Feasibility study of in vitro drug sensitivity assay of advanced non-small cell lung adenocarcinomas

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ABSTRACT

Background Despite improved screening techniques, diagnosis of lung cancer is often late and its prognosis is poor. In the present study, in vitro chemosensitivity of solid tumours and pleural effusions of lung adenocarcinomas were analysed and compared with clinical drug response. **Methods** Tumour cells were isolated from resected solid tumours or pleural effusions, and cryopreserved. Three-dimensional (3D) tissue aggregate cultures were set up when the oncoteam reached therapy decision for individual patients. The aggregates were then treated with the selected drug or drug combination and in vitro chemosensitivity was tested individually measuring ATP levels. The clinical response to therapy was assessed by standard clinical evaluation over an 18 months period. **Results** Based on the data, the in vitro chemosensitivity test results correlate well with clinical treatment response. **Conclusions** Such tests if implemented into the clinical decision making process might allow the selection of an even more individualised chemotherapy protocol which could lead to better therapy response.

INTRODUCTION

While the recently improved treatment strategies have resulted in better survival statistics in many cancers, in non-small cell lung cancer (NSCLC) the 1-year overall survival at a locally advanced or metastatic stage barely exceeds 20%.¹ Although key mutations aid clinical decision-making and facilitate the application of targeted therapies, radical improvements have not been observed and the 5-year survival rate remains at approximately 5%.¹ Although next-generation sequencing has become a cornerstone of therapy guidance,² clinical decision-making remains difficult due to the histological diversity of NSCLC (adenocarcinoma, squamous, large cell) and the variation of the mutation characteristics of the different subtypes.³ Based on clinical guidelines, patients with advanced lung adenocarcinoma (AC) are tested for the presence of Kirsten rat sarcoma 2 viral oncogene homolog (KRAS) and epidermal growth factor receptor (EGFR)

mutations and rearrangements involving anaplastic lymphoma kinase (ALK).⁴ Unfortunately, analysis of tumour mutations can only describe the mutations existing at the specific location where the sample was taken from and at the time of sample taking. By the time therapy is selected, additional mutations may have occurred.^{5,6} Due to the diversity of histology as well as mutations in NSCLC, the first-line treatment of locally advanced or inoperable cancer is platinum based, which can in itself dramatically increase the mutation rate.⁷ The relatively slow acting immunotherapies are only considered as an alternative in specific cases⁸; therefore, chemotherapy remains the principal treatment modality in advanced NSCLC. To improve chemotherapy response rates, drug sensitivity assays have been under intense investigation.⁹ It was recognised that tissues derived from the original tumour represent the tumour composition suitably well to test chemosensitivity on freshly isolated tumour cells in vitro.¹⁰ Most published tests, however, have not been performed on advanced NSCLC.⁹ While the statistical analysis of data in the current literature involving different tumours looks convincing, clinicians remain wary of such tests due to the clinical complexity of individual treatment responses. In the present study, we have performed in vitro drug sensitivity analysis in advanced NSCLC AC samples, to investigate sensitivity to currently recommended drugs and compared the results to clinical therapy response.

MATERIALS AND METHODS

Resected solid tissue samples were digested using a Miltenyi Tumor Dissociation Kit (Miltenyi Biotec, Auburn, USA). Cells from pleural fluid were centrifuged (600g, 10 min), then isolated by Ficol separation. Red blood cells were removed by Red Blood Cell Lysis Buffer (Roche, Mannheim, Germany). Cells

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were cryopreserved using Cryo-SFM (PromoCell, Heidelberg, Germany) and stored at -80°C until used.

Cryopreserved tumour cells were combined with normal human lung fibroblast cells (1:1) and then aggregated in a low-attachment 96-well plate (Corning, New York, USA).¹⁰

Tissue aggregates were treated with chemotherapeutic compounds (Selleckem, Munich, Germany) selected by the oncoteam.

Concentrations for in vitro treatments were based on the literature^{11 12} and in vitro concentration tests performed in our laboratory. Drug concentrations were as follows: cisplatin ($7\mu\text{M}$),^{13 14} carboplatin (CBP; $100\mu\text{M}$),¹⁴ vinorelbine (150nM),¹⁵ gemcitabine ($30\mu\text{M}$),¹⁶ paclitaxel (100nM),¹⁷ pemetrexed ($10\mu\text{M}$),¹⁶ erlotinib (100nM)¹⁸ and gefitinib (100nM).¹⁹ Treatments were carried out for 48 hours at 37°C in 5% CO_2 atmosphere in four parallels.

In vitro viability assay was performed using the three-dimensional (3D) CellTiter Glo (Promega, Madison, USA) kit, measured in a PerkinElmer Plate Reader (PerkinElmer, Waltham, USA).

