTP markers, including AXL, KDM5A, and SOX2.

[0110] Following identification of responder DTP cells (ALPP^{high}), and optional distinguishing from non-responder cells (ALPP^{low}), the present inventors also found that upon a longer period of drug exposure, a small proportion of the initial responder DTP cells (ALPP^{high}) will re-enter a faster

proliferative state (fast-cycling DTEPs) while the remainder remains slow-cycling (slow-cycling DTEPs). At this DTEP stage, ALPP expression level may also be used to further distinguish and differentiate slow-cycling DTEPs and fast-cycling DTEPs. In particular, ALPP expression level was found to be higher in slow-cycling DTEPs compared to fast-cycling DTEPs, the latter might be responsible for faster tumour recurrence and therapy failure. In various embodiments, slow-cycling DTEPs may be distinguished from fast-cycling DTEPs based on surface ALPP expression levels of cells detected and quantified using fluorescence-activated cell sorting (FACS), with fluorescence intensity measured in one or more detection channels appropriate for the fluorophore employed. For example, the threshold values to distinguish these cells may be approximately 10^3 units and lower to determine fast-cycling DTEPs ($ALPP^{low}$) and fluorescence intensity greater than 10^4 to determine slow-cycling DTEPs ($ALPP^{high}$).

[0111] Accordingly, the method disclosed herein may further comprise distinguishing fast-cycling DTEP cells from slow-cycling DTEP cells based on the detected expression level of ALPP, wherein the expression level of ALPP is higher in slow-cycling DTEP cells relative to fast-cycling DTEP cells.

[0112] In various embodiments, non-responder cells may refer to a subpopulation of fast cycling cells that maintain a higher rate of cell division even in the presence of therapeutic drugs. This characteristic is attributed to the $ALPP^{low}$ cells upon 7 days of TKI treatment. The term "slow-cycling DTP cells" refers to a subpopulation of cells that enter a state of reduced proliferation, characterized by significantly slower or near-quiescent cell cycles. This population corresponds to the $ALPP^{high}$ cells upon 7 days of TKI treatment. DTEPs (Drug-Tolerant Expanded Persisters) refer to expanded, proliferating cells that have evolved from slow-cycling DTPs, when continuously exposed to drugs, demonstrating a more advanced and robust state of drug tolerance and adaptation. In this regard, the inventors observed that slow-cycling DTPs which evolve into DTEPs, can be further distinguished by ALPP surface expression levels. DTEPs expressing low levels of ALPP, termed as $DTEP^{low}$ cells, represent a faster proliferative, more advanced state of drug tolerance with acquired resistance to TKI treatment. In contrast, DTEPs expressing high levels of ALPP, termed as $DTEP^{high}$ cells, are much slower proliferative cells in the continuous presence of TKI. In summary, DTPs or DTEPs which maintain high ALPP expression remain in a reversible, quiescent-like state of drug tolerance and are capable of regaining TKI sensitivity upon drug withdrawal (i.e. drug holiday).

[0113] In various embodiments, slow cycling DTP cells may be termed as responder cells and fast cycling cells as non-responder cells.

[0114] In various embodiments, the fast-cycling non-responder cells and slow-cycling DTP cells may be distinguished using Fluorescence-Activated Cell Sorting (FACS) based on the detected expression level of ALPP. In various embodiments, the method may further comprise isolating the fast-cycling non-responder cells and/or slow-cycling DTP cells in the sample.

[0115] In various embodiments, the method disclosed herein may further comprise classifying the likelihood of the subject being responsive or non-responsive to TKI treatment and/or anti-ALPP therapies based on the expression level of ALPP and detection of DTP cells, DTEPs and/or intrinsically resistant cells. The rationale is that ALPP expression and the detection of distinct drug-tolerant or resistant cell subpopulations provide predictive value for treatment outcome. DTP cells, as well as DTEPs and intrinsically resistant cells, are known to contribute to therapeutic failure and disease relapse under TKI regimens. ALPP expression levels may thus further indicate sensitivity to anti-ALPP targeted therapies, and when considered together with the abundance of DTP- or resistant-like cell states in a sample, it may allow stratification of patients into groups more or less likely to benefit from TKI treatment and/or anti-ALPP therapies. Accordingly, classification of a subject based on these combined markers provides a framework for personalised treatment selection, early identification of non-responders, and the development of combination regimens to overcome resistance mechanisms.

[0116] The term "anti-ALPP therapies" refers to the use of antibodies or Antibody-Drug Conjugates (ADCs) that target the ALPP protein, CAR T-cell therapy involving genetically engineering a patient's T-cells to express a receptor that targets ALPP, enabling them to specifically attack ALPP-expressing cancer cells, therapeutic cancer vaccines that target ALPP are designed to stimulate the immune system to recognize and attack ALPP-expressing tumour cells, small-molecule inhibitors targeting ALPP's enzymatic activity, and other known therapies that target the ALPP gene expression or gene product.

[0117] In various embodiments, the TKI treatment comprises EGFR TKI treatment with one or more EGFR TKIs selected from 1st generation EGFR TKIs, 2nd generation EGFR TKIs, 3rd generation EGFR TKIs, and 4th Generation EGFR TKIs.

[0118] The term "1st generation EGFR TKIs" refers to EGFR TKIs that are reversible inhibitors of the ATP-binding site of the EGFR tyrosine kinase. They bind to both the wild-type and mutant forms of the EGFR, but they are less specific to mutations and can cause side effects by inhibiting the normal (wild-type) EGFR. These drugs were the first to be approved for EGFR-mutant NSCLC and showed effectiveness in tumours with common activating mutations (e.g., exon 19 deletions and L858R point mutations in exon 21). However, resistance often develops, commonly due to a secondary mutation in EGFR, such as T790M. In various embodiments, the 1st generation EGFR TKIs may comprise Gefitinib and Erlotinib.

[0119] The term "2nd generation EGFR TKIs" refer to EGFR TKIs that are irreversible inhibitors that form a covalent bond with the ATP-binding site, leading to prolonged inhibition of EGFR. They are designed to overcome resistance due to the T790M mutation, but they are less specific and can also

inhibit other members of the HER family, like HER2. These TKIs are more potent than 1st-generation inhibitors and have shown efficacy in patients who have failed prior 1st-generation therapy. However, due to their broader action, they may have more significant side effects. In various embodiments, the 2nd generation EGFR TKIs may comprise Afatinib and Dacomitinib.

[0120] The term "3rd generation EGFR TKIs" refer to EGFR TKIs that are designed to specifically target the T790M resistance mutation while sparing wild-type EGFR. This selectivity aims to reduce toxicity and provide a better safety profile. Osimertinib, the most widely used 3rd-generation TKI, is effective against both T790M-positive and T790M-negative mutations and is currently considered a standard first-line treatment for patients with EGFR-mutant NSCLC. In various embodiments, the 3rd generation EGFR TKIs may comprise Osimertinib and Nazartinib.

