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Multiplexed Profiling of CDK Kinase Activities in Tumour Biopsies with Fluorescent Peptide Biosensors.

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ABSTRACT: Detection of disease biomarkers constitutes a major challenge for development of personalized and predictive diagnostics as well as companion assays. Protein kinases (PK) involved in coordination of cell cycle progression and proliferation that are hyperactivated in human cancers constitute attractive pharmacological targets and relevant biomarkers. Although it is relatively straightforward to assess the relative abundance of PKs in a biological sample, there is not always a direct correlation with enzymatic activity, which is regulated by several posttranslational mechanisms. Studies of relative abundance therefore convey limited information and the lack of selective, sensitive and standardized tools together with the inherent complexity of biological samples makes it difficult to quantify PK activities in physio-pathological tissues. To address this challenge, we have developed a toolbox of fluorescent biosensors that report on CDK activities in a sensitive, selective, dose-dependent, and quantitative fashion, which we have implemented to profile CDK activity signatures in cancer cell lines and biopsies from human tumours. In this study we report on a standardized and calibrated biosensing approach to quantify CDK1,2,4 and 6 activities simultaneously through combination of four different biosensors in a panel of 40 lung adenocarcinoma and 40 follicular lymphoma samples. CDK activity profiling highlighted two major patterns which were further correlated with age, sex of patients, tumour size, grade, genetic and immunohistochemical features of the biopsies. Multiplex CDKACT biosensing technology provides new and complementary information relative to current genetic and immunohistochemical characterization of tumour biopsies, which will be useful for diagnostic purposes, potentially guiding therapeutic decision. These fluorescent peptide biosensors offer promises for personalized diagnostics based on kinase activity profiling.

Protein Kinases (PKs) are important and established game-players in biological signalling pathways and a wide variety of genetic, transcriptomic and proteomic studies point to PK dysregulation and rewiring of kinase networks in disease [1–9]. PK signalling pathways, activities and functions undergo numerous alterations in disease. A large body of evidence has revealed significant rewiring within PK networks and altered crosstalk between signalling pathways [5–9]. PKs involved in coordination of cell proliferation are notoriously hyperactivated in cancer [10–14]. As such they constitute attractive pharmacological targets for an arsenal of FDA-approved inhibitors and established biomarkers in human cancers [15–21]. In particular PKs involved in cell cycle regulation are often amplified, over-expressed or hyperactivated in cancer, due to constitutively activating mutations or modulators from upstream regulatory pathways [10, 13, 14].

Detection of disease biomarkers for diagnostics and companion assays is of major importance for improving early and personalized therapeutics. Despite their attractiveness as pathological targets, PKs remain largely underexploited as biomarkers for diagnostics. Indeed, although it is relatively straightforward to assess the relative abundance of PKs in biological samples through antigenic approaches such as Western blotting or ELISA, there is not always a direct correlation between PK pro-

tein expression levels and their functional activity, which is subject to numerous posttranslational modifications, interactions with regulatory partners, and stimuli from upstream signalling pathways [22]. The lack of selective and sensitive tools enabling direct quantification PK activities in complex samples, such as patient biopsies, constitutes a serious limitation to exploitation of PK activities as cancer biomarkers, for predictive diagnostics and therapeutic purposes.

Technologies that report on PK activity are essential for complete appreciation of their behaviour in physio-pathological settings [23–25]. Tools tailored to profile the activity of PKs in tumour biopsies and precancerous lesions could provide precious functional information which cannot be obtained by genetic, transcriptomic or immunohistochemical analysis of samples. Moreover, since cancers are not monofactorial diseases and result from combinations of molecular alterations, technologies that would report on a panel of relevant PK activities in the same sample would not only be cost- and time-effective, but also enable identification of more global biomarker signatures associated with disease.

Fluorescent Biosensors constitute attractive tools for probing biomolecules in complex environments and are well suited for highlighting biomarkers and molecular alterations associated with pathological disorders [26–32]. In particular, a wide variety

of fluorescent biosensors have been developed to report on PK activities in vitro or in cell-based assays, thereby enabling to monitor the dynamics and quantify the activity of these enzymes [30-37]. Genetically-encoded biosensors have been described for imaging oncogenic kinase activities in living cells and tumours and for multiplex imaging in cultured cells [34, 35]. Likewise multiplex assays based on synthetic biosensors have been designed to assess PK activities in cell extracts by several groups [36-40]. However, so far, no multiplex approach has been implemented to profile PK activities in pathological samples, such as human biopsies from patients with cancer, with a diagnostic perspective.

Cyclin-dependent kinases (CDK/Cyclins) form a family of heterodimeric Ser/Thr protein kinases that were initially identified for their roles in cell cycle progression [41, 42]. They are notoriously dysregulated in many human cancers, due to genetic amplification of CDK or cyclin genes, to truncation, point mutation or protein overexpression, which generally leads to constitutive hyperactivation of CDK activity, and several drugs have been approved by the FDA as targeted therapeutics [43, 44]. In order to monitor CDK/Cyclin activities in complex biological samples, our group has developed a toolbox of fluorescent biosensors through conjugation of environmentally-sensitive probes to synthetic peptide scaffolds derived from CDK substrates, associated with a short phospho amino acid binding domain (PAABD) derived from a WW domain [45, 46]. Upon phosphorylation by an active kinase, these biosensors convert from an open to a closed conformation in which the PAABD binds the phosphorylated substrate moiety, thereby altering the local environment of the fluorescent dye, and consequently promoting a significantly detectable and quantifiable change in fluorescence emission. Specifically, we have engineered a CDK4-specific biosensor which enables quantification CDK4 hyperactivity in skin cancer cell lines, biopsies and melanoma xenografts [47], a CDK6 biosensor, derived from which was implemented to compare CDK6 and CDK4 activities in lung cancer [48], a CDK5 biosensor for neuronal disorders such as glioblastoma [49], and a CDK1 biosensor conjugated to carbon nanotubes for in vivo imaging in tumour xenografts in mice [50]. These biosensors were not only carefully designed and engineered from substrates that are selective for each kinase of interest, their selectivity and sensitivity were thoroughly characterized using recombinant CDKs, and a combination of siRNA and of CDK and PK inhibitors. CDKs are present at subnanomolar concentrations in cultured cell lines and the exquisite sensitivity of these biosensors enables detection of CDK activities in 40-100 μ g total protein lysates from cultured cells, with a dynamic range between 20-60% fluorescent enhancement. These synthetic biosensors offer straightforward means of quantifying differences in PK activities between healthy and cancer cells and report on alterations in response to therapeutics in a sensitive and selective fashion. Moreover, we have successfully shown that we could combine two different fluorescent peptide biosensors to characterize CDK2 and CDK4 activities in psoriasis, on the one hand [51], and to quantify the relative CDK4 and CDK6 activities in cell lines derived from patients with mesothelioma on the other [48].

In the present study, we have combined an ensemble of CDKACT biosensors to profile CDK1, 2, 4 and 6 activities simultaneously first in lysates from cultured cell lines to establish a proof-of-concept for a multiplex approach, which we then implemented to profile these four kinase activities in a panel of human lung adenocarcinoma and lymphoma biopsies.

RESULTS and DISCUSSION

Multisensing CDK activities in lysates from different cell lines. In order to monitor several CDK activities simultaneously in the same sample and establish a proof-of-concept for multiplexing PK activities in lysates derived from cell extracts, we first developed an assay enabling implementation of four CDKACT biosensors we previously engineered and validated [45-51] to quantify relative CDK1, 2, 4 and 6 activities simultaneously in one same cell extract (**Figure 1A**). To this aim, 150 nM TAMRA-labelled CDKACT1, 2, 4 and 6 were incubated with 100 μ g lysates from different cell lines (A375, A549, PC9, H1299, HeLa, MCF7, MDA, HCT116) and relative fluorescence enhancement was monitored over time. Statistical analyses of n=5-8 activity assays revealed differences in CDK activity profiles in different cell lines of different origins, as well as between cell lines with different genetic backgrounds, as summarized in the bar plots in **Figure 1B (Suppl. Fig. S1-5)**. For instance, comparison of three different lung cancer cell lines, A549, PC9 and H1299 reveals different CDK activity profiles, with significantly greater CDK4 hyperactivity in A549 cells (>40% enhancement of CDKACT4 fluorescence), relative to CDK1 and 2 activities (<20%), and an intermediate level of CDK6 activity (25%). In PC9 and H1299 cells, CDK1 activities were greater than in A549 cells, and in PC9 cells CDK2 activity was much greater than in A549, in which CDK2 activity was similar to that of H1299. The melanoma A375 cell line displayed similar CDK4 and 6 activities to PC9 cells, with however lower CDK1 and intermediate CDK2 activities. In the HCT116 cancer colorectal, cell line CDK1,4,6 activities were all quite high (30-35% fluorescence enhancement), whilst CDK2 activity was somewhat lower, resembling that of H1299 and A549 cells. In HeLa cells, CDK2 activity was very low, whereas CDK4 and CDK6 activities were very high and similar profiles were found in breast cancer cell lines MCF7 and MDA.

Tumours and cancer cells are characterized by a significantly different metabolism. We therefore addressed whether CDKACT biosensors might respond differently to CDK activities in the presence of metabolites in cancer cells. To this aim, we compared overall CDKACT fluorescence enhancement incubated with A549 and A375 cell lysates in the presence of 100 μ M, 1mM or 10mM glucose, CaCl₂, glucose, lactate or glutamate. However, there were no significant differences in fluorescence emission (**Suppl. Fig. S6**).

To establish the most adapted protocol for profiling CDK activities and calibrate response relative to background, we performed dose-dependent fluorescence-based assays in which TAMRA-labelled CDKACT biosensors were incubated with different protein concentrations of A549 cell extracts. BSA was used as a negative control in these experiments, to verify there was little if any non-specific fluorescence emission. Moreover, incubation of TAMRA-labelled peptide substrates of CDK1, 2, 4 and 6 derived from each biosensor did not induce any significant fluorescence enhancement (**Figure 2A**). The best response for all four CDK biosensors was observed with 60-100 μ g total cell extract. The lack of significant fluorescence enhancement of the labelled peptide substrates makes them ideal probes to normalize response of the biosensors in complex samples.

