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A strategy to analyse activity-based profiling of tyrosine kinase substrates in OCT-embedded lung cancer tissue☆

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ABSTRACT

The use of optimal cutting temperature (OCT) medium has served to improve the long-term preservation of surgical tissue specimens. Unfortunately, the presence of polymers in OCT has been found to generate signal interference in proteomic-based techniques. Indeed the presence of OCT medium in tissue lysates precludes the analysis of activity based proteomic profiles obtained from lung adenocarcinoma (LuAdCa) resection specimens. In order to probe this question further tissue lysates were prepared from 47 lung non-neoplastic and tumour, node, metastasis (TNM) stage 1 LuAdCa resection specimens embedded with or without OCT, and data of activity based multiplex profiles of protein tyrosine kinase peptide substrates were obtained. We found that changes in overall phosphorylation level coincided with the use of OCT and subsequently developed an OCT per peptide median correcting strategy by performing median centering on the values of each peptide. Application of this post-analytical strategy not only can identify changes in kinase activity but can also assist in identifying novel targets for therapeutic intervention against LuAdCa.

Introduction

Adenocarcinoma, squamous cell carcinoma and large cell carcinoma represent the main subtypes of non-small cell lung cancer (NSCLC) [1]. Unfortunately there are few therapeutic options currently available for the treatment of these diseases. What is more, a broad search for reliable molecular biomarkers of early stage NSCLC still remains unsuccessful. The scarce number of reliable biomarkers can be attributed to a lack of correlation between mRNA expression levels and the cellular abundance of their corresponding translated proteins [2,3]. Against this background, we developed Activity-Based Protein discovery platforms [4–6] and characterized lung adenocarcinoma (LuAdCa) resection specimens to identify biomarkers for diagnosis, prognosis [7] and prediction of response to therapeutic intervention with tissue preserved with or without optimal cutting temperature (OCT) medium.

OCT medium is a viscous compound of relatively conserved proportions of polyvinyl alcohol (0.24%), polyethylene glycol (4.26%) with 85.5% of non-reactive chemical ingredients [8]. The OCT medium-embedding protocols present several advantages for long-term biobanking [9], and as a means to protect surgical resection specimens. Firstly, when compared with a conventional protocol of direct snap freezing of samples, OCT medium confers an additional long-term protection for fragile organs such as air-filled lung [10]. Secondly, during sample handling, OCT medium embedded tissue are protected by both an oxygen-free and a thermally-stabilized environment [10]. Thirdly, OCT embedded tissues do not fall apart when processed for histological assessment [11], and are well protected from desiccation for long-term storage periods in a nitrogen gas-phase tank at −195 °C. Finally, OCT medium embedded resection specimens remain oriented with a pre-defined area facing the cryostat blade, and this reduces the implications of heterogeneity observed in tumour versus stroma ratio that may occur when different parts of a tumour block from the same patient are cryosectioned. Similarly, this observation applies when evaluating the absence or presence of tumour necrotic area. All the confounding factors contributing to tumour heterogeneity may influence the outcome of proteomic analysis [11] and are reduced when the exact same part of the OCT-embedded tumour block is analysed. As a

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means to carefully evaluate cryosections, a 'pool' of patient tumour blocks is an important prerequisite to obtain a uniform population of samples with well defined composition, especially regarding tumour versus stroma ratio.

A number of proteomic publications report important signal interferences in the presence of OCT medium for the different types of signals analysed [8,12–14]. When OCT-embedded surgical resection specimens are used in RNA or DNA analysis approaches, the OCT interferences during PCR enzymatic reaction are prevented, without apparent qualitative and quantitative losses, by prior steps of sample column or solvent separations [15,16]. Enzymatic activity studies are devoid of both classical PCR signal amplification and column or solvent purification steps. OCT-removal approaches are generally difficult to implement in proteomic or protein purification protocols, and OCT medium interferes in subsequent quantitative or qualitative proteomic analysis [17].

In this study we describe protein tyrosine kinase (PTK) activities in tumour, node metastasis (TNM) stage 1 lung adenocarcinoma (LuAdCa) with a multiplex profiling approach of well-characterized PTK peptide substrates. We prepared lung protein tissue lysates (from non-neoplastic or malignant tissues) from the same patient and we also analysed the lysates obtained from malignant tissues in the presence of the PTK inhibitor gefitinib [18]. Treatment of LuAdCa lysates with geftinib represents a supplemental assay tool applied in combination with clinical follow-up to test the discriminative power of our molecular signature for low-risk versus high-risk patients with respect to treatment response and survival. In the past we have embedded specimens in our bio-bank either with or without OCT medium. To design this study with both training and validation cohorts and relevant 5-years survival clinical data, we had to rely upon all the LuAdCa resection specimens available that were either embedded in or not embedded in OCT.

In this study we observed OCT medium interferences on the data recorded from PTK peptide substrate microarrays. We applied a post-analytical OCT medium per peptide median correcting procedure for all our processed samples. This OCT correcting procedure may be well suited to study mixed sources of surgical resection specimens (i.e., with or without OCT or other related embedding support). The molecular prognosis signature based on PTK activity differences found in our OCT or non-OCT medium-embedded heterogeneous group of LuAdCa resection specimens has identified several novel targets for future anti-lung cancer therapies [7].