[0121] The term "4th-generation EGFR TKIs" refer to EGFR TKIs that are still in development. They are designed to overcome resistance mutations that arise after 3rd-generation TKIs, such as the C797S mutation, which can lead to resistance to Osimertinib. They aim to maintain efficacy against a broad range of EGFR mutations while avoiding resistance mechanisms that may arise against earlier-generation inhibitors. These TKIs are in early clinical trials and aim to provide further options for patients who have developed resistance to earlier-generation EGFR inhibitors. In various embodiments, the 4th generation EGFR TKIs may comprise BLU-945 and BDTX-189.

[0122] Detecting the presence or absence of DTP cells in a subject's cancer, and optionally intrinsically resistant cells, and/or DTEPs, may provide an opportunity for early therapeutic intervention before clinically significant resistance occurs. Therefore, the combined use of anti-ALPP therapies alongside TKIs may provide a strategy to selectively target ALPP-expressing cells, including drug-tolerant states, thereby delaying or preventing the onset of resistance and relapse.

[0123] Accordingly, in one aspect, there is also provided a method for delaying and/or preventing the development of drug-tolerant persister (DTP) cells resistant to TKI treatment in a subject with cancer, comprising administering to the subject an anti-ALPP therapy, thereby preventing or delaying relapse and/or the emergence of acquired resistance to TKI treatment. The presence/absence of drug-tolerant persister (DTP) cells resistant to TKI treatment may have been detected in the subject using the method disclosed herein.

[0124] The term "administering" and variations of that term, including "administer" and "administration", include contacting, applying, delivering or providing a TKI to the subject by any appropriate means.

[0125] In various embodiments, the anti-ALPP therapy may be used as an adjuvant therapy in combination with the TKI treatment, to improve disease-free survival of the subject. In various

embodiments, administering the anti-ALPP therapy may be concurrent with the TKI treatment, sequentially before or after the TKI treatment, or in alternating cycles, in order to maximize depletion of ALPP-expressing DTP cells while maintaining inhibition of the target kinase pathway.

[0126] In various embodiments, administration of the anti-ALPP therapy is guided by a prior step of detecting ALPP expression and/or the abundance of DTP cells in a biological sample or in vivo. Treatment may be initiated upon detection of a threshold level of ALPP expression or DTP burden, or continued in subjects identified as being at risk of relapse based on these biomarkers.

[0127] In various embodiments, the administration of anti-ALPP therapy may delay or prevent the emergence of acquired resistance mutations (e.g., EGFR T790M, EGFR C797S, ALK G1202R) that typically arise from the expansion of drug-tolerant populations under selective pressure of TKI treatment.

[0128] Accordingly, the invention also provides anti-ALPP therapies as an adjuvant treatment for EGFR mutant LUAD, thereby eliminating residual DTP cancer cells that persist following TKI therapy. In further embodiments, detection of ALPP expression levels in a sample, such as blood, may be employed to identify patients harbouring residual DTP cells after EGFR TKI treatment, thereby enabling patient stratification and guiding the administration of anti-ALPP therapies to prevent relapse and disease progression.

[0129] In various embodiments, the method may further comprise stratifying the subject into responsive or non-responsive categories based on the detection of ALPP expression levels in the subject, and optionally in combination with detecting the amount of DTP, DTEPs, or intrinsically resistant cells, thereby enabling a precision medicine approach.

[0130] The efficacy of TKI treatment varies among patients due to the heterogeneous nature of tumor cell populations and the emergence of drug-tolerant states. Within the tumor, subsets of cells respond differently to TKI pressure: responder cells, such as slow-cycling drug-tolerant persister (DTP) cells, can survive initial treatment but are often characterized by high expression of ALPP and may remain sensitive to therapeutic intervention. By contrast, non-responder cells/ intrinsically resistant cells, and fast-cycling subset of drug-tolerant expanded persisters (DTEPs), have been shown here to exhibit low ALPP expression and drive therapeutic resistance and eventual relapse.

[0131] In addition, ALPP is also secreted into the bloodstream via exosomes from ALPP expressing cells. This allows subjects who are likely to benefit from anti-ALPP therapy to be easily identified as well as monitor their response over time with ELISA-based blood tests. There are currently no clinically approved therapies to target these drug persisters, nor methods to detect them.

[0132] In considering the discovery that ALPP-positive residual cancer cells, particularly ALPP^{high} cells retain sensitivity to TKIs, ALPP expression may be used as a biomarker of treatment response. In this regard, monitoring the balance between responder and non-responder cell populations in cancer patients may provide a functional readout of how effectively a TKI treatment is suppressing resistant subclones. Thus, the detection of ALPP expression levels, which distinguish these cellular states, may be used to enable real-time assessment of TKI treatment efficacy.

[0133] Accordingly, there is also provided a method to evaluate patient response, guide therapeutic decisions, and identify early signs of treatment failure, thereby facilitating precision management of TKI therapy in cancer. In various embodiments, there is provided a method of monitoring the efficacy of an TKI treatment in treating cancer in a subject, comprising: administering the TKI treatment to the subject; obtaining a sample from the subject; and detecting the presence/absence, and optionally quantity, of responder cells (i.e. slow cycling drug-tolerant persister (DTP) cells characterized by high expression levels of ALPP) and non-responder cells (i.e. intrinsically resistant cells, DTEP cells characterized by low expression levels of ALPP) to TKI treatment in the sample based on detected expression levels of ALPP of cells in the sample using the method disclosed herein. The presence/absence, and optionally quantity, of responder cells and/or non-responder cells in the sample may be indicative of the efficacy of the TKI treatment in treating the cancer in the subject.

[0134] In various embodiments, the method may further comprise detecting expression levels of ALPP of cells in a reference sample obtained from the subject prior to the administration step, wherein the expression level of ALPP in the detection step is compared to the expression level of ALPP in the reference sample obtained prior to the administration step.

[0135] In various embodiments, the steps may be repeated two or more times within a predetermined time-frame pre-treatment, during and post-treatment, and the classification at each time point is compared against each other to assess the progression of the cancer and efficacy of the cancer treatment based on the detection, and optional quantification, of DTP cells, DTEPs, and/or intrinsically resistant cells in the sample at each time point. The method may comprise serial monitoring of ALPP expression in the subject over the course of TKI therapy.

[0136] In various embodiments, the method may further comprise selecting a cancer treatment regimen for the subject comprising a TKI treatment and/or an anti-ALPP therapy based on the presence/absence of responder and/or non-responder cells detected in the sample.