Profiling CDK activities in biopsies from human patients with lung adenocarcinoma Human biopsies were obtained from the Biobank of Montpellier Hospital (CRB-CHU). We

first performed three sets of experiments with 10 lung adenocarcinoma biopsies using three different concentrations: 20, 60 and 100µg total protein, which were incubated with CDKACT biosensors and each of their cognate control peptides derived from the substrate moiety, so as to subtract any non-specific environmental perturbations of fluorescence unrelated to kinase activities (**Figure 2B, Suppl. Fig. S7, Table 1**). These experiments revealed that 60µg yielded optimal results. Based on these results, we established a systematic protocol to measure response of 200nM CDKACT-TAMRA biosensors to CDK activities in 60µg total protein lysates, from which we subtracted fluorescence emission of 200nM cognate substrate peptides incubated with the same concentration of total protein, as shown for biopsy n° 16 (**Figure 3A, B, Suppl. Fig. S8**). Signals were measured in triplicate for CDKACT biosensors and control substrate peptides and overall response and standard deviation were calculated from the averages of CDKACT biosensor responses from which averages of control peptides were subtracted (**Figure 3B, C Suppl. Fig. S9 and Table 2**). CDKACT responses in lung adenocarcinoma biopsies were ranked to represent relative CDK activity in heatmaps as shown in **Tables 1 and 2**. Profiling CDK activities in human lung adenocarcinoma biopsies with CDKACT biosensors revealed that most samples presented high levels of CDK4 activity, low levels of CDK1 and 2, and low to intermediate CDK6 activities, in line with the activities observed in A549 cell lines. Whilst high CDK4 activity was detected most of the time, high CDK1 was more rarely detected. Comparative analysis of CDK activity profiles revealed two distinct patterns found in a majority of samples. Average and representative examples are shown in **Figure 3** (all profiles shown in **Suppl. Fig. S10-12**). The first most frequent profile, in which all CDK activities were detected, ie “All CDKs” were observed in 11 biopsies (**Figure 3D and Suppl. Fig S10**). The second profile, in which CDK1 was very low or undetectable compared to CDK2,4,6 activity, ie “No CDK1” was observed in 15 biopsies (**Figure 3E and Suppl. Fig S11**). Other samples yielded signatures that were not observed frequently and therefore considered outliers with unique patterns, either lacking CDK2 activity (3 biopsies, **Suppl. Fig S12A**), only displaying CDK2 and CDK4 activities (3 biopsies, **Suppl. Fig S12B**), lacking CDK6 activity (2 biopsies, **Suppl. Fig S12C**), displaying CDK2 and CDK6 activities (2 biopsies, **Suppl. Fig S12D**) as well as three individual biopsies with unique profiles, presenting respectively no CDK4 activity, CDK1 and CDK6 activities, and CDK1 and CDK2 activities (**Suppl. Fig. 12E**).

Patient age and sex, medical history from individual case reports and associated molecular profiles for EGFR, and KRAS mutations were compiled and correlations between CDK activities were plotted in box-plots shown in **Figure 4** (summarized in **Table 3, Suppl. Table S1, 2, 3**). 41% of adenocarcinoma samples were from female patients, 59% from male patients. However, 73% of samples with “All CDK” activity were female, and only 33% of samples from the “No CDK1” group were female, indicating that more males lacked CDK1 activity. Samples were grouped into patient age groups younger than 50 years old, between 51-60, 61-70, and older than 70. The percentage of samples with “All CDK” activities reflected the overall percentage of samples per group, indicating a fairly even distribution of CDK activity profiles, irrespective of age. In contrast there was a lower percentage of “No CDK1” samples in the two younger groups (<50 and 51-60) and a much higher percentage of samples from patients aged 61 and above, indicating that the lack of CDK1 activity was associated with age.

Lung adenocarcinoma biopsies from tumours smaller than 3mm lacked CDK1 activity, whereas most samples from 3-5mm tumour group (55% of all samples) exhibited “all CDK” activities and very few lacked CDK1 activity. In all larger tumours the relative percentage of “All CDK” activities reflected the proportion of tumours. Moreover, the larger proportion of tumours with “all CDK” activities had metastasized to distal sites and there was also a correlation between the lack of CDK1 activity in tumours that had not metastasized.

Finally, analysis of genetic mutations in EGFR and K-RAS did not reveal any obvious correlations with CDK activities. In fact, only 10% of adenocarcinoma samples had EGFR mutations, but these did not exhibit significantly different “all CDK” or “no CDK1” activities; likewise for 41% of samples with a K-RAS mutation.

Profiling CDK activities in biopsies from human patients with follicular B lymphoma. Total extracts were prepared from follicular lymphoma tumours and CDK activities were measured in triplicate with 200nM CDKACT 1,2, 4 or 6 and 60ug total protein (**Figure 5, Suppl. Fig S13, 14, Tables 4 and 5**). The response of 200nM CDKACT-TAMRA biosensors to CDK activities in 60ug total protein lysates, from which we subtracted fluorescence emission of 200nM cognate substrate peptides incubated with the same concentration of total protein, is shown for biopsy n° 28 (**Figure 5A, Suppl. Fig S13**). Signals were measured in triplicate for both CDKACT biosensors and control substrate peptides and overall response and standard deviation were calculated from the average fluorescence of CDKACT biosensor responses from which average fluorescence of control peptides were subtracted (**Figure 5B Suppl. Fig S14 and Tables 4 and 5**).

CDKACT responses in lymphoma biopsies were ranked to represent relative CDK activity in heatmaps summarized in **Tables 4 and 5** (**Table 4**: 9 follicular lymphoma biopsies without substrate subtraction; **Table 5**: follicular lymphoma biopsies following subtraction). As observed for lung adenocarcinoma biopsies, two large groups with distinctive CDK profiles were identified in follicular lymphoma samples. Most samples were either characterized with “all CDK” activities or “no CDK1” activity, the latter group comprising a subset of “high CDK2,4,6” and another subset of “low CDK2,4,6” activities, which was not observed in lung adenocarcinoma biopsies. Average and representative examples of these most frequent CDK activity profiles are shown in **Figure 5** (all profiles shown in **Suppl. Fig. S15-17**). A first group of 9 biopsies displayed all CDKs activities (**Figure 5C and Suppl. Fig S15**). A second group of 14 biopsies did not display any CDK1 activity and was subdivided into 2 subgroups: with either high CDK2, CDK4 or CDK6 activity (7 biopsies) or low CDK2, CDK4 or CDK6 activity (7 biopsies) (**Figure 5D and Suppl. Fig S16**). Outlier profiles, that were not frequently observed, include a lack of CDK2 activity (3 biopsies, **Suppl. Fig S17A**), no CDK6 activity (3 biopsies, **Suppl. Fig S17B**), only CDK4 and CDK6 activities (3 biopsies, **Suppl. Fig S17C**), and only CDK2 and CDK4 activities (3 biopsies, **Suppl. Fig S17D**), as well as three individual, unique CDK activity profiles, one with only CDK2 and CDK6 activities, another with only CDK4 activity, and one altogether lacking any significant CDK activity (**Suppl. Fig. S17E**).

Patient age and sex, medical history from individual case reports and associated immunohistochemistry analyses of cyclin D, E and p16 expression were compiled and correlations with

CDK activities were plotted in box-plots **Figure 6** and summarized in **Table 6, Suppl. Table S4-6**. 50% of lymphoma samples were from female patients, 50% from male patients. In contrast to lung adenocarcinoma, the relative proportion of biopsies from males and females was not significantly different in the “All CDKs” group (56% female, 44% male) and in the “No CDK1” group (50% female and 50% male). As for lung adenocarcinoma biopsies, samples were grouped into four age groups: less than 50 years old, between 50-60, 60-70, and older than 70. Amongst all lymphoma biopsies, 30% of samples were from patients younger than 50, 18% between 51-60, 25% between 61-70, 28% aged over 70. In the “All CDKs” group, 44% of samples were from patients aged <50, 33% between 51-60, 11% between 61-70, 11% aged over 70, indicating greater CDK activities in younger patients. Conversely, the lack of CDK1 was more often found in older patient groups; since 29% of samples in the “No CDK1” group were from patients aged <50, 7% between 51-60, 36% between 61-70, 29% aged over 70. In the “No CDK1 low CDK2,4,6” subgroup, 14% of samples were from patients aged <50, none between 51-60, 57% between 61-70, 29% aged over 70. In the “No CDK1 high CDK2,4,6” subgroup, 43% of samples were from patients aged <50, 14% between 51-60 and 14% between 61-70, 29% aged over 70. Regarding tumour staging of follicular lymphoma biopsies, we found that grade 1 samples (10% of all samples) did not exhibit any “All CDKs” activities, and constituted 29% of samples with “No CDK1” activity. Grade 1-2 samples (63% of samples) constituted 67% of samples with “All CDK” activity and 43% samples with “No CDK1” activity. Grade 2 samples (5% samples) constituted 7% of “No CDK1” activity. Grade 3 samples (15%) constituted 33% of samples with “All CDK” activity, and 7% of “No CDK1” activities. These results did not reveal any significant correlation between tumour grade and CDK activity profiles.

Immunohistochemical analysis of samples revealed that 60% of follicular lymphoma biopsies did not express p16; they constituted 78% samples with “All CDK” activities and 64% of samples with “No CDK1” activity. Samples expressing low levels of p16 (25% samples) constituted 11% samples with “all CDK” activities and 29% samples with “No CDK1”. Samples expressing higher levels of p16 (8%) constituted a minority of all activity groups, ie 11% “all CDK” activities and only 7% “No CDK1”. These results are consistent with CDK4/6 activities being greater in cancer patients in which p16 is deleted. Interestingly 88% of samples did not express cyclin D1; yet they constituted 78% samples with “All CDK activities” and 93% samples with “No CDK1” activity. Cyclin E expression was more variable and there did not appear to be any significant correlation with the proportion of “All CDK” or “No CDK1” samples.