CT, MRI, chest X-ray and abdominal ultrasound methods were used for Response Evaluation Criteria

In Solid Tumours (RECIST V.1.1) evaluations.²⁰ In the selected patient populations, only stable (SD) and progressive (PD) diseases were distinguished. The in vitro chemosensitivity results were compared with the patients' clinical responses to chemotherapy at 2–3 months and patients were monitored over 18 months. One-way analysis of variance was used for statistical analysis and $p < 0.05$ was considered as significant.

RESULTS

The study design and patient exclusion criteria are summarised in figures 1 and 2, respectively. Patient information is summarised in table 1.

Cells from cancer tissue or pleural effusion (PE) samples obtained from each patient were limited; therefore, the in vitro chemosensitivity tests were performed based on the clinical decision for treatment. The cryopreserved samples were thawed (viability routinely exceeded 90%; online supplementary S. figure 1), aggregates were made, then drug sensitivity tests were performed with drugs or drug combinations selected by the oncoteam (online supplementary S. figure 2). The in vitro viability

Figure 1 Study design. All the samples were freshly cryopreserved as single cell suspensions, then thawed when the oncoteam made a decision for therapy. Both the resected tissue and the tumour-enriched pleural effusion stained positive for TTF1 and both sample types were routinely tested for Kirsten rat sarcoma 2 viral oncogene homolog, epidermal growth factor receptor and anaplastic lymphoma kinase mutations. Single cell suspensions were cryopreserved and stored at -80°C until used. Samples were thawed and placed into three-dimensional (3D) aggregate cultures, then treated with the corresponding chemotherapeutic agent(s) selected by the oncoteam. Patient therapy responses were monitored and compared with the in vitro assay results. PD, progressive disease; SD, stable disease; TTF1, Thyroid transcription factor 1.

Figure 2 Exclusion criteria. Out of the 120 patients only 14 met the inclusion criteria, 10 for solid tumours and 4 for pleural effusions. The exclusion criteria are named in the boxes along with the number of patients excluded from the study. RECIST, Response Evaluation Criteria In Solid Tumours.

test results were compared with the RECIST1.1 data.¹⁶ In vitro mean viability values at and below 0.8 (induction of cell death 0.2<) corresponded to patients with clinically SD, while a mean viability value of 0.9 and above (no or low level (<0.1) induction of cell death) corresponded to patients with clinically PD (figure 3A; online supplementary table 1). Cut-off values are explained in online supplementary S. figure 3. Sample numbers on the figure correspond to the patient numbers in table 1. The patient who donated the KRAS mutant solid tumour sample (S1) was PD during clinical observation, then following cisplatin+pemetrexed combination therapy became stable (SD). S1 patient became PD again after changing the treatment to pemetrexed monotherapy and the RECIST result correlated with the in vitro chemosensitivity analysis. The change in therapy was forced by severe adverse reactions to cisplatin. Patients S2 (wild type (WT)) and S9 (KRAS) responded well to cisplatin+vinorelbine combination as they were both SD at clinical examination and in vitro testing. Patients S4 (KRAS), S7 (KRAS), S8 (undisclosed mutation status) and S10 (EGFR) were SD correlating to the in vitro analysis. Patient samples S3 (KRAS), S5 (KRAS) and S6 (KRAS) remained firmly non-responsive to therapy and clinically PD after evaluation (figure 3A). The in vitro test results correlated well with the clinical data (figure 3A). Among the PE samples, donor of PE sample 2 (PE2) was initially SD after cisplatin+pemetrexed combination treatment but became PD when due to severe reactions to cisplatin, treatment was

changed to pemetrexed monotherapy (figure 3A). The same chemosensitivity response was detected also in vitro. Discrepancies between clinical and in vitro evaluation were detected in some cases. Correcting the corresponding in vitro data with the time course of progression information (figure 3B), a stronger association between the in vitro viability analysis and patient response to therapy (figure 3C) was detected.

To investigate the possibility whether the above-mentioned in vitro drug sensitivity test could supplement the clinical decision-making process, a PE sample was selected for further studies. The patient who donated the sample did not respond to the clinically offered CBP-paclitaxel combination therapy (PD; figure 4). The in vitro chemosensitivity analysis using CBP-paclitaxel matched the clinical response (relative cell viability values were above 0.9, no induction of cell death; figure 4). Another, clinically approved combination for therapy in this particular case could have been CBP-pemetrexed. In vitro analysis of the sample using CBP-pemetrexed treatment of cell aggregates reduced cell viability below 0.8 (effective induction of cell death) that is in the SD range of the therapy response (figure 4).