[0137] The term "cancer treatment regimen" as used herein refers to a cancer treatment regimen the selection of which may be indicated by the methods of the invention and may comprise provision to the subject of TKI treatment and/or an anti-ALPP therapy. Suitably the TKI treatment may be provided as the only (or primary) anti-cancer agent in a treatment regimen, or an anti-ALPP therapy

may be provided as the only (or primary) anti-cancer agent in a treatment regimen, or the anti-ALPP therapy may be used as an adjuvant therapy to the TKI treatment. The cancer treatment regimen further comprises provision of one or more chemotherapeutic agents. The term "chemotherapeutic agent" as used herein refers to a chemotherapeutic agent suitable for use in a cancer treatment regimen selection may be one that is conventionally used for treatment of the cancer in question.

[0138] Detecting the presence, and optional quantity, of responder cells in a subject's cancer following TKI treatment provides an opportunity for therapeutic intervention before resistant clones expand. By administering an anti-ALPP therapy after TKI treatment, the ALPP-expressing DTP population can be selectively targeted and eliminated, thereby reducing the likelihood of relapse and delaying or preventing the emergence of acquired resistance. This sequential treatment strategy leverages TKI therapy to debulk the bulk tumor while employing anti-ALPP therapy to eradicate residual persister cells.

[0139] Accordingly, in another aspect, there is provided a method for treating cancer in a subject, comprising administering to the subject an Anti-ALPP therapy after the subject has received TKI treatment, wherein the presence of responder cells (i.e. drug-tolerant persister (DTP) cells or slow cycling drug-tolerant expanded persister (DTEP) cells characterized by high expression levels of ALPP) resistant to TKI treatment has been detected in the subject using the method disclosed herein.

[0140] As used herein, the terms "treating" and "treatment" refer to reduction in severity and/or frequency of symptoms, elimination of symptoms and/or underlying cause, prevention of the occurrence of symptoms and/or their underlying cause, and improvement or remediation of damage. As used herein, the term "preventing" refers to the prophylactic or preventative measures that prevent and/or slow the development of a targeted pathologic condition or disorder. Thus, those in need of treatment include those already with the disorder; those prone to have the disorder; and those in whom the disorder is to be prevented and those in whom reoccurrence of the disorder needs to be prevented. In various embodiments, the terms "treating", "ameliorating", "delaying" or "preventing", as used herein refer to achieving one or more of the following in the subject: (a) reducing the severity of a given condition; (b) limiting or preventing the development of a condition; (c) removing a given condition; (d) limiting or preventing the recurrence of a given condition; (e) alleviation of the condition and/or its symptoms; and (f) delay the onset of a condition. Any one or more of these effects may be achieved in a subject who previously had or currently has or is suspected to have cancer.

[0141] It is also contemplated that a kit is provided for use in carrying out the method disclosed herein. All embodiments disclosed above in relation to the methods similarly apply to the kit and *vice versa*. In particular, the kit may be used for (i) detecting the presence or absence of drug-tolerant persister (DTP) cells resistant to tyrosine kinase inhibitor (TKI) treatment and/or intrinsically resistant cells to TKI treatment in a subject having cancer, (ii) delaying and/or preventing the development of

drug-tolerant persister (DTP) cells resistant to TKI treatment in a subject with cancer, (iii) monitoring the efficacy of an TKI treatment in treating cancer in a subject, or (iv) treating cancer in a subject.

[0142] There is also contemplates the use of the biomarker ALPP in the preparation of a diagnostic product for (i) detecting the presence or absence of drug-tolerant persister (DTP) cells resistant to tyrosine kinase inhibitor (TKI) treatment and/or intrinsically resistant cells to TKI treatment in a subject having cancer, (ii) delaying and/or preventing the development of drug-tolerant persister (DTP) cells resistant to TKI treatment in a subject with cancer, (iii) monitoring the efficacy of an TKI treatment in treating cancer in a subject, or (iv) treating cancer in a subject.

[0143] In various embodiments, the diagnostic product may be selected from the group consisting of a kit, a diagnostic device and a computer system.

[0144] In various embodiments, the kit may comprise one or more binding agents directed to ALPP; and instructions for use.

[0145] In various embodiments, the one or more binding agent is for detecting and quantifying expression levels of ALPP. The binding agent may be selected from a polypeptide, a polynucleotide probe, or a fragment thereof. Preferably, the polypeptide is an antibody that specifically binds to ALPP, which may be surface-expressed on tumour cells. Optionally, the antibody may be an antibody-based conjugate.

[0146] In various embodiments, the binding agents may be selected and designed for use in immunoassays, including ELISA, flow cytometry, lateral flow assays, or dipstick formats, for detection of ALPP in liquid samples, such as blood or cell culture supernatants. In various embodiments, antibody-based conjugates may be used for imaging or detection as a molecular complex. These conjugates may specifically bind to ALPP in the sample, including ALPP that is surface expressed on tumour cells. In the case of lateral flow assays, such binding results in a visual signal, typically appearing as a test line, while in flow cytometry or ELISA formats, the binding enables quantification of ALPP expression levels.

[0147] In various embodiments, the kit may further include a detectable label. The term "detectable label" refers to an atom or molecule that specifically detects a molecule, including a label among the same type of molecules without the label. The detectable label may be one attached to an antibody, interacting protein, ligand, nanoparticle, or aptamer that specifically binds to the protein or fragment thereof. The detectable label may include a radionuclide, a fluorophore, or an enzyme. The kit may be used according to various immunoassays, immunostaining methods, or sequencing-based techniques known in the art. The immunoassays or immunostaining may include radioimmunoassay, radioimmunoprecipitation, immunoprecipitation, ELISA, capture-ELISA, inhibition or competition

assays, sandwich assays, flow cytometry, immunofluorescence, and immunoaffinity purification. Additionally, the kit may be applied in next-generation sequencing (NGS)-based approaches, including RNA sequencing (RNA-seq) for transcriptomic analysis and chromatin immunoprecipitation sequencing (ChIP-seq) for detecting protein-DNA interactions, both of which can facilitate biomarker discovery. Preferably, the kit may be a reverse transcription polymerase chain reaction (RT-PCR) kit, a DNA chip kit, an enzyme-linked immunosorbent assay (ELISA) kit, a protein chip kit, a rapid kit, a multiple reaction monitoring (MRM) kit, or an NGS-based kit optimized for RNA-seq or ChIP-seq applications.

EXAMPLES

Materials and Methods

[0148] Cell culture and materials: PC9 and PC9FT cells were maintained in RPMI 1640, with L-Glutamine, with 25 mM HEPES (Catalog No.SH30255.01) supplemented with 10%(v/v) fetal bovine serum (FBS) (Catalog No. SV30160.03), 1% (v/v) penicillin/streptomycin (Catalog No. LS15140122) and 1%(v/v) L-glutamine (Catalog No. 25030081).

[0149] 293T and MDA-MB-231 were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Catalog No. SH30022.01) supplemented with 10%(v/v) foetal bovine serum (FBS) (Catalog No. SV30160.03), 1% (v/v) penicillin/streptomycin (Catalog No. LS15140122) and 1%(v/v) L-glutamine (Catalog No.25030081).