Unsupervised principal component analysis (PCA) performed on all samples, then on adenocarcinoma samples and lymphoma samples, separately, with the aim of highlighting correlations between CDK activities, sex and age, did not reveal any major differences in CDK activities overall between tumour types, nor between sexes, nor age groups (**Suppl. Fig S18**), nor between CDK activities, sexes or age groups within lung adenocarcinoma biopsies, or within lymphoma biopsies, respectively (**Suppl. Fig S19 and S20**). PCA analysis was further performed for tumour size, metastasis, KRAS mutation and EGFR mutation in the adenocarcinoma group (**Suppl. Fig. S19**) and for grade, p16 cyclin D and cyclin E expression in the lymphoma group (**Suppl. Fig. S20**). Unsupervised PCA did not

identify any of these factors as variables impacting CDK activities, as shown in the heatmap representations (**Suppl. Fig S21**). However, comparison of the differences in “All CDK” and “No CDK1” profiles in lung adenocarcinoma and follicular lymphoma samples revealed a net difference between female and male biopsies in lung adenocarcinoma samples (**Figure 7A**). Moreover, comparison of CDK1 activities in both lung adenocarcinoma and follicular lymphoma revealed a net reduction in older patients (**Figure 7B**). In agreement with this observation, PCA of lymphoma samples revealed an inverse correlation between age and CDK1 activity (**Figure 7C and Suppl. Fig S22**).

Several reports have examined CDK protein levels or transcripts in cancer. Liu et al. [52] investigated 108 proteins and phosphoproteins in 30 paired non-small cell lung cancer (NSCLC) samples with the aim of identifying protein signatures for prognosis. They report differential expression of CDK1 and CDK4 between NSCLC and benign lung tumour. Another study by Hou et al. 2010 [53] addressed gene expression-based classification of non-small cell lung carcinomas and survival prediction. Two more recent studies by Liu et al. [54] and Yang et al. [55] performed an integrative human pan-cancer analysis of CDK1. In these studies CDK1 upregulation was observed in a wide variety of tumours, including lymphoma and lung cancers. However, these studies do not account for the functional activity of CDKs.

CDK1 is involved in coordination of mitosis [56, 57] and high CDK1 activity is therefore considered as an indicator of high mitotic index. In most of the biopsies characterized with CDK1 biosensor however CDK1 activity was rather low, in agreement with other reports in the literature which do not highlight CDK1 as a biomarker upregulated in lung cancer or lymphoma. However, some of the biopsies did reveal high CDK1 levels. CDK1 expression has been reported to be increased in tumours of different origins and associated with tumorigenesis. A recent pan-cancer study by Liu et al. [54] using several well-established databases including the Cancer Genome Atlas Database, the Clinical Proteomic Tumor Analysis Consortium and the Human Protein Atlas reveals that CDK1 expression increases on more than 20 different tumours and is highly correlated with oncogenic features as well as lower survival rates.

CDK2 is a kinase involved in replication, associated with Cyclin E and A. Active phospho-CDK2 and low molecular forms of cyclin E are considered biomarkers of aggressive breast cancer [58]. Moreover, CDK2 activity was previously reported to constitute a predictive feature in several cancers [59]. Conversely, loss of CDK2 and cyclin A was reported to impair cell proliferation and tumorigenesis, in line with its central function in DNA replication and DNA repair [60–62]. Moreover, centrosome abnormalities associated with aneuploidy and cyclin E overexpression, inferring high CDK2 activity, are frequently observed in non-small cell lung cancer [63]. In this respect, several recent reviews highlight CDK2 as a challenging yet attractive therapeutic target [64, 65].

CDK4/CDK6 hyperactivities have been associated with several forms of cancers and in particular lung cancer and lymphoma. In lung cancer, amplification of the cyclin D1 locus is observed in 5-30% of non-small cell lung cancer (NSCLC) and high levels of cyclin D1 protein were found in 18-76% of invasive NSCLC [66] and correlate with a worse outcome [67]. CDK4 overexpression in lung cancer may accelerate tumour progression and is associated with an overall shorter survival time in lung cancer patients [68]. In particular, KRAS-driven

NSCLC is particularly dependent on CDK4 and its targeting has been proposed as a therapeutic strategy in KRAS-mutant lung cancer that is resistant to conventional therapies [69]. Several studies have also highlighted the role of CDK6 in regulation of lung cancer growth, metastasis and angiogenesis [70-71]. Wang et al. [72] identified a key metabolic function of CDK6-cyclin D3 in cancer cell survival and Bellutti et al. [73] found that CDK6 antagonizes p53-induced responses during tumorigenesis. Taken together these studies point to CDK6 as an attractive pharmacological target [74].

Whilst the CDK activity profiling studies we performed on 40 lung adenocarcinoma and 40 follicular lymphoma biopsies led us to identify two major patterns with “All CDK” and “No CDK1” activity profiles in both types of cancers, it also highlighted unique and distinctive features in individual samples that may be informative for guiding the choice of therapeutics. For instance, lymphoma biopsy n° 23 was from a patient who had undergone 6 cures of R-CHOP then Rituximab. Together with biopsies 14 and 15, this sample did not display any CDK2 activity. Likewise, adenocarcinoma biopsy n° 36 had no CDK2 activity, and this patient had undergone chemotherapy. Adenocarcinoma samples n° 26 and n° 33 did not display any CDK1 activity. Patients from which these biopsies were removed had undergone neoadjuvant chemotherapy for the former and surgical removal of brain metastasis and radiotherapy for the latter. Adenocarcinoma sample n° 9 did not display any CDK4 activity. This biopsy was removed from a patient who had undergone brain surgery and chemotherapy. Biopsy n°20, removed from a patient who had undergone radiotherapy and hormone therapy, presented neither CDK1 nor CDK6 activity.

Taken together, these results highlight the heterogeneity of kinase activities in biopsies from patients with different genetic backgrounds and medical histories and emphasize the attractiveness and utility of kinase activity biosensors for personalized diagnostics, especially in cases when cognate kinase inhibitors are available for targeted therapy.

CONCLUSIONS and PERSPECTIVES

Several technologies are currently implemented to gain insight into the pathways that contribute to cancer pathogenesis, including genetic, transcriptomic and proteomic approaches, with particular focus on PKs that undergo molecular alterations. Despite the wealth of information obtained concerning PK expression levels, mutations and associated pathways that are dysregulated in cancer, these approaches do not report directly on PK activity. This functional information is therefore not accounted for and is not implemented for diagnostic purposes.

We have developed a fluorescent peptide biosensor technology that offers means of profiling CDK activities both qualitatively (dynamic curves) and quantitatively (maximal fluorescence emission intensity), thereby enabling identification of characteristic signatures in complex samples such as lysates derived from cultured cells or biopsies. In this study, we have implemented four different CDK peptide biosensors to report on the kinase activities of CDK1, CDK2, CDK4 and CDK6 respectively, first in lysates from a panel of cancer cell lines, and then in human biopsies from patients with lung cancer (adenocarcinoma) and lymphoma (follicular B), respectively. We have established a protocol to profile and quantify CDK activities in lysates from cultured cancer cell lines in a standardized fashion,

based on an internal calibration strategy accounting for non-specific, background fluorescence thanks to a pair of CDKACT biosensor / CDK substrate peptide, both conjugated to the same fluorophore, response of the latter being subtracted from the former. Heatmap and activity profile analyses enabled us to identify two major patterns and several outliers with unique signatures. This multiplex optical biosensing approach highlights distinctive PK signatures in different cell lines and in patient samples, thereby providing entirely new information which could be useful for diagnostics purposes, complementing immuno-histological and genetic information, and thereby contributing to improve patient stratification and guiding therapeutic decision with more precision and in a more personalized fashion.

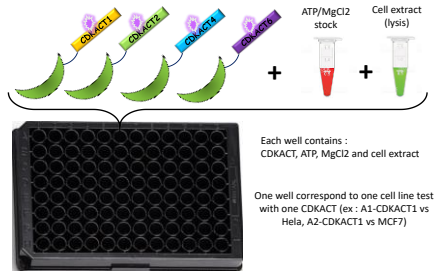
An emerging trend in the fields of diagnostics concerns development and implementation of technologies that enable rapid screening of multiple biomarkers in one single sample, thereby contributing to improve early detection and stratification, to guide treatment and potentially predict outcome. PKs are notoriously dysregulated in human cancers. However genetic profiling and immunohistochemical approaches currently implemented for diagnostics do not provide any functional information relative to PKs activities that may constitute disease biomarkers. Hence, profiling PK activities to complement current technologies could pave the way for the next generation of molecular diagnostics and enable personalized detection of functional PK alterations with therapeutic implications and development of companion assays.

The CDKACT multisensing approach described in this study provides unique means of profiling PK activities in human biopsies and of highlighting PK biomarker signatures that can be exploited for personalized predictive diagnostics. It will be interesting to implement this approach to profile CDK activities in biopsies from other cancers, to address whether similar signatures or different patterns of CDK activities may be identified. From a diagnostics perspective, acquisition of CDK signatures in a multisensing approach provides an overview of the different PK biomarkers of interest which may be dysregulated. Such information could be precious to guide towards potential treatment options when targeted therapies are available, such as CDK4/CDK6 inhibitors approved by the FDA (Abemaciclib, Palbociclib, Ribociclib). Such an approach may also enable to study interconnections or compensation mechanisms that may occur following stimuli or therapeutic intervention.

Fluorescent biosensors implemented for early-stage detection of biomarker signatures could also be used to monitor progression of disease and response to therapeutics, through development of companion assays. Hence, beyond contributing to improve molecular diagnostics through PK activity profiling to complement genetic screens for mutations and antigenic detection of seric or tissular biomarkers, the potential of this biosensing technology may further be harnessed for assessing the benefits of therapeutic intervention, drug efficiency, target and off-target response as well as emergence of resistance following treatment. Last but not least, this technology can be expected to evolve towards a multi-probe assay combining several biosensors responding to kinase activity through fluorescence changes in the emission of different fluorophores.

FIGURES

A.



B.