DISCUSSION

According to the US Precision Medicine Initiative,²¹ the arsenal of precision medicine should be at the fingertips of every oncologist. The clinical reality, however,

Table 1 Clinical data (Part A contains data of patients donating solid tumours, while part B contains a list of samples obtained from pleural effusions)

Sample number	Mutations	Diagnosis	T	N	M	Intervention	Date of sampling	Clinical therapy	In vitro	Type of treatment	Beginning of therapy	End of therapy	Treatment before/after sampling	RECIST date	RECIST	PD-L1
A. Patient data of solid tumours																
Solid tumours																
1	KRAS	NSCLC-Adenoc.	pT1b	pN1	Mx	Complete tumour resection	27 October 2015	Observation	–		27 October 2015	22 May 2017	After	PD	29 March 2017	>50% (Positive)
			pT1b	N3	M1a			Cisplatin + pemetrexed	Tested	Palliative	23 May 2017	09 August 2017	After	SD	25 August 2017	
			T3	N3	M1c			Pemetrexed mono	Tested	Palliative	17 October 2017	28 November 2017	After	PD	14 December 2017	
2	WT	NSCLC-Adenoc.	pT2a	N1	Mx	Complete tumour resection	01 December 2015	Cisplatin + vinorelbine	Tested	Adjuvant	06 January 2016	24 June 2016	After	SD	–	Negative
3	KRAS	NSCLC-Adenoc.	pT3	pN2	Mx	Complete tumour resection	28 April 2016	Observation	–		29 April 2016	29 June 2016	After	PD	26 May 2016	Negative
			pT3	pN2	M1a			Cisplatin + pemetrexed	Tested	Palliative	30 June 2016	30 June 2016	After	PD	05 July 2016	
4	KRAS	NSCLC-Adenoc.	pT3	N0	M1b	Complete tumour resection	03 May 2016	Cisplatin + gemcitabine	Tested	Adjuvant	07 July 2016	08 August 2016	After	SD	06 September 2017	Negative
5	KRAS	NSCLC-Adenoc.	pT3a	N1	Mx	Complete tumour resection	26 June 2016	Cisplatin + vinorelbine	Tested	Adjuvant	08 August 2016	30 September 2016	After	SD	27 April 2017	Negative
6	KRAS	NSCLC-Adenoc.	pT2a	pN2	M1c	Complete tumour resection	12 September 2016	Carboplatin + gemcitabine	Tested	Adjuvant	12 October 2016	07 December 2016	After	PD	24 January 2017	<1% (Negative)
7	KRAS	NSCLC - Adeno cc	pT2	pN1	Mx	Complete tumour resection	05 December 2016	Cisplatin+pemetrexed	Tested	Adjuvant	21 February 2017	03 May 2017	After	SD	23 May 2017	
8		NSCLC - Adeno cc	pT2a	pNx	Mx	Complete tumour resection	05 January 2017	Carboplatin – paclitaxel	Tested	Adjuvant	28 March 2017	15 June 2017	After	SD	20 July 2017	
9	KRAS	NSCLC - Adeno cc	pT2	pN1	Mx	Complete tumour resection	14 December 2015	Cisplatin + vinorelbine	Tested	Adjuvant	18 February 2016	08 April 2016		SD	08 July 2016	
10	EGFR	NSCLC - Adeno cc	pT2a	pN2	Mx	Complete tumour resection	19 December 2016	Cisplatin + vinorelbine	Tested	Adjuvant	09 February 2017	02 March 2017	After	SD	20 March 2017	
			pT2a	pN2	Mx			Erlotinib mono	Tested	Palliative	18 October 2017	13 August 2018	After	PD	13 August 2018	

Continued

Table 1 Continued

Sample number	Mutations	Diagnosis	T	N	M	Intervention	Date of sampling	Clinical therapy	In vitro	Type of treatment	Beginning of therapy	End of therapy	Treatment before/after sampling	RECIST date	PD-L1
B. Patient data of pleural effusion donors															
Pleural effusions															
1	EGFR	NSCLC - Adeno cc	T3	Nx	M1b	Thoracic aspiration	20 February 2017	Gefitinib	Tested	Palliative	04 February 2015	14 June 2017	During	PD	Negative
2	KRAS	NSCLC - Adeno cc	T2a	N2	M1a	Thoracic aspiration	06 May 2017	Cisplatin + pemetrexed	Tested	Palliative	20 May 2016	28 July 2017	After	SD	Negative
			T2a	N2	M1a			Pemetrexed mono	Tested	Palliative	19 September 2016	03 November 2016	After	PD	17 November 2016
3	EGFR	NSCLC - Adeno cc	T4	N2	M1	Thoracic aspiration	25 February 2016	Erlotinib mono	Tested	Palliative	26 January 2016	28 July 2016	Before	PD	13 July 2017
			T4	N2	M1			Osimeitinib	-	Palliative	31 August 2016	30 June 2017	After	PD	01 August 2017
			T4	N2	M1			Carboplatin + paclitaxel	Tested	Palliative	09 August 2017	30 August 2017	After	PD	18 September 2017
			T4	N3	M1c			Pemetrexed mono	Tested	Palliative	11 October 2017	05 January 2018	After	PD	25 January 2018
4	WT	NSCLC - Adeno cc	T2a	N2	M1a	Thoracic aspiration	27 July 2016	Carboplatin + paclitaxel	Tested	Palliative	15 June 2016	26 August 2016	During	PD	08 September 2016
			T4	N3	M1b			Erlotinib mono	Tested	Palliative	04 October 2016	12 December 2016	After	PD	12 December 2016