[0150] Generation of reporter cell line: Reporter plasmid was purchased from Addgene (pSCMV-H2B-FT-Medium (#157670)) and cloned into a lentiviral backbone with a doxycycline inducible system. 293T cells were seeded in a 10cm dish. Upon 70% confluency, cells were transfected with 20ug of plasmids containing both FT reporter plasmid and packaging plasmids. FuGENE(R) HD Transfection Reagent (Catalog No. E2312) was used for the transfection of 293t cells and media was changes after 24hours. Fresh media was added and collected after 48 fours which contains lentivirus. PC9 cells were transduced with lentivirus for 48 hours with polybrene transfection reagent (Catalog no. TR-1003-G). Selection of cells with FT reporter was done using puromycin (1ug/ml) (Catalog No. A11138-03)

[0151] Generation of DTPs: For PC9 FT DTP population: 6 x 10^6 PC9FT cells were seeded in 15cm dishes one day before addition of gefitinib at a final concentration of 0.2uM. Respective drug depending on the cell type were added for 7 days with a change of culture media and top up of drug after the first three days. At day 7, cells were sorted using FACS for RNA sequencing or continuous culture in drug holiday.

[0152] For PC9 DTP population: 6 x 10^6 PC9FT cells were seeded in 15cm dishes one day before addition of osimertinib. Osimertinib was added to make a final concentration of 0.2uM for 7 days with a change of culture media and top up of drug after the first three days. At day 7, cells were either sorted and cultured in drug holiday or continuous drug exposure for another 14 days. After which, DTP population in both drug holiday and continuous drug exposure was were sorted again based on their ALPP expression.

[0153] **Preparation of cell lysates and western blots:** Western blot samples were harvested and washed with phosphate buffered saline (PBS). Cell pellets were collected and lysed with urea lysis buffer (50mM TRIS pH 7.9, 8M Urea, 1% (w/v) CHAPS) containing protease inhibitors. Protein assay dye reagent concentrate (Bio-Rad; Catalog No. 5000006) was used to measure the lysate protein concentration. 10-30 µg of boiled protein lysates were separated on an 8% or 10% Bis-Tris PAGE gel before trans-blotting onto a PVDF membrane. The membrane was blocked with 5% (w/v) milk for 1 hour before incubating with respective primary antibodies overnight at 4 degrees with slow shaking. Blots were washed with PBST (PBS +0.1% tween 20) and then incubated with respective HRP-conjugated secondary antibodies for 1 hour at room temperature. Blots were washed again with PBST before they were developed by the ECL-based chemiluminescence method (Catalog No. 170-5061) and images were taken by iBright1500 (Invitrogen).

[0154] **Flow cytometry:** For FACS analysis and sorting of DTPs, cells were harvested and washed with PBS. Cells were then resuspended in FACS buffer (made from PBS with 10% FBS). Cells were sorted based on their baseline expression or upregulation upon drug treatment.

[0155] For antibody staining, antibodies were diluted in FACs buffer. Cells were first washed after harvesting and incubated with primary antibody for 30 mins at room temperature. Antibody dilution used was 1ug per 1million cells in 100ul of FACS buffer. After primary antibody incubation, cells were washed with PBS and incubated at room temperature with secondary antibody in the dark for 30 minutes. After secondary antibody staining, cells were washed with PBS and resuspended with FACs buffer with DAPI (Catalog No. 75004) at a 1:1000 dilution. Cells were passed through 40um or 70um strainer before analysis or sorting.

[0156] For FACS analysis to investigate cell cycle profiles, cells were harvested and washed with PBS before fixation with cold 70% ethanol for minimally 30 minutes at 4 degrees. Cells were spun down and resuspended with PBS to rehydrate for at least 30 minutes. After rehydrating, cells were spun down and resuspended in FACS buffer. Propidium iodide (PI) (Catalog No. 75002) and RNAse A (Catalog No. R1253) was added to the samples to stain the DNA content.

[0157] **Immunofluorescence assay:** Cells were seeded in a 35 mm imaging dish with four compartments and the ibidi Polymer Coverslip Bottom (Catalog No. 80416).

[0158] For permeabilised staining: After treatment with DMSO or stated drug, chambers were washed with PBS before fixation using 4% PFA (Catalog No. sc- 281692) for 10 mins. Chambers were then washed with PBS before blocking using IF blocking buffer [PBS +BSA (Catalog No A-420-1) + triton-X (Catalog No T8787-250ml)]. After blocking, chambers were incubated with primary antibody at 4 degrees overnight incubation. Chambers were washed again using PBS and incubated with secondary antibody for 1.5 hours. After secondary antibody incubation, chambers were washed and DAPI (Catalog No. 75004) was added in IF blocking buffer at least 30 mins before imaging.

[0159] **RNA isolation and real-time quantitative PCR (qPCR):** Following manufacturers protocol, total RNA was extracted using TRIzol and Direct-zol RNA miniprep Kit (Catalog No. R2052). Complementary DNA (cDNA) was attained using SensiFAST cDNA synthesis kit (Catalog No. BIO-65054). Real-Time Quantitative PCR(RT-qPCR) was conducted using powerup SYBR green PCR master mix (Catalog No. A25776) on Quantistudio5 real time PCR System (applied biosystems).

[0160] **RNA Sequencing:** RNA was extracted from DTP cells using AllPrep DNA/RNA Micro Kit (Catalog No. 80284), and the RNA integrity number was determined using RNA screen tape and sample buffer following manufacturer's protocol (Catalog No. 5067-5579 and 5067-5580). Extracted RNA was used for preparing libraries using the NEBNext Ultra II RNA library Prep Kit for Illumina (Catalog No. #E770L) and the average fragment length was checked by D1000 ScreenTape (Catalog No. 5067-5582) with D1000 reagents (Agilent; Catalog No. 5067-5583). Concentration of libraries were measured using Equalbit 1x dsDNA HS Assay Kit (Catalog No. EQ121-02). These samples with unique index tags were pooled and sequenced by HiSeq-PE150. The raw sequencing files were demultiplexed. The raw fastq files and data analysis were processed by our bioinformatician (Dr. Oleg Grinchuk, WWT lab).

[0161] For RNAseq analysis: Briefly, paired-end raw sequencing reads were trimmed with Trim Galore (version 0.6.7; https://www.bioinformatics.babraham.ac.uk/projects/ trim_galore/) with default parameters. Cleaned reads were then mapped to the human hg19 reference genome (downloaded from Illumina website: https://sapac.support.illumina.com/sequencing/sequencing_software/igenome.html) using the RSEM pipeline (v1.3.1) with option for reads aligner --bowtie2. DeSeq2 (1.36.0) was applied to differential gene expression analyses with default settings. Genes were considered to be differentially expressed if they showed more than 1.5-fold difference in expression with adjusted P-value less than 0.05 after correcting for multiple testing by FDR (Benjamini and Hochberg false discovery rate).