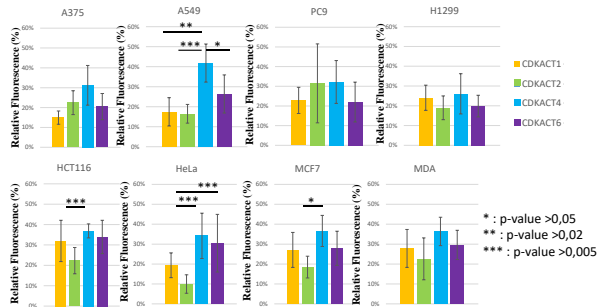
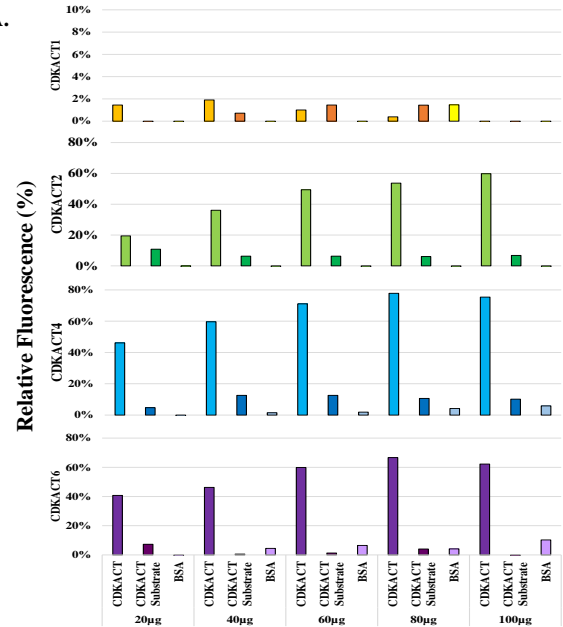


Figure 1. Establishing a multisensing assay to profiling CDK1,2,4 and 6 activities simultaneously in lysates from different cell lines. (A) Design of fluorescent peptide biosensor based multiplex detection of CDK activities: plate layout with four different CDKACT biosensors labelled with TAMRA. (B) Characterization of CDK activities in lysates from different cell lines : Relative changes in fluorescence of 150 nM TAMRA-labelled CDKACT1 (yellow), CDKACT2 (green), CDKACT4 (blue) and CDKACT6 (purple) incubated with 100ug lysates from different cell lines : A375, A549, PC9, H1299, HCT116, HeLa, MCF7, MDA

A.



B.

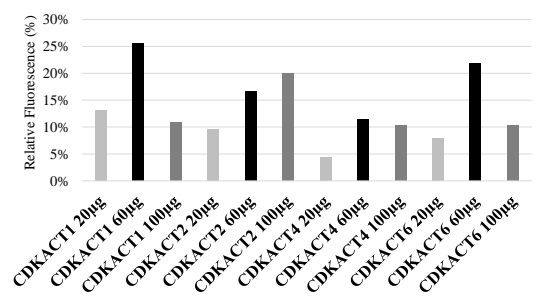


Figure 2. Optimization and Calibration of CDKACT biosensor assay in A549 cells and in human lung adenocarcinoma biopsies. (A) Fluorescence enhancement of 150nM TAMRA-labelled CDKACT1 (yellow), CDKACT2 (green), CDKACT4 (blue) and CDKACT6 (purple) or substrate peptides following incubation with increasing concentrations of total proteins extracted from A549 cells or 150nM CDKACT1, 2, 4 or 6 incubated with increasing concentrations of BSA. (B) Relative fluorescence enhancement of TAMRA-labelled CDKACT1, 2, 4, 6 following incubation with 20, 60 and 100µg cell human lung adenocarcinoma biopsies, from which fluorescence response of cognate TAMRA-labelled peptide substrates incubated with lysates was subtracted (biopsy n°8).

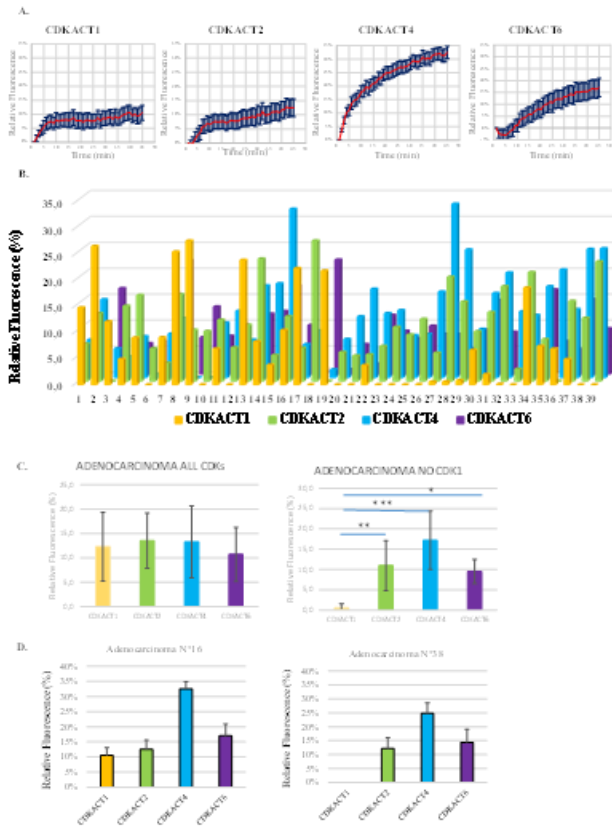


Figure 3. Relative CDK1, 2, 4 and 6 activities in human lung adenocarcinoma biopsies. (A) Relative fluorescence enhancement of 150nm TAMRA-labelled CDKACT1, CDKACT2, CDKACT4 and CDKACT6 following incubation with 60µg protein lysates human lung adenocarcinoma biopsies, from which fluorescence of cognate TAMRA-labelled peptide substrates incubated with lysates were subtracted (biopsy 16): fluorescence curves corresponding to triplicate activity assays are shown (B) Bar plot representation of CDKACT1,2,4,6 fluorescence enhancement corresponding to relative CDK1 (yellow), CDK2 (green), CDK4 (blue) and CDK6 (purple) activities in human lung adenocarcinoma biopsies 1-40 (derived from fluorescence curves as in A). (D) CDK1, 2, 4, 6 ie “All CDKs” (11 patients) and CDK2,4,6 activity, ie “No CDK1” (15 patients). Activity of each of the four CDKs was not significantly different among biopsies of “All CDKs” (Kruskal Wallis global test pvalue 0.60), whereas in the “No CDK1” group, CDK1 activity was different from the three other CDK activities (Kruskal Wallis global test pvalue 2.732 e-06) (E) Representative examples of “All CDKs”: biopsy n° 16 and “No CDK1”: biopsy n° 38

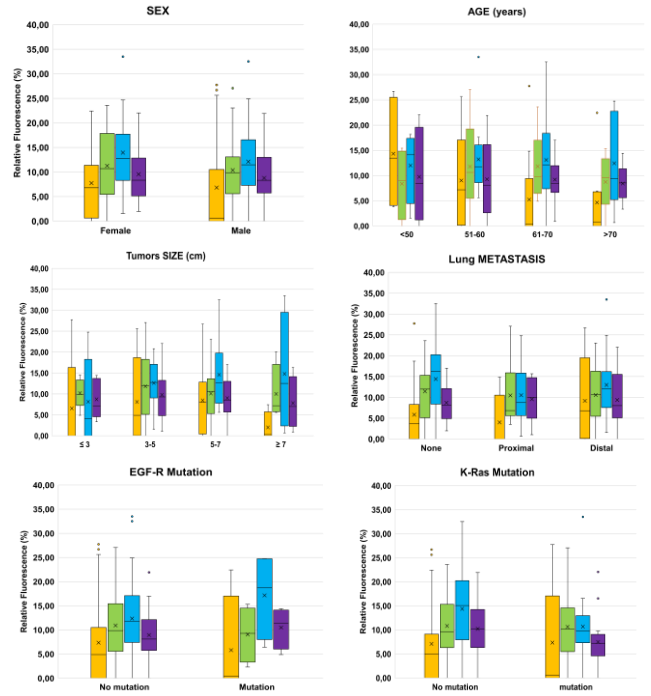


Figure 4. Correlation of CDK activities in adenocarcinoma biopsies with age, sex, tumour size, grade and genetic mutations. Relative fluorescence of CDKACT1 (yellow), CDKACT2 (green), CDKACT4 (blue) and CDKACT6 (purple) determined in lung adenocarcinoma biopsies

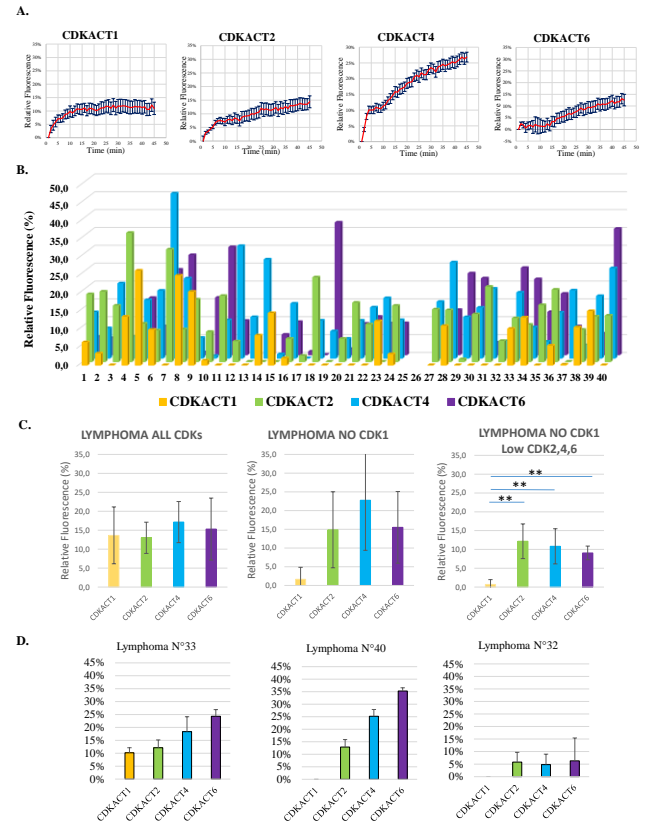


Figure 5. Relative CDK1, 2, 4, 6 activities in human lymphoma biopsies. (A) Relative fluorescence enhancement of 150nm TAMRA-labelled CDKACT1, CDKACT2, CDKACT4 and CDKACT6 following incubation with 60µg protein lysates human lymphoma biopsies, from which fluorescence emission of cognate

TAMRA-labelled peptide substrates incubated with lysates was subtracted (biopsy 28): fluorescence curves corresponding to triplicate activity assays (B) Bar plot representation of CDKACT1,2,4,6 fluorescence enhancement corresponding to relative CDK1 (yellow), CDK2 (green), CDK4 (blue) and CDK6 (purple) activities in human lymphoma biopsies 1-40 (derived from fluorescence curves as shown in A). (C) 9 biopsies have CDK1,2,4,6 activity “All CDKs”; 14 patients display CDK 2,4,6 activity “no CDK1”, with either high activities of the other CDKs or low activities. Overall activity of each of the four CDKs was not significantly different among biopsies of “All CDKs” (Kruskal Wallis global test pvalue 0.42), whereas in the “No CDK1” group, CDK1 activity was different from the three other CDK activities (Kruskal Wallis global test pvalue 0.00125) (D) Representative examples of “All CDKs”: biopsy n° 33 and “No CDK1” with high CDK2,4,6 activities: biopsy n° 40 and “No CDK1 low CDKs” biopsy n°32