The information ranges from the actual method of obtaining the sample, mutation analysis, diagnosis, staging (TNM=tumour, node, metastasis status) of the disease and applied treatment or treatments and clinical responses.
EGFR, epidermal growth factor receptor; KRAS, Kirsten rat sarcoma 2 viral oncogene homolog; NSCLC, non-small cell lung cancer; PD, progressive disease; PD-L1, programmed death ligand-1; RECIST, Response Evaluation Criteria In Solid Tumours; SD, stable disease.

Figure 3 Chemosensitivity analysis. (A) Three-dimensional (3D) aggregate cultures were treated with patient-specific chemotherapeutic agents as determined by the oncoteam. After incubation at 37°C for 48 hours in a 96-well plate, ATP levels corresponding to cell viability were determined using a 3D CellTiter Glo kit. In vitro viability data were compared with Response Evaluation Criteria In Solid Tumours (RECIST1.1) data when it became available. Patient-specific data are shown individually and marked with the patient identifying number used in the study. (B) Individual patient data shown in association of time laps to disease progression. (C) Percentage of correspondence between clinical RECIST1.1 information and in vitro analysis results. PD, progressive disease; SD, stable disease.

is different. Traditional chemotherapies are still the remaining treatment options for patients with stage III B or IV NSCLC tumours, that carry no targetable mutations, and are not positive for PD-L1. However, decision-making as chemosensitivity of the tumour is currently not tested routinely. Oncologists, who are bound by specific clinical guidelines, still need more than one treatment option to offer a patient. Even if the approved guidelines provide some choices, the fast progressing disease allows little time to select additional tests for the presence or absence of biomarkers indicating previously unpredicted therapeutic targets. The in vitro drug sensitivity analysis in the above study was performed to test whether the clinical effect of chemotherapeutic drug combinations could be tested using a simple and fast method where targeted therapy was not available for the patients. Compared with previous studies and test methods,²² we intentionally remained within the current routine clinical boundaries. Instead of using a vast number of mutation analyses²³ and artificial intelligence²⁴ to achieve better accuracy, we simply selected the patient population more carefully. For example, patients who were subjected to Avastin or immune checkpoint treatment were excluded from

the test, as the test tissue was lacking blood supply and contained no immune cells. Such strict selection criteria had the consequence of a drastically reduced number of patients whose in vitro and clinical data were suitable for comparison. Although the number of patients were limited in the study, the in vitro data indicate that in vitro chemosensitivity tests could aid clinical drug selection and potentially expand survival even for patients with advanced lung AC. The fact that we are able to test more than one chemotherapy combination in vitro (figure 4) raises the possibility that an approved in vitro drug sensitivity test could make clinical decision-making easier. The in vitro process is feasible and could be easily added to the decision-making process. Partly, because it does not require additional sample taking from the patient and does not increase the workload of clinical staff.

In conclusion, chemosensitivity tests could supplement the clinical diagnostic arsenal as: (1) the assays can be performed from a small number of cells (1000 cell/well); therefore, even samples from distinct metastatic sites can be tested if sample taking is clinically feasible. (2) Cryopreservation of tumour cells allows sufficient time to perform additional diagnostic tests. (3) The analysis

Figure 4 Testing optional drug sensitivity. Clinical application of carboplatin–paclitaxel combination therapy resulted in progressive disease with a matching in vitro chemosensitivity analysis of cell viability values above 0.9. Treatment of tissue culture with carboplatin–pemetrexed in vitro reduced cell viability below 0.8 that is in the stable disease range of therapy response. Viability compared with the untreated control was significantly lower when cell cultures were incubated with carboplatin–pemetrexed combination ($p < 0.01$). PE, pleural effusion; N.S., not significant.

provides information within 48 hours, which is vital for patients with fast progressing tumours.

Additionally, the above system could also be introduced into drug development. To reduce systemic toxicity, novel prodrug systems are being developed.²⁵ Although the 3D tissue aggregate is not suitable to test most prodrugs, the toxic effects of the active metabolite can be tested in the above system.

The prediction of the clinical response to chemotherapeutic drugs remains a major challenge in clinical oncology. If our simple and fast in vitro method were to be used to test chemosensitivity and if that test result is added to the patient's full clinical assessment, a decision for therapy could be made with increased confidence.

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