[0162] **Drug sensitivity titrations/curves:** 2000-3000 cells were seeded in each well of a 96-well plate in triplicates. Drug titrations were performed with the maximum effective concentration of the respective drugs stated in the figures below. Drug exposures were performed for a duration of 72

hours unless otherwise stated. Measurement of cell viability was performed using CellTiter-Glo® 3D cell viability assay reagent (Catalog No. G9683) or cell counting lite 2.0 (Catalog No. DD1101-02).

Results and Discussion

[0163] Therapeutic resistance is a major challenge in targeted therapies. Specifically, the drug-tolerant persister (DTP) state was of interest, wherein a small population of cancer cells employs non-genetic mechanisms to evade cell death and enter a reversible drug-tolerant state in response to treatment. Notably, DTPs exhibit a slow cycling dormant state similar to embryonic diapause**[1,2]**, underscoring cancer cells' appropriation of early developmental programs to shape their distinctive states (**FIG. 1A)[9].**

[0164] Several approaches have been devised to study DTPs, mostly leveraging on single-cell RNA sequencing, coupled to lineage tracing and label retention. However, an inherent limitation is that such assays can only provide information on the divisional history, but not the current cycling state of the cells**[3-6]**. To address this limitation, it was sought to identify a complementary approach that will enable us to identify and prospectively isolate DTPs with distinct cycling rates in *real time*.

[0165] To achieve this, a genetically encoded H2B-fluorescent timer (FT) reporter was used that can capture the cell cycle speed of live cells in a single measurement**[7]** (**FIG. 1B**). This approach offers significant advantages over existing methods and allows the capture of different cell states upon drug treatment such as intrinsically resistant cells, DTPs and those that re-enter the cell cycle and resume proliferation (termed 'drug-tolerant expanded persisters', DTEP). Importantly, the ability to isolate distinct populations of DTPs from non-responders in real-time based on their proliferative capacity has enabled a thorough characterization of DTPs to be performed, leading to the discovery of novel biomarkers and therapeutic opportunities.

[0166] The H2B-FT reporter system was first applied to study resistance mechanisms in lung cancer cells subjected to standard-of-care tyrosine kinase inhibitors (TKIs). PC9-FT were treated with gefitinib for 3 days and 7 days and dox was added 48hours prior to FACs analysis. The reporter illustrates an increase in the population of cells exhibiting higher red fluorescence after 3 days of gefitinib treatment. As the FT protein is constitutively synthesised, cells that start to get arrested, would exhibit an accumulation of red signal within the cells since the half-life of the red FT conformation is much longer than the blue FT conformation. Meanwhile, actively proliferating cells would have lesser red FT protein retained in the cells due to dilution by cell division thus emitting lesser red signal. FACS analysis of PC9FT upon gefitinib treatment for 3 days revealed that there is an increase in the number of cells in the Slow (red) gating which corresponds to the increase in number of cells undergoing growth arrest (from 0.82% to 5.71% when compared to +Dox) while there is a loss of cells in the Fast (blue) gating which corresponds to cells actively proliferating persisters (from 21.9% to 0.57% when compared to +Dox). By day 7 of gefitinib treatment, the increase in

proportion of Slow (red cells) is smaller (from 1.25% to 3.69% when compared to +Dox) and the decrease in proportion of Fast (blue) cells is smaller (from 21.5% to 5.07% when compared to +Dox) indicating that the persisters are starting to re-enter the cell cycle after being arrested at day 3 **(FIG. 1C)**.

[0167] RNA sequencing (RNAseq) was performed on the PC9FT cells sorted from +dox only and after 7 days treatment of gefitinib to investigate the transcriptional differences between the red and blue cells upon TKI treatment. Notably from the sequencing results, we identified 318 downregulated and 511 upregulated genes with corrected p-value < 0.05 and log2-fold change of >1.5 and <−1.5 amongst the slower proliferative red cells than the faster proliferative blue cells.

[0168] Pathway and process enrichment analysis was performed using Metascape and based on the Gene ontology (GO) biological processes and Reactome gene set sources, several pathways associated with cell cycle were enriched. In particular, amongst the downregulated genes in the slower proliferative red cells, 99 genes were associated with the term "mitotic cell cycle" while amongst the upregulated genes, 43 genes were associated with the term "negative regulation of cell population proliferation" (**FIG. 1D**). Furthermore, we noticed that the faster proliferative persisters had higher transcription levels of genes associated with S-phase and G2/M-phase (**FIG. 1E**) providing further support that our reporter can accurately distinguish faster proliferative cells from the slower ones. Q1-Q4 are quadrants where the numbers below each quadrant represents the percentage of the population which lies within the quadrant.

[0169] Elevated expression levels of ALPP, is observed in various embryonic stages where the ALPP expression peaks at the 8 cell embryo stage and decreases thereafter (**FIG. 2A**). In normal adult tissues, ALPP is predominantly expressed in the placenta during pregnancy, but is ectopically upregulated in various cancers**[8]**. Nonetheless, the precise roles of ALPP in tumorigenesis and therapeutic resistance remain elusive. Interestingly, when PC9 cells were treated with gefitinib, elevated expression of ALPP was observed in the DTPs. Quantitative polymerase chain reaction (qPCR) was performed on PC9 cells upon Gefitinib treatment (0.2uM) for 3,7,4,21 days and the results were normalised to heterogenous nuclear ribonucleoprotein L (HNRNPL), as a loading control. Compared to the DMSO treatment, cells that were treated with gefitinib had much higher mRNA transcript levels (**FIG. 2B**). Protein expression was also compared by performing western blot and ALPP expression levels were also seen to be upregulated **(FIG. 2C)**. Immunofluorescence imaging was also conducted to see the localisation and expression of ALPP upon Gefitinib treatment which further illustrates the increase in ALPP expression upon gefitinib treatment (**FIG. 2D**).

[0170] To translate these findings in a clinical setting, collaboration with NCCS was carried out to leverage a TKI-NSCLC clinical trial (PROGRESS), and demonstrated that ALPP is indeed upregulated in neoadjuvant Gefitinib (1st generation TKI) compared to treatment-naïve tumour

resections (**FIG. 2E**).

[0171] In particular, **FIG. 2E** shows a comparative analysis of ALPP expression in treatment-naïve lung tumour samples versus tumours exposed to neoadjuvant gefitinib. ALPP expression levels are represented as LogCPM (CPM is Counts Per Million) values derived from transcriptomic analysis. Treatment-naïve tumours display low to undetectable ALPP expression, with mean value below zero on the LogCPM scale, consistent with minimal or absent ALPP activity at baseline. By contrast, tumours treated with neoadjuvant gefitinib exhibit a marked increase in ALPP expression, with mean LogCPM value closer to zero and a broader distribution of expression across samples. Statistical testing confirmed this upregulation to be significant ($p = 0.00027$). These results demonstrate that ALPP is strongly induced by TKI treatment *in vivo*, supporting its role as a marker of drug-tolerant persister cells that emerge in response to therapy.