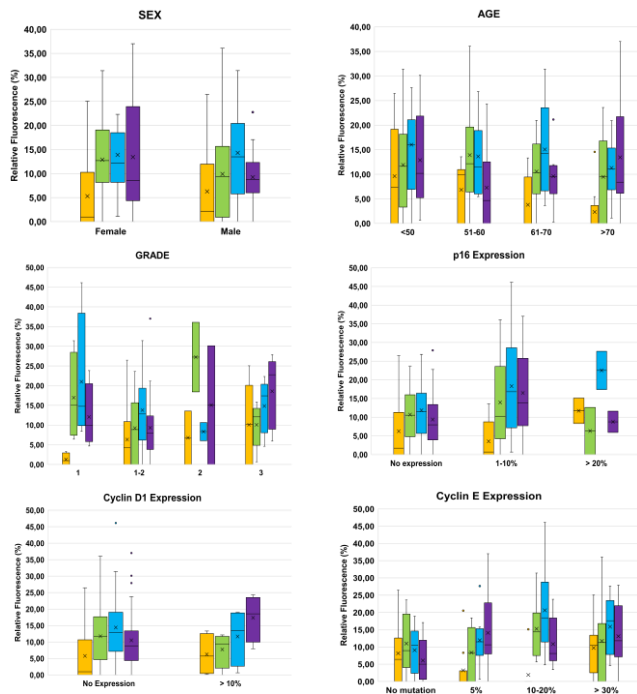


Figure 6. Correlation of CDK activities in lymphoma biopsies with age, sex, tumour size and grade and immunohistochemical features (p16, cyclin D1 and cyclin E expression). Relative fluorescence of CDKACT1 (yellow), CDKACT2 (green), CDKACT4 (blue) and CDKACT6 (purple) determined in follicular lymphoma biopsies

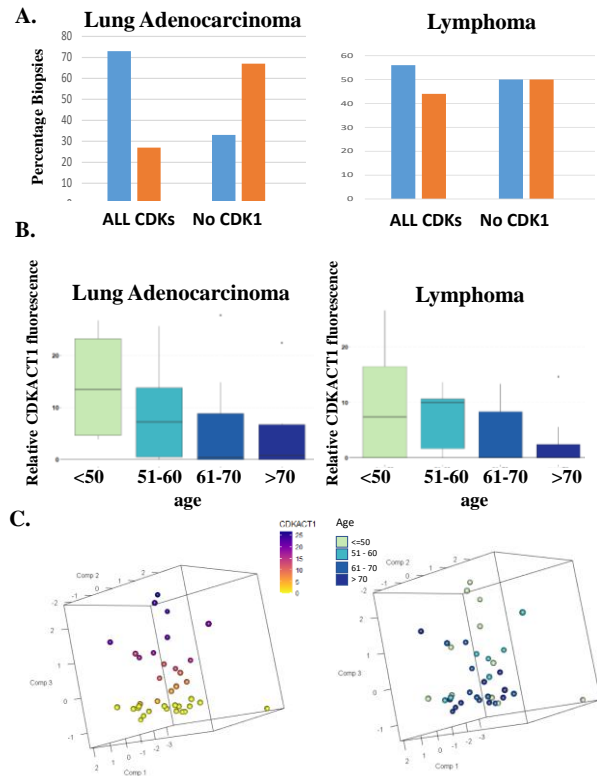


Figure 7. Differences in CDK1 Activity according to sex and age. (A) Percentage of female (blue) and male (orange) biopsies with “All CDK” and “No CDK1” activities in lung adenocarcinoma (left panel) and lymphoma (right panel) (B) Box plots of CDK1 activity in lung adenocarcinoma (left panel) and follicular lymphoma (right panel) according to different age groups. (C) 3D representation of unsupervised principal component analysis for CDK1 activity (left) and age (right) in lymphoma biopsies: projection of samples onto the 3D space generated by the first three principal components.

ASSOCIATED CONTENT

Supporting Information. Experimental Procedures, Supplementary Figures S1-22 and Tables S1-S6

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Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1 - CDK1 kinase activities in lysates from different cell lines, Figure S2 CDK2 kinase activities in lysates from different cell lines, Figure S3 CDK4 kinase activities in lysates from different cell lines, Figure S4 CDK6 kinase activities in lysates from different cell lines, Figure S5 Relative CDK1,2,4,6 kinase activities in lysates from different cell lines, Figure S6 Relative CDK1,2,4,6 kinase activities in lysates from A549 and A375 cells incubated with ATP/MgCl₂, CaCl₂, glucose, lactate and glutamate, Figure S7 Fluorescence enhancement of CDKACT1,2,4,6 and of cognate substrate peptides in 20, 60, 100µg total lysates from adenocarcinoma biopsy N°8, Figure S8 CDKACT1,2,4,6 fluorescence enhancement in 60µg total lysates from adenocarcinoma biopsy N°16, Figure S9 CDKACT1,2,4,6 fluorescence emission curves in 60µg total lysates from adenocarcinoma biopsies (11-

39), Figure S10 Lung Adenocarcinoma CDKACT1,2,4,6 Activity Profiles: All CDKs, Figure S11 Lung Adenocarcinoma CDKACT1,2,4,6 Activity Profiles: no CDK1, Figure S12 Other Lung Adenocarcinoma CDKACT1,2,4,6 Activity Profiles, Figure S13 CDKACT1,2,4,6 fluorescence enhancement in 60µg total lysates from lymphoma biopsy 28, Figure S14 CDKACT1,2,4,6 fluorescence enhancement in 60µg total lysates from lymphoma biopsies (10-40), Figure S15 Lymphoma CDKACT1,2,4,6 Activity Profiles : All CDKs, Figure S16 Lymphoma CDKACT1,2,4,6 Activity Profiles : No CDK1, Figure S17 Other Lymphoma CDKACT1,2,4,6 Activity Profiles, Figure S18 unsupervised PCA for all samples (lung adenocarcinoma & lymphoma), Figure S19 unsupervised PCA for lung adenocarcinoma samples, Figure S20 unsupervised PCA for lymphoma samples, Figure S21 Heatmap Plots of CDK activities clustered based on PCA analysis, Figure S22 Supervised PCA on lymphoma samples : age and CDK1, Table S1 Age, Sex, TNM stage, Genetic features of lung adenocarcinoma biopsies, Table S2 Age, Sex, TNM stage, Genetic Features of lung adenocarcinoma biopsies with “All CDK” activities, Table S3 Age, Sex, TNM stage, Genetic Features of lung adenocarcinoma biopsies with “No CDK1” activities Table S4 Age, Sex, TNM stage, immunohistochemical features of lymphoma biopsies Table S5 Age, Sex, TNM stage, immunohistochemical features of lymphoma biopsies with “All CDK” activities Table S6 Age, Sex, TNM stage, immunohistochemical features of lymphoma biopsies with “No CDK1” activities.

Author Contributions

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ABBREVIATIONS

CDK, cyclin-dependent kinase; CDKACT, CDK activity biosensor; PAABD : phospho amino acid binding domain ; PCA: principal component analysis

REFERENCES

- (1) Manning, B.D. Challenges and Opportunities in Defining the Essential Cancer Kinome. *Sci. Signal.* **2009**, *2* (63). Doi.org: 10.1126/scisignal.263pe15.
- (2) Fabbro, D.; Cowan-Jacob, S.W.; Moebitz, H. Ten things you should know about protein kinases: IUPHAR Review 14: Ten things you should know about protein kinases. *Br J Pharmacol.* **2015**, *172* (111), 2675–2700. Doi: 10.1111/bph.13096.
- (3) Vogelstein, B.; Papadopoulos, N.; Velculescu, V.E.; Zhou, S.; Diaz, L.A.; Kinzler, K.W. Cancer Genome Landscapes. *Science.* **2013**, *339*, 1546–1558. Doi: 10.1126/science.1235122.
- (4) The Cancer Genome Atlas Research Network, Weinstein, J.N.; Collisson, E.A.; Mills, G.B.; Shwa K.R.M.; Ozenberger, B.A.; Ellrott, K.; Shmulevich, I.; Sander, C.; Stuart, J.M.. The Cancer Genome Atlas Pan-Cancer analysis project. *Nat Genet.* **2013**, *45* (10), 1113–1120. Doi: 10.1038/ng.2764.
- (5) Creixell, P.; Schoof, E.M.; Simpson, C.D.; Longden, J.; Milller, C.J.; Lou, H.J.; Perryman, L.; Cox, T.R.; Zivanovic, N.; Palmeri, A.; et al. Kinome-wide Decoding of Network-Attacking Mutations Rewiring Cancer Signaling. *Cell.* **2015**, *163* (1), 202–217. Doi: 10.1016/j.cell.2015.08.056.
- (6) Campbell, J.; Ryan, C.J.; Brough, R.; Bajrami, I.; Pemberton, H.N.; Chong, I.Y.; Costa-Cabral, S.; Frankum, J.; Gulati, A.; Holme, H.; et al. Large-Scale Profiling of Kinase Dependencies in Cancer Cell Lines. *Cell Rep.* **2016**, *14* (10), 2490–2501. Doi: 10.1016/j.celrep.2016.02.023.
- (7) Buljan, M.; Ciuffa, R.; van Drogen, A.; Vichalkovski, A.; Mehnert, M.; Rosenberger, G.; Lee, S.; Varjosalo, M.; Pernas, L.E.; Spegg, V.; et al. Kinase Interaction Network Expands Functional and Disease Roles of Human Kinases. *Molecular Cell.* **2020**, *79* (3), 504-520.e9. Doi: 10.1016/j.molcel.2020.07.001.
- (8) Sacco, F., Perfetto, L. and Cesareni, G. Combining phosphoproteomics datasets and literature information to reveal the functional connections in a cell phosphorylation network. *Proteomics.* **2018**, *18* (5-6), 1700311. Doi: 10.1002/pmic.201700311.
- (9) Hijazi, M.; Smith, R.; Rajeeve, V.; Bessant, C.; Cutillas, P.R. Reconstructing kinase network topologies from phosphoproteomics data reveals cancer-associated rewiring. *Nat Biotechnol.* **2020**, *38* (4), 493–502. Doi: 10.1038/s41587-019-0391-9.
- (10) Malumbres, M.; Barbacid, M. Cell cycle kinase in cancer. *Current Opinion in Genetics and Development.* **2007**, *17* (1), 60-5. Doi: 10.1016/j.gde.2006.12.008.
- (11) Asghar, U.; Witkiewicz, A.K.; Turner, N.C.; Knudsen, E.S. The history and future of targeting cyclin-dependent kinases in cancer therapy. *Nat Rev Drug Discov.* **2015**, *14* (2), 130–146. Doi: 10.1038/nrd4504.
- (12) Peyressatre, M.; Prével, C.; Pellerano, M.; Morris, M. Targeting Cyclin-Dependent Kinases in Human Cancers: From Small Molecules to Peptide Inhibitors. *Cancers.* **2015**, *7* (1), 179–237. Doi: 10.3390/cancers7010179.
- (13) Fleuren, E.D.G.; Zhang, L.; Wu, J.; Daly, R.J. The kinome ‘at large’ in cancer. *Nat Rev Cancer.* **2016**, *16* (2), 83–98. Doi: 10.1038/nrc.2015.18.