Materials and methods

Management of the collected LuAdCa resection specimens, histopathological assessment and patient clinical data

The Ethical Review Board of the University Hospital Zürich (UHZ) approved the use of human tissue in the present study. Informed consent was obtained from each subject prior to participation in the study. This study was conducted in accordance with the Declaration of Helsinki. Fresh frozen lung malignant and non-neoplastic tissues were collected from the same patients dated between 2003 up to 2006. All of the specimens were cryopreserved in the vapour phase of liquid nitrogen at −196 °C. As of January 2007 up until the censoring date of January 2013, the procedure of snap-freezing lung specimens in liquid nitrogen following surgical resection was updated to embedding this tissue in OCT medium (OCT, Tissue-Tek, Miles Inc., Elkhart, IN, USA) [9].

We obtained and archived all the mandatory informed consent approval forms with all clinical interventions and follow up treatment decisions from each of the participating patients. 47 LuAdCa surgical resection specimens were collected with clinical characteristics of TNM stage 1 (1A and 1B) and we selected 53.6 months as a long-versus short-term survivor cut-off value [7]. Histopathological examinations were conducted both at the time of surgical resection specimen collection and before starting the sample processing of this study. We stained our cryosections with haematoxylin and eosin for a second blinded pathological assessment according to the 6th TNM classification of malignant lung [19,20] of all our surgical resection specimens. We mounted the LuAdCa resection specimens included in this study in OCT medium and stained them with haematoxylin and eosin for a second blinded pathological assessment.

Preparation of LuAdCa surgical resection specimens for multiplex profiling with an ex vivo PTK assay

For each of the individual 47 TNM stage 1 LuAdCa OCT blocks specimens, we prepared two well-defined tissue area cryosections of 30-µm thickness (1–1.5 mm^3 of tissue). The protein lysates were quickly thawed by hand and immediately extracted on ice in 100 µL of ice cold mammalian extraction buffer (M-PER) which contained both Halt Phosphatase and Halt Protease Inhibitor Cocktail (Thermo Fisher Pierce, Rockford, IL, USA). We used wide bore pipet tips in order to resuspend the lysates 30 times and subsequently all samples were pelleted at 3000 rpm for 0.5 min in a microcentrifuge set at 4 °C. The samples were then re-extracted again using the wide bore pipet tips 30 times and finally lysed for 15 min on ice. After centrifugation at 10000 rpm for 15 min at 4 °C, each lysate supernatant was aliquoted and stored at −80 °C. The protein concentration was estimated with a micro BCA kit (Thermo Fisher Pierce, Rockford, IL, USA). The malignant protein lysates were also analysed with a 10 µM final concentration of gefitinib (Cayman Biochemicals, Ann Arbor, MI). The 40 µL final volume of kinase master mix contained the kinase assay buffer (50 mM Tris-HCl pH 7.5, 10 mM MgCl2, 1 mM EGTA, 2 mM dithiothreitol, 0.01% Brij 35, 1 mg/mL BSA, and 12.5 µg/mL FITC-labelled antibody) was prepared according to the instructions provided by the manufacturer (PamGene's-Hertogenbosch, The Netherlands) and assayed with the following modifications: 1) we tested 5 µg of the extracted protein lysate in a 1–5 µL final volume of M-PER extraction buffer and, 2) we included either 2 µL of a 200 µM stock of gefitinib in DMSO or 2 µL DMSO. To synchronise all the kinase reactions, we added 4 µL of a 4 mM ATP stock solution to reach a 0.4 mM final ATP concentration.

Ex-vivo multiplexed profiling of PTK substrates

Multiplexed *ex-vivo* profiling of PTK substrates from human lung protein lysates was performed with a PTK PamChip®4microarrays (PamGene, 's-Hertogenbosch, The Netherlands) [21]. The time course of substrate tyrosine phosphorylation at 30 °C was detected using fluorescein-labelled anti-phosphotyrosine antibodies. During a 60 min incubation with 92 pumping cycles through the microarray, we collected fluorescent image of each array at a rate of one array's image taken for every fifth pump cycle with a CCD camera. We applied a randomisation scheme for duplicate measurements of samples: two distinct runs of the PamStation®12 (PamGene, 's-Hertogenbosch, The Netherlands) were performed on two independent PTK PamChip®4 microarrays. Each run was performed with two freshly thawed lysate aliquots. We established predefined inclusion and exclusion criteria before the experiment and removed patient assay results in case they did not meet these criteria: 1) very low or highly saturated peptide tyrosine phosphorylation resulting in extensive image saturation, 2) clear outlier from the rest of the data set in the Principal Component Analysis (PCA).

Image processing, data and statistical analysis

Quality control investigations and quantitation of the tyrosine phosphorylation signals was performed with the software package BioNavigatoR (v.5.2–6.2; PamGene, 's-Hertogenbosch, The Netherlands). Measurements were inspected and repeated for one or both replicates in case of evident technical or mechanical problems. The