[0172] As shown in **FIG. 2F,** PC9FT cells treated with gefitinib for 7 days at 0.2uM were sorted based on the gating on the left panel where 5.07% of the surviving population were fast proliferating blue DTPs and 3.69% were the slow proliferating red persisters. Q1-Q4 represents quadrants where the numbers directly under the Q1-Q4 header represent the percentage of the population within each quadrant. Histograms of the FITC-A channel (ALPP surface staining; $\log_{10}$ arbitrary units, 10^0–10^6) demonstrate a shift from baseline to post-treatment. Baseline level of ALPP was set to 10^4 based on the DMSO treatment for the PC9FTcells where 99.5% of the blue fast proliferating cells and 100% of the red slow proliferating cells are clustered. Upon gefitinib treatment for 7 days at 0.2uM, 83.9% of the fast-proliferating blue cells remain in the baseline gating of signal below 10^4 while 83.8% of the slow proliferating red cells have increased levels of surface ALPP expression beyond the 10^4 range. These thresholds enable prospective separation of persister subpopulations by FACS and establish ALPP as a robust surface biomarker enriched in Slow DTPs following EGFR-TKI treatment.

[0173] Further, western blot analysis (**FIG. 2G**) was performed to compare ALPP protein expression between red slow-cycling DTP cells and fast-cycling blue non-responder cells in continuous drug exposure. A distinct ALPP band was detected in the slow-cycling DTP population in continuous drug treatment, whereas ALPP was absent or only minimally detectable in the fast-cycling non-responder population. GAPDH was used as a housekeeping control and was consistently expressed at comparable levels across both lanes, confirming equal protein loading. These results demonstrate that ALPP expression is selectively enriched and increased in slow-cycling DTPs relative to fast-cycling cells, supporting the role of ALPP as a biomarker distinguishing DTP subpopulations.

[0174] Importantly, taking advantage of ALPP as a cell surface marker, **FIG. 2F-2G** evidences that ALPP is highly expressed in the slow DTPs, allowing distinction from the fast-cycling non-responder cells using FACS. This finding is significant as it positions ALPP as novel biomarker for identifying slow-cycling DTPs in clinical specimens, independent of the FT reporter, and paves the way for the

development of ALPP-ADC directed therapy.

[0175] A key feature of DTPs is their ability to undergo reversible resistance upon a drug holiday. It was found that ALPP^{high} cells indeed regained sensitivity to Gefitinib after a drug holiday, confirming their classification as DTPs. Interestingly, ALPP^{low} cells did not exhibit this reversal, indicating that they are likely intrinsically resistant cells that already pre-exist in the bulk population. Similar observations were made using Osimertinib (Osi), a 3rd generation TKI, emphasizing the generality of these findings (**FIG. 3A-3B**).

[0176] In this regard, it was determined that ALPP surface marker alone was sufficient to differentiate DTPs with reversible resistance from non-reversible pre-existing resistance.

[0177] Specifically, **FIG. 3A** provides flow cytometry analysis to define and isolate ALPP^{high} and ALPP^{low} subpopulations of PC9 lung adenocarcinoma cells following 7 days of treatment with gefitinib (ALPP surface staining; $\log_{10}$ arbitrary units, 10^0–10^5). In the DMSO control condition (7 days), the vast majority of cells (≈95.7%) expressed no or minimal surface ALPP ($<10^3$), with no cells detected to express high levels of ALPP above the threshold of 10^4]. In contrast, after 7 days of gefitinib treatment, a marked shift in ALPP expression was observed: approximately 49.4% of cells were classified as ALPP^{high}, while only 0.6% remained ALPP^{low}. This demonstrates a strong induction of ALPP expression upon drug treatment and validates ALPP as a marker of drug-tolerant persister cells. In these analyses, ALPP^{low} was defined as cells with fluorescence intensity below 10^3 FITC-A units, consistent with background staining levels determined by DMSO controls and isotype/FMO gating. Conversely, ALPP^{high} was defined as cells with fluorescence intensity of $\geq 10^4$ FITC-A units, representing cells with robust surface ALPP expression. This threshold enables the reproducible distinction and prospective isolation of ALPP^{high} persisters from ALPP^{low} cells by fluorescence-activated cell sorting (FACS).

[0178] **FIG. 3B** provides results of cell viability assays of ALPP^{high} and ALPP^{low} PC9 cells sorted after 7 days of gefitinib or osimertinib treatment (following strategy outlined in FIG. 3A) and subsequently placed on drug holiday, which revealed distinct responses upon drug rechallenge. After a 14-day drug holiday, ALPP^{low} cells maintained higher viability across a range of drug concentrations compared to ALPP^{high} cells, which showed a greater reduction in viability and a left-shifted dose-response, indicating that ALPP^{low} cells retain a more drug-resistant phenotype whereas ALPP^{high} cells are more sensitive to rechallenge with TKI treatment. Similar results were also found following an extended 1 or 1.25-month drug holiday, where ALPP^{low} cells maintained higher viability across a range of drug concentrations compared to ALPP^{high} cells. Similar trends were observed for both gefitinib and osimertinib, with ALPP^{low} cells showing higher drug resistance. This demonstrates that ALPP^{high} cells possess reversible resistance and are once again sensitive to the same TKI treatment after a period of drug holiday whereas ALPP^{low} do not.

[0179] These result holds significant implications, as it introduces the use of ALPP as a method to sort and identify responders ($ALPP^{high}$) from non-responders ($ALPP^{low}$), the latter representing pre-existing resistant clones in tumours prior to therapy. $ALPP^{high}$ cells represent the DTPs with reversible resistance upon drug holiday, and $ALPP^{low}$ cells are likely pre-existing resistant cells. There is no current literature on a biomarker that can indicate if a drug persister cell is capable of reversible resistance.

[0180] Importantly, this discovery provides a means to distinguish *bona fide* DTPs from intrinsically resistant cells, a capability not previously available. This differentiation opens a specific avenue for investigating the full spectrum of resistance trajectories, allowing the exploration of how DTPs evolve to acquire proliferative capability, culminating in the formation of DTEPs, and ultimately permanently resistant cells.

[0181] To translate these findings to a clinical setting, and given that ALPP can be secreted, it was first validated by ELISA assays that ALPP can be readily detected in the supernatant derived from TKI-treated $ALPP^{high}$ cells, but not in the case of $ALPP^{low}$ and treatment-naïve cells (**FIG. 3C**).