- (14) Wilson, L.J.; Linley, A.; Hammond, D.E.; Hood, F.E.; Coulson, J.M.; MacErwan, D.J.; Ross, S.; Slupsky, J.R.; Evers, P.A.; Smith, P.D.; et al. New Perspectives, Opportunities, and Challenges in Exploring the Human Protein Kinome *Cancer Res.* **2018**, *78* (1), 15-29. Doi: 10.1158/0008-5472.CAN-17-2291.
- (15) Cohen, P. Protein kinases — the major drug targets of the twenty-first century? *Nat Rev Drug Discov.* **2002**, *1* (4), 309–315. Doi: 10.1038/nrd773.
- (16) Cohen, P.; Cross, D.; Jänne, P.A. Kinase drug discovery 20 years after imatinib: progress and future directions. *Nat Rev Drug Discov.* **2021**, *20* (7), 551–569. doi:10.1038/s41573-021-00195-4.
- (17) Brognard, J.; Hunter, T. Protein kinase signaling networks in cancer. *Current Opinion in Genetics and Development.* **2011**, *21* (1), 4–11. Doi: 10.1016/j.gde.2010.10.012.
- (18) Lapenna, S.; Giordano, A. Cell cycle kinases as therapeutic targets for cancer. *Nat Rev Drug Discov.* **2009**, *8* (7), 547–566. Doi: 10.1038/nrd2907.
- (19) Wu, P.; Nielsen, T.E.; Clausen, M.H. FDA-approved small-molecule kinase inhibitors. *Trends in Pharmaceutical Sciences.* **2015**, *36* (7), 422–439. Doi: 10.1016/j.tips.2015.04.005.
- (20) Wu, P.; Nielsen, T.E.; Clausen, M.H. Small-molecule kinase inhibitors: an analysis of FDA-approved drugs. *Drug Discovery Today.* **2016**, *21* (1), 5–10. Doi: 10.1016/j.drudis.2015.07.008.
- (21) Roskoski R. Properties of FDA-approved small molecule protein kinase inhibitors: A 2021 update. *Pharmacological Research.* **2021**, *165*, 105463. Doi: 10.1016/j.phrs.2021.105463.
- (22) Hahn, W.C.; Weinberg, R.A. Modelling the molecular circuitry of cancer. *Nat Rev Cancer.* **2002**, *2* (5), 331–341. Doi: 10.1038/nrc795.
- (23) Wang, Y.; Ma, H. Protein kinase profiling assays: a technology review. *Drug Discovery Today: Technologies.* **2015**, *18*, 1–8. Doi: 10.1016/j.ddtec.2015.10.007.
- (24) Baharani, A.; Trost, B.; Kusalik, A.; Napper, S. Technological advances for interrogating the human kinome. *Biochem. Soc. Trans.* **2017**, *45* (1), 65-77. Doi: 10.1042/BST20160163.
- (25) Cann, M.L.; McDonald, I.M.; East, M.P.; Johnson, G.L.; Graves, L.M. *Measuring Kinase Activity-A Global Challenge.* *J Cell Biochem.* **2017**, *118* (11), 3595-3606. Doi: 10.1002/jcb.26103.
- (26) Zhang, J.; Campbell, R.E.; Ting, A.Y.; Tsien, R.Y. Creating New Fluorescent Probes for Cell Biology. *Nat. Rev. Mol. Cell Biol.* **2002**, *3* (12), 906-918. Doi: 10.1038/nrm976.
- (27) Pazos, E.; Vázquez, O.; Mascareñas, J.L.; Vázquez, M.E. Peptide-based fluorescent biosensors. *Chem. Soc. Rev.* **2009**, *38* (12), 3348. doi:10.1039/b908546g.
- (28) Morris, M.C. Fluorescent biosensors of intracellular targets from genetically encoded reporters to modular polypeptide probes. *Cell Biochem Biophys.* **2010**, *56* (1), 19-37. Doi: 10.1007/s12013-009-9070-7.
- (29) New, E.J. Harnessing the Potential of Small Molecule Intracellular Fluorescent Sensors. *ACS Sensors*, **2016**, *1* (4), 328-333. Doi: 10.1021/acssensors.6b00148.
- (30) Nhu Ngoc Van, T.; Morris, M.C. Fluorescent sensors of protein kinases: from basics to biomedical applications. *Prog Mol Biol Transl Sci.* **2013**, *113*, 217-274. Doi: 10.1039/b908546g.
- (31) Morris, M.C. Spotlight on fluorescent biosensors-tools for diagnostics and drug discovery. *ACS Med Chem Lett.* **2013**, *5* (2), 99-101. Doi: 10.1021/ml400472e.
- (32) Prével, C.; Pellerano, M.; Van, T.N.N.; Morris, M.C. Fluorescent Biosensors for High Throughput Screening of Protein Kinase Inhibitors. *Biotechnology J.* **2014**, *9*, (2) 253-265. Doi: 10.1002/biot.201300196.
- (33) Zhang, J.; Allen, M. D.. FRET-based biosensors for protein kinases: Illuminating the kinome. *Mol. Biosystems.* **2007**, *3* (11), 759–765. Doi: 10.1039/b706628g.
- (34) Lin, W.; Mehta, S.; Zhang, J. Genetically encoded fluorescent biosensors illuminate kinase signalling in cancer. *J. Biol. Chem.* **2019**, *294* (40), 14814–14822. Doi: 10.1074/jbc.REV119.006177.
- (35) Mehta, S.; Zhang, Y.; Roth, R.H.; Zhang, J.F.; Mo, A.; Tenner, B.; Haganir, R.L.; Zhang, J. Single-fluorophore biosensors for sensitive and multiplexed detection of signalling activities. *Nat Cell Biol.* **2018**, *20* (10), 1215-1225. Doi: 10.1038/s41556-018-0200-6.
- (36) González-Vera, J.A. Probing the kinome in real time with fluorescent peptides. *Chem Soc Rev.* **2012**, *41* (5), 1652-1664. Doi: 10.1039/C1CS15198C.
- (37) González-Vera, J.A.; Morris, M.C. Fluorescent Reporters and Biosensors for Probing the Dynamic Behavior of Protein Kinases. *Proteomes.* **2015**, *3* (4), 369-410. Doi: 10.3390/proteomes3040369.
- (38) Shults, M.D.; Janes, K.A.; Lauffenburger, D.A.; Imperiali, B. A multiplexed homogeneous fluorescence-based assay for protein kinase activity in cell lysates. *Nat. Methods.* **2005**, *2* (4), 277-283. Doi: 10.1038/nmeth747.
- (39) Wang, Q.; Zimmerman, E.I.; Touchkine, A.; Martin, T.D.; Graves, L.M.; Lawrence, D.S. Multicolor monitoring of dysregulated protein kinases in chronic myelogenous leukemia. *ACS Chem Biol.* **2010**, *5* (9), 887-895. Doi: 10.1021/cb100099h.
- (40) Jena, S.; Damayanti, N.P.; Tan, J.; Pratt, E.D.; Irudayaraj, J.M.K.; Parker, L.L. Multiplexable fluorescence lifetime imaging (FLIM) probes for Abl and Src-family kinases. *Chem Commun (Camb).* **2020**, *56* (87), 13409-13412. Doi: 10.1039/d0cc05030j.
- (41) Malumbres, M.; Harlow, E.; Hunt, T.; Hunter, T.; Lahti, J.M.; Manning, G.; Morgan, D.O.; Tsai, L.H.; Wolgemuth, D.J. Cyclin-dependent kinases: a family portrait. *Nat Cell Biol.* **2009**, *11* (11), 1275–1276. Doi: 10.1038/ncb1109-1275.
- (42) Lim, S.; Kaldis, P. Cdks, cyclins and CKIs: roles beyond cell cycle regulation. *Dev Biol.* **2013**, *140* (15), 3079–3093. Doi: 10.1242/dev.091744.

- (43) Sherr, C.J.; Beach, D.; Shapiro, G.I. Targeting CDK4 and CDK6: From Discovery to Therapy. *Cancer Discov.* **2016**, *6* (4), 353–367. Doi: 10.1158/2159-8290.CD-15-0894.
- (44) Malumbres, M. CDK4/6 Inhibitors: What Is the Best Cocktail? *Clin Cancer Res.* **2019**, *25* (1), 6–8. Doi: 10.1158/1078-0432.CCR-18-2177.
- (45) Morris, M.C. Shining Light on Protein Kinase Biomarkers with Fluorescent Peptide Biosensors. *Life.* **2022**, *12* (4), 516. Doi: 10.3390/life12040516.
- (46) Morris, M.C. A Toolbox of Fluorescent Peptide Biosensors to Highlight Protein Kinases in Complex Samples: Focus on Cyclin-Dependent Kinases. *European J Organic Chem.* **2022**, *21*. Doi: 10.1002/ejoc.202200120.
- (47) Prével, C.; Pellerano, M.; González-Vera, J.A.; Henri, P.; Meunier, L.; Vollaie, J.; Josserand, V.; Morris, M.C. Fluorescent peptide biosensor for monitoring CDK4/cyclin D kinase activity in melanoma cell extracts, mouse xenografts and skin biopsies. *Biosensors and Bioelectronics.* **2016**, *85*, 371–380. Doi: 10.1016/j.bios.2016.04.050.
- (48) Soamalala, J.; Diot, S.; Pellerano, M.; Blanquart, C.; Galibert, M.; Jullian, M.; Puget, K.; Morris, M.C. Fluorescent Peptide Biosensor for Probing CDK6 Kinase Activity in Lung Cancer Cell Extracts. *ChemBioChem.* **2021**, *22* (6), 1065–1071. Doi: 10.1002/cbic.202000677.
- (49) Peyressatre, M.; Laure, A.; Pellerano, M.; Boukhadaoui, H.; Soussi, I.; Morris, M.C. Fluorescent Biosensor of CDK5 Kinase Activity in Glioblastoma Cell Extracts and Living Cells. *Biotechnol J.* **2020**, *15* (9), 1900474. Doi: 10.1002/biot.201900474.
- (50) Tilmaciu, C.M.; Dinesh, B.; Pellerano, M.; Diot, S.; Guidetti, M.; Vollaie, J.; Bianco, A.; Ménard-Moyon, C.; Josserand, V.; Morris, M.C. Nanobiosensor Reports on CDK1 Kinase Activity in Tumor Xenografts in Mice. *Small.* **2021**, *17* (9), 2007177. Doi: 10.1002/sml.202007177.
- (51) Henri, P.; Prevel, C.; Pellerano, M.; Lacotte, J.; Stoebner, P.E.; Morris, M.C.; Meunier, L. Psoriatic epidermis is associated with upregulation of CDK2 and inhibition of CDK 4 activity. *Br J Dermatol.* **2020**, *182* (3), 678–689. Doi: 10.1111/bjd.18178.
- (52) Liu, W.; Wu, Y.; Wang, L.; Gao, L.; Wang, Y.; Liu, X.; Zhang, K.; Song, J.; Wang, H.; Bayer, T.; et al. Protein signature for non-small cell lung cancer prognosis. *Am J Cancer Res.* **2014**, *4* (3), 256–269.
- (53) Hou, J.; Aerts, J.; den Hamer, B.; Van IJcken, W.; Den Bakker, M.; Riegman, P.; Van Der Leest, C.; Van Der Spek, P.; Foekens, J.A.; Hoogsteden, H.C.; et al. Gene Expression-Based Classification of Non-Small Cell Lung Carcinomas and Survival Prediction. *PLoS ONE.* **2010**, *5* (4), e10312. Doi: 10.1371/journal.pone.0010312.
- (54) Liu, X.; Wu, H.; Liu, Z. An Integrative Human Pan-Cancer Analysis of Cyclin-Dependent Kinase 1 (CDK1). *Cancers (Basel).* **2022**, *14* (11), 2658. Doi: 10.3390/cancers14112658.
- (55) Yang, Y.; Liu, Q.; Guo, X.; Yuan, Q.; Nian, P.; Xu, Z.; Li, L.; Ye, Y.; Li, X. Systematic Pan-Cancer Analysis Identifies CDK1 as an Immunological and Prognostic Biomarker. *J Oncol.* **2022**, 8115474. Doi: 10.1155/2022/8115474.
- (56) Enserink, J.M.; Kolodner, R.D. An overview of Cdk1-controlled targets and processes. *Cell Div.* **2010**, *5*, 11. Doi: 10.1186/1747-1028-5-11.
- (57) Kalous, J.; Jansová, D.; Šušor, A. Role of Cyclin-Dependent Kinase 1 in Translational Regulation in the M-Phase. *Cells.* **2020**, *9* (7), e1568. Doi: 10.3390/cells9071568.
- (58) Karakas, C.; Biernacka, A.; Bui, T.; Sahin, A.A.; Yi, M.; Akli, S.; Schafer, J.; Alexander, A.; Adjapong, O.; Hunt, K.K.; et al. Cytoplasmic Cyclin E and Phospho-Cyclin-Dependent Kinase 2 Are Biomarkers of Aggressive Breast Cancer. *Am J Pathol.* **2016**, *186* (7), 1900–1912. Doi: 10.1016/j.ajpath.2016.02.024.
- (59) McCurdy, S.R.; Pacal, M.; Ahmad, M.; Bremner, R. A CDK2 activity signature predicts outcome in CDK2-low cancers. *Oncogene.* **2017**, *36* (18), 2491–2502. Doi: 10.1038/onc.2016.409.
- (60) Gopinathan, L.; Tan, S.L.W.; Padmakumar, V.C.; Coppola, V.; Tessarollo, L.; Kaldis, P. Loss of Cdk2 and cyclin A2 impairs cell proliferation and tumorigenesis. *Cancer Res.* **2014**, *74* (14), 3870–3879. Doi: 10.1158/0008-5472.CAN-13-3440.
- (61) Tsai, L.H.; Lees, E.; Faha, B.; Harlow, E.; Riabowol, K. The cdk2 kinase is required for the G1-to-S transition in mammalian cells. *Oncogene.* **1993**, *8* (6), 1593–1602.
- (62) Deans, A.J.; Khanna, K.K.; McNees, C.J.; Mercurio, C.; Heierhorst, J.; McArthur, G.A. Cyclin-dependent kinase 2 functions in normal DNA repair and is a therapeutic target in BRCA1-deficient cancers. *Cancer Res.* **2006**, *66* (16), 8219–8226. Doi: 10.1158/0008-5472.CAN-05-3945.
- (63) Koutsami, M.K.; Tsantouliss, P.K.; Kouloukoussa, M.; Apostolopoulou, K.; Pateras, I.; Spartinou, Z.; Drougou, A.; Evangelou, K.; Kittas, C.; Bartkova, J.; et al. Centrosome abnormalities are frequently observed in non-small-cell lung cancer and are associated with aneuploidy and cyclin E overexpression. *J Pathol.* **2006**, *209* (4), 512–521. Doi: 10.1002/path.2005.
- (64) Caruso, J.A.; Duong, M.T.; Carey, J.P.W.; Hunt, K.K.; Keyomarsi, K. Low-Molecular-Weight Cyclin E in Human Cancer: Cellular Consequences and Opportunities for Targeted Therapies. *Cancer Res.* **2018**, *78* (19), 5481–5491. Doi: 10.1158/0008-5472.CAN-18-1235.
- (65) Tadesse, S.; Anshabo, A.T.; Portman N, Lim, E.; Tilley, W.; Caldon, C.E.; Wang, S. Targeting CDK2 in cancer: challenges and opportunities for therapy. *Drug Discov Today.* **2020**, *25* (2), 406–413. Doi: 10.1016/j.drudis.2019.12.001.
- (66) Musgrove, E.A.; Caldon, C.E.; Barraclough, J.; Stone, A.; Sutherland, R.L. Cyclin D as a therapeutic target in cancer. *Nat Rev Cancer.* **2011**, *11* (8), 558–572. Doi: 10.1038/nrc3090.
- (67) Gautschi, O.; Ratschiller, D.; Gugger, M. Betticher, D.C.; Heighway, J. Cyclin D1 in non-small cell lung cancer: a key driver of malignant transformation. *Lung Cancer.* **2007**, *55* (1), 1–14. Doi: 10.1016/j.lungcan.2006.09.024.

- (68) Dobashi, Y.; Goto, A.; Fukayama, M.; Abe, A.; Ooi, A. Overexpression of cdk4/cyclin D1, a possible mediator of apoptosis and an indicator of prognosis in human primary lung carcinoma. *Int J Cancer*. **2004**, *110* (4), 532–541. Doi: 10.1002/ijc.20167.
- (69) Puyol, M.; Martín, A.; Dubus, P.; Mulero, F.; Pizcueta, P.; Khan, G.; Guera, C.; Santamaria, D.; Barbacid, M. A synthetic lethal interaction between K-Ras oncogenes and Cdk4 unveils a therapeutic strategy for non-small cell lung carcinoma. *Cancer Cell*. **2010**, *18* (1), 63–73. Doi: 10.1016/j.ccr.2010.05.025.
- (70) Geng, B.; Liang, M.; Qin, L.; Zhao, W.; Wang, H.; Wang, L.; Pan, X.; Chen, X. An TRIM59-CDK6 axis regulates growth and metastasis of lung cancer. *J Cellular Molecular Medi*. **2019**, *23* (2), 1458-1469. Doi: 10.1111/jcmm.14052.
- (71) Kollmann, K.; Heller, G.; Scheneckenleithner, C.; Warsch, W.; Scheicher, R., Ott, R.G, Schäfer, M.; Fajmann, S.; Schlederer, M.; Schiefer, A.-I.; et al. A Kinase-Independent Function of CDK6 Links the Cell Cycle to Tumor Angiogenesis. *Cancer Cell*. **2013**, *24* (2), 167–181. Doi: 10.1016/j.ccell.2016.07.003.
- (72) Wang, H.; Nicolay, B.N.; Chick, J.M.; Gao, X.; Geng, Y.; Ren, H.; Gao, H.; Yang, G.; Williams, J.A.; Suski, J.M.; et al. The metabolic function of cyclin D3-CDK6 kinase in cancer cell survival. *Nature*. **2017**, *546* (7658), 426-430. Doi: 10.1038/nature22797.
- (73) Bellutti, F.; Tigan, A.S.; Nebenfuehr, S.; Dolezal, M.; Zojer, M.; Grausenburger, R.; Hartenberger, S.; Kollman, S.; Doma, E.; Prchal-Murphy, M.; et al. CDK6 antagonizes p53-induced responses during tumorigenesis. *Cancer Discov*. **2018**, *8* (7), 884–897. Doi: 10.1158/2159-8290.CD-17-0912.
- (74) Tadesse, S.; Yu, M.; Kumarasiri, M.; Le, B.T.; Wang, S. Targeting CDK6 in cancer: State of the art and new insights. *Cell Cycle*. **2015**, *14* (20), 3220-3230. Doi: 10.1080/15384101.2015.1084445.