[0182] In particular, **FIG. 3C** shows the results of an ELISA assay demonstrating that secreted ALPP protein was upregulated in the supernatant of gefitinib-treated PC9 cells compared with DMSO controls. PC9 Gef (Neat) showed the highest optical density (OD), confirming robust ALPP secretion, while the same supernatant assayed at 2-fold dilution (PC9 Gef) produced a lower but still elevated signal comparable to the 2000 pg/mL standard. The $ALPP^{high}$ Gef population also exhibited strong ALPP secretion, with OD values higher than the diluted PC9 Gef and clearly above background, indicating high levels of ALPP expression in this persister subpopulation. In contrast, $ALPP^{low}$ Gef, PC9 DMSO, and controls ($ALPP^{high}$/ $ALPP^{low}$ treated with DMSO) displayed minimal or undetectable ALPP secretion, with OD values close to the negative controls (0 pg/mL standard and blank media). These findings establish that ALPP secretion is specifically induced by gefitinib treatment and is particularly enriched in DTP cells, supporting its role as a secreted biomarker for drug-tolerant persisters. The OD values provide a quantitative surrogate readout of ALPP protein abundance in cell culture supernatants or biological fluids.

[0183] The above findings have consistently shown that ALPP-positive cells (at different stages of resistance development) exhibit high and reversible sensitivity to TKIs, supporting its use as a biomarker of treatment response.

[0184] **FIG. 4A** illustrates a model of lung cancer cell fate under TKI treatment and represents a sorting strategy of ALPP high and low cells, highlighting the role of ALPP expression as a biomarker distinguishing reversible drug-tolerant persisters (DTPs) from resistant cell populations (i.e.

responders v non-responders). In the untreated state (Step 1), PC9 lung adenocarcinoma cells represent the drug-sensitive baseline population. Upon TKI treatment with gefitinib or osimertinib, the cell population diverges into two distinct groups: ALPP^{high} responders (Step 2) and ALPP^{low} non-responders (Step 3). ALPP^{high} responders enter a drug-tolerant persister state, whereas ALPP^{low} cells represent non-responders (intrinsically resistant cells).

[0185] The model of **FIG. 4A** further demonstrates the effect of drug withdrawal ("drug holiday") on these populations. ALPP^{high} DTPs may revert to the naïve drug-sensitive state when treatment is discontinued (Step 4), confirming their reversibility. In contrast, ALPP^{low} non-responders fail to revert following drug withdrawal (Step 5), consistent with an intrinsically resistant phenotype. Under continuous drug exposure, ALPP^{high} DTPs may re-enter the cell cycle and form dividing persisters (DTEPs) (Step 6). ALPP^{high} DTEPs are slower-cycling and able to regain sensitivity to TKI treatment after drug withdrawal (Step 9). By contrast, ALPP^{low} DTEPs that divide faster under drug pressure (Step 7) develop acquired resistance, which persists after drug withdrawal (Step 10). A further population of ALPP^{low} cells remains viable under continuous TKI treatment without transitioning through a reversible persister state (Step 8). These cells correspond to pre-existing resistant clones that continue to proliferate despite therapy and remain resistant even after drug withdrawal (Step 11).

[0186] Taken together, the model of **FIG. 4A** shows that ALPP expression is dynamically regulated and provides a discriminating biomarker for different tumour cell fates: ALPP^{high} expression marks reversible persisters capable of regaining sensitivity, whereas ALPP^{low} expression identifies both non-responders and resistant populations (intrinsic or acquired). This highlights ALPP as a key biomarker for distinguishing reversible drug-tolerant states from resistant phenotypes and for monitoring therapeutic response trajectories in NSCLC. Based on the FACS gating strategy, cells within the ALPP^{low} gating ($<10^3$), determined by baseline expression levels of cells treated with DMSO, would be considered as cells expressing low levels of ALPP. This constitutes populations 1,3,4,5,7,8,9,10,11 in **FIG. 4A**. Cells within the gating of ALPP^{high} ($>10^4$) would be considered cells expressing high levels of ALPP. This constitutes populations 2 and 6 in **FIG. 4A**.

[0187] In **FIG. 4B**, it is shown that ALPP^{high} DTEPs sorted from ALPP^{high} DTPs that were placed on additional 14 days of osimertinib (Osi.) treatment still maintain the ability to revert to a drug-sensitive state after a period of drug holiday. As illustrated by the dose-response curves, ALPP^{high} DTEP drug holiday cells (ALPP^{high} DTEP DH) demonstrated the most similar sensitivity to Osimertinib as naïve PC9 cells. In contrast, ALPP^{low} DTEP drug holiday cells (ALPP^{low} DTEP DH), cells initially sorted from ALPP^{high} DTPs but had since downregulated ALPP after prolonged drug exposure, retained memory of drug resistance and exhibited markedly reduced sensitivity to the same drug. Finally, the initial non-responders (pre-existing resistance), remained the most resistant after being further cultured in the presence of drug for the same additional 14-day duration before drug holiday and rechallenge (ALPP^{low} CD->DH). The bar graph quantifies cell viability at the 0.5 μM Osi. concentration of the dose-

response curves, showing reduced viability of ALPP^{high} DTEP DH cells upon Osi. rechallenge compared to resistant cells. Together, these findings support that ALPP^{high} DTEP cells represent a drug-tolerant persister state with reversible resistance, distinguishing them from ALPP^{low} DTEPs and intrinsically resistant ALPP^{low} populations that fail to revert.

[0188] **FIG. 4C** shows the same cell populations as in FIG. 4B but placed on an extended drug holiday of 1 month. Once again, ALPP^{high} DTEP DH cells demonstrated the most similar sensitivity to Osimertinib as naïve PC9 cells while ALPP^{low} DTEP DH cells and ALPP^{low} CD->DH remained highly resistant to the drug. Together, these findings reinforce that ALPP^{high} cells represent a drug-tolerant persister state capable of reverting to sensitivity after drug withdrawal, whereas ALPP^{low} resistant cells retain stable insensitivity to Osi.

FIG. 5A-5B shows the growth rate of PC9 DTP populations. ALPP^{high} and ALPP^{low} that were sorted after 7 days of osimertinib treatment at 0.2uM concentration and were cultured in drug holiday for a minimum of 14 day before investigating their growth rate in the presence of osimertinib pressure at 0.2uM concentration. In DMSO control conditions, ALPP^{high} and ALPP^{low} populations exhibited similar proliferation rates, indicating comparable baseline growth in the absence of drug pressure. However, upon treatment with osimertinib, ALPP^{low} cells continued to proliferate robustly, whereas ALPP^{high} cells showed markedly impaired growth and failed to expand over time. The images of **FIG. 5B** further support this conclusion.