Table 1. CDKACT1, 2, 4, 6 response in 20ug, 60, 100µg first 10 lung adenocarcinoma

	CDKACT1			CDKACT2			CDKACT4			CDKACT6		
	20µg	60µg	100µg	20µg	60µg	100µg	20µg	60µg	100µg	20µg	60µg	100µg
Adenocarcinoma N°1	5,15	14,84	14,22	6,43	7,18	5,09	0,00	7,27	0,00	0,00	10,22	0,00
Adenocarcinoma N°2	12,93	26,69	23,63	0,00	13,06	14,09	5,71	15,06	16,06	3,96	0,00	11,49
Adenocarcinoma N°3	0,00	12,15	4,24	0,00	0,00	0,00	16,14	5,60	7,44	7,18	16,53	16,32
Adenocarcinoma N°4	1,60	4,92	4,24	0,23	14,50	8,35	13,71	4,05	0,00	2,62	3,34	13,24
Adenocarcinoma N°5	0,00	9,03	19,17	9,79	16,57	7,87	0,32	7,94	9,61	0,00	5,92	6,30
Adenocarcinoma N°6	0,00	0,00	0,00	0,89	6,30	11,94	5,42	0,67	3,42	0,00	6,32	13,37
Adenocarcinoma N°7	20,47	9,10	9,03	14,32	3,50	11,44	8,14	8,45	20,53	12,52	15,66	16,00
Adenocarcinoma N°8	13,07	25,62	10,96	9,67	16,71	20,01	4,44	11,45	10,25	7,98	21,91	10,40
Adenocarcinoma N°9	1,41	27,73	13,39	27,48	9,86	3,75	0,00	0,00	0,00	11,15	7,08	14,17
Adenocarcinoma N°10	0,00	0,00	10,28	12,95	9,62	1,13	13,71	0,00	0,00	9,98	13,00	8,28

Table 2. Relative CDK activities in a Panel of Adenocarcinoma Biopsies

	CDKACT1	CDKACT2	CDKACT4	CDKACT6
Adenocarcinoma N°11	6,9	11,8	10,6	7,4
Adenocarcinoma N°12	0,0	6,5	12,8	9,8
Adenocarcinoma N°13	24,0	10,8	7,3	0,0
Adenocarcinoma N°14	8,3	23,6	17,7	11,6
Adenocarcinoma N°15	3,8	5,0	18,1	12,1
Adenocarcinoma N°16	10,5	12,5	32,5	17,0
Adenocarcinoma N°17	22,4	6,4	6,4	9,4
Adenocarcinoma N°18	0,0	27,1	9,0	1,0
Adenocarcinoma N°19	22,0	0,0	1,5	22,0
Adenocarcinoma N°20	0,0	5,6	7,4	0,9
Adenocarcinoma N°21	0,0	4,9	11,8	5,7
Adenocarcinoma N°22	3,7	5,1	17,1	2,0
Adenocarcinoma N°23	0,0	6,7	12,4	11,4
Adenocarcinoma N°24	0,0	10,4	13,0	8,3
Adenocarcinoma N°25	0,0	8,9	8,2	6,9
Adenocarcinoma N°26	0,0	12,0	8,4	9,3
Adenocarcinoma N°27	0,6	5,4	16,6	7,7
Adenocarcinoma N°28	0,6	20,1	33,5	7,7
Adenocarcinoma N°29	0,8	15,3	24,7	4,9
Adenocarcinoma N°30	6,7	9,6	9,3	8,5
Adenocarcinoma N°31	2,0	13,3	16,3	14,2
Adenocarcinoma N°32	0,1	18,3	20,2	8,1
Adenocarcinoma N°33	0,0	2,3	12,7	13,2
Adenocarcinoma N°34	18,7	21,0	12,1	4,5
Adenocarcinoma N°35	7,4	8,1	17,6	16,3
Adenocarcinoma N°36	6,9	0,0	20,8	6,3
Adenocarcinoma N°37	4,9	15,4	13,2	4,8
Adenocarcinoma N°38	0,0	12,2	24,8	14,4
Adenocarcinoma N°39	0,0	23,0	24,9	8,8

Table 3. Correlation CDK activities with age, sex, TNM stage and genetic profile of adenocarcinoma biopsies

Age group	% adenocarcinoma samples	% samples with "All CDK" activities	% samples with "No CDK1" activity
<50	10	9	7
51-60	31	36	20
61-70	36	36	47
>70	23	18	27
SEX			
F	41	73	33
M	59	27	67
Tumour Size			
≤ 3	13	0	13
3-5	38	55	33
5-7	36	36	40
≥ 7	10	9	7
Metastasis			
None	38	27	47
Proximal	15	9	13
Distal	41	55	33
EGFR Mutation			
None	88	91	80
Mutation	10	9	20
KRAS Mutation			
None	59	73	53
Mutation	41	27	47

Table 4. CDKACT1,2,4,6 fluorescence response in 60µg first 9 lymphoma biopsies (triplicate)

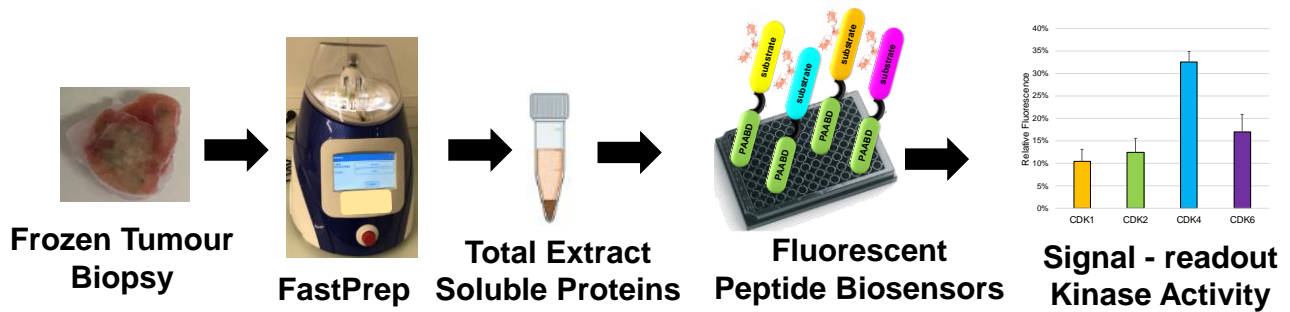
	CDKACT1	CDKACT2	CDKACT4	CDKACT6
Lymphoma N°1	6,38	18,88	12,88	5,00
Lymphoma N°2	3,27	19,62	8,40	4,67
Lymphoma N°3	0,00	15,67	20,93	7,52
Lymphoma N°4	13,56	36,07	6,01	0,00
Lymphoma N°5	26,46	10,64	16,28	15,91
Lymphoma N°6	9,88	8,74	18,90	7,96
Lymphoma N°7	0,00	31,40	46,12	23,81
Lymphoma N°8	25,04	9,05	22,32	27,90
Lymphoma N°9	20,52	17,40	5,71	0,67

Table 5. Relative CDK activities in a Panel of Lymphoma Biopsies

	CDKACT1	CDKACT2	CDKACT4	CDKACT6
Lymphoma N°10	1,3	8,3	0,6	16,0
Lymphoma N°11	0,0	18,4	10,6	30,1
Lymphoma N°12	0,0	5,6	31,4	9,5
Lymphoma N°13	0,0	0,0	11,5	0,0
Lymphoma N°14	8,3	0,0	27,6	11,6
Lymphoma N°15	14,6	0,0	1,1	5,6
Lymphoma N°16	1,9	6,4	15,3	9,2
Lymphoma N°17	0,0	1,6	0,0	0,9
Lymphoma N°18	0,0	23,6	10,5	0,0
Lymphoma N°19	0,0	0,0	7,5	37,0
Lymphoma N°20	0,0	6,4	5,5	1,6
Lymphoma N°21	0,0	16,5	10,5	8,7
Lymphoma N°22	0,0	10,4	14,2	10,6
Lymphoma N°23	12,3	0,0	16,8	8,8
Lymphoma N°24	3,1	15,6	10,7	8,8
Lymphoma N°25				0,0
Lymphoma N°26				0,0
Lymphoma N°27	0,0	14,6	15,8	8,0
Lymphoma N°28	11,0	14,4	26,8	12,5
Lymphoma N°29	0,0	0,7	11,5	22,8
Lymphoma N°30	0,0	13,3	14,1	21,4
Lymphoma N°31	0,0	21,0	19,5	3,4
Lymphoma N°32	0,0	5,8	4,8	6,3
Lymphoma N°33	10,2	12,2	18,4	24,3
Lymphoma N°34	13,3	10,3	8,7	21,1
Lymphoma N°35	0,0	15,8	4,6	12,0
Lymphoma N°36	5,5	20,2	12,8	17,1
Lymphoma N°37	0,2	0,0	19,0	8,0
Lymphoma N°38	10,6	8,9	3,6	0,3
Lymphoma N°39	15,1	12,6	17,4	5,9
Lymphoma N°40	0,0	12,9	25,2	35,3

Table 6. Correlation CDK activities with age, sex, TNM stage, immunohistochemical features of lymphoma biopsies

Age group	% lymphoma samples	"All CDK" activities	"No CDK1" activity	"No CDK1 - low CDK2,4,6"	"No CDK1 - high CDK2,4,6"
<50	30	44	29	14	43
51-60	18	33	7	0	14
61-70	25	11	36	57	14
>70	28	11	29	29	29
SEX					
F	50	56	50	43	57
M	50	44	50	57	43
TNM Staging					
1	10	0	29	29	29
1-2	63	67	43	43	43
2	5	0	7	0	14
3	15	33	7	14	0
p16 expression					
No expression	60	78	64	86	43
1-10	25	11	29	14	43
> 20	8	11	7	0	14
cyclin D1 expression					
No expression	88	78	93	86	100
> 10	13	22	7	14	0
cyclin E expression					
No expression	23	44	0	0	0
>5%	30	0	36	57	14
10-20%	20	11	43	29	57
> 30	28	44	21	14	29



Multiplexed Profiling of CDK Kinase Activities in Tumour Biopsies with Fluorescent Peptide Biosensors.

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Detection of disease biomarkers constitutes a major challenge for development of personalized and predictive diagnostics as well as companion assays. Protein kinases (PK) involved in coordination of cell cycle progression and proliferation that are hyperactivated in human cancers constitute relevant biomarkers. In this study we have implemented four fluorescent biosensors that report on CDK activities to establish a standardized and calibrated multiplex assay to profile CDK activity signatures in biopsies from human lung adenocarcinoma and follicular lymphoma tumours