Discussion

[0189] Although blood-based methods such as detection of circulating tumor DNA (ctDNA) by Next Generation Sequencing have been used to monitor TKI treatment response, these approaches have important limitations. Clearance of ctDNA in plasma correlates with improved outcomes following TKI treatment, however, ctDNA levels primarily reflect overall tumor burden, and absence of detectable ctDNA may indicate either complete clearance of cancer cells or the persistence of a small number of residual cells below detection thresholds. Moreover, ctDNA analysis cannot discriminate between DTPs and intrinsically resistant tumor cells, nor distinguish between slow-cycling DTEPs and fast-cycling DTEPs, the latter being drivers of tumor recurrence and therapy failure.

[0190] In contrast, the present invention overcomes these limitations by utilizing ALPP as a selective biomarker to enable blood-based detection of residual cancer cells after EGFR TKI treatment, to isolate and distinguish bona fide DTPs from intrinsically resistant cells, and to further differentiate slow-cycling DTEPs from fast-cycling DTEPs. In addition, the invention provides anti-ALPP adjuvant therapies for EGFR mutant LUAD patients undergoing EGFR TKI treatment, thereby offering both a diagnostic tool and a therapeutic strategy to improve patient stratification, predict treatment efficacy, and reduce the risk of relapse.

[0191] The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein. Other embodiments are within the following claims.

[0192] One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. Further, it will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention. The methods, kits and uses described herein are presently representative of preferred embodiments are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention are defined by the scope of the claims. The listing or discussion of a previously published document in this specification should not necessarily be taken as an acknowledgement that the document is part of the state of the art or is common general knowledge.

[0193] The invention illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, it should be understood that although the present invention has been specifically disclosed by exemplary embodiments and optional features, modification and variation of the inventions embodied therein herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

[0194] The content of all documents and patent documents cited herein is incorporated by reference in their entirety.

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CLAIMS

1. A method of detecting the presence or absence of drug-tolerant persister (DTP) cells resistant to tyrosine kinase inhibitor (TKI) treatment, and/or intrinsically resistant cells to TKI treatment in a subject having cancer, comprising:

detecting an expression level of ALPP in the subject, wherein the expression level of ALPP is indicative of the presence or absence of the DTP cells and/or the intrinsically resistant cells in the subject's cancer.

2. The method of claim 1, wherein the subject has previously received TKI treatment, optionally the TKI treatment comprises treatment of the subject by administration of one or more TKIs.

3. The method of claims 1 or 2, wherein the expression level of ALPP is detected in a sample obtained from the subject, wherein the sample is a blood or tumour sample.

4. The method of any one of claims 1-3, wherein the step of detecting the ALPP expression level comprises quantifying the amount of a gene product of ALPP.

5. The method of any one of claims 1-4, wherein the expression level of ALPP in the sample is compared with a reference ALPP expression level, optionally the reference ALPP expression level may be derived from a control or comparative sample.

6. The method of any one of claims 1-5, wherein a detected differential expression level of ALPP in the sample is indicative of the presence or absence of the DTP cells, and/or intrinsically resistant cells, in the subject.

7. The method of any one of claims 6, wherein an increased expression level of ALPP is indicative of the presence of the DTP cells in the sample.

8. The method of any one of claims 1-7, further comprising distinguishing DTP cells from intrinsically resistant cells in the sample based on a detected surface expression level of ALPP, wherein a high surface expression level of ALPP ($ALPP^{high}$) is indicative of DTP cells and a low surface expression level of ALPP ($ALPP^{low}$) is indicative of intrinsically (innate) resistant cells.

9. The method of any one of claims 1-8, further comprising distinguishing fast-cycling DTEP cells, from slow-cycling DTEP cells based on a detected surface expression level of ALPP, wherein the surface expression level of ALPP is higher in slow-cycling DTEP cells relative to fast-cycling DTEP cells.

10. The method of any one of claims 1-9, further comprising classifying the likelihood of the subject being responsive or non-responsive to TKI treatment and/or anti-ALPP therapies based on the expression level of ALPP and detection of DTP cells, DTEPs, and/or intrinsically resistant cells.

11. The method of claim 1, wherein the cancer is lung cancer, and wherein the lung cancer is classified as a lung adenocarcinoma (LUAD), optionally the LUAD is an EGFR mutated LUAD.

12. The method of claim 1, wherein the cancer is non-small cell lung cancer (NSCLC).

13. The method of any one of claims 1-12, wherein the TKI treatment comprises EGFR TKI treatment with one or more EGFR TKIs selected from 1st generation EGFR TKIs, 2nd generation EGFR TKIs, 3rd generation EGFR TKIs, and 4th Generation EGFR TKIs, preferably the TKI treatment comprises treatment with Gefitinib or Osimertinib.

14. The method of claim 2, wherein the detecting step is performed at one or more pre-determined time points before, during, and after a period of withdrawal of the TKI treatment ("drug holiday"), and the expression level of ALPP at each pre-determined time point is compared to provide a differential expression level of ALPP,

wherein a decrease in the expression level of ALPP during the period of withdrawal, followed by an increase in the expression level of ALPP after re-administration of the TKI treatment to the subject, is indicative of the presence, and quantity, of DTP cells, and optionally the absence of intrinsically resistant cells.

15. A method for delaying and/or preventing the development of drug-tolerant persister (DTP) cells resistant to TKI treatment in a subject with cancer, comprising administering to the subject an anti-ALPP therapy, thereby preventing or delaying relapse and/or the emergence of acquired resistance to TKI treatment, wherein the presence or absence of drug-tolerant persister (DTP) cells resistant to TKI treatment has been detected in the subject using the method of claim 1.

16. The method of claim 15, wherein the anti-ALPP therapy is used as an adjuvant therapy in combination with the TKI treatment, to improve disease-free survival of the subject.

17. A method of monitoring the efficacy of a TKI treatment in treating cancer in a subject, comprising:

(a) administering the TKI treatment to the subject;

(b) obtaining a sample from the subject; and

(c) detecting the presence or absence, and optionally quantity, of responder cells and non-responder cells to TKI treatment in the sample based on detected expression levels of ALPP of cells in the sample using the method of claim 1,

wherein the presence or absence, and optionally quantity, of responder cells and/or non-responder cells in the sample is indicative of the efficacy of the TKI treatment in treating the cancer in the subject.

18. The method of claim 17, further comprising detecting expression levels of ALPP of cells in a reference sample obtained from the subject prior to step (a), wherein the expression level of ALPP in step (c) is compared to the expression level of ALPP in the reference sample obtained prior to step (a).

19. The method of claim 17 or 18, further comprising selecting a cancer treatment regimen for the subject comprising a TKI treatment and/or an anti-ALPP therapy based on the presence or absence of responder and/or non-responder cells detected in the sample.

20. A method for treating cancer in a subject, comprising administering to the subject an Anti-ALPP therapy after the subject has received TKI treatment, wherein the presence of responder cells resistant to TKI treatment has been detected in the subject using the method of claim 1.