Poor response to platinum-based chemotherapy is associated with KRAS mutation and concomitant low expression of BRAC1 and TYMS in NSCLC

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Abstract

Objective: To evaluate treatment response, survival, and the associations between KRAS mutation status and tumour expression levels of BRCA1, TYMS and SRC retrospectively in a cohort of patients with non-small cell lung cancer (NSCLC), treated exclusively with conjunctive platinum-based doublet chemotherapy.

Methods: KRAS mutation status was determined via amplification refractory mutation and multiple quantitative polymerase chain reaction (PCR) analysis. Tumour expression levels of BRCA1, TYMS and SRC were determined via real time quantitative PCR.

Results: Patients with KRAS mutations (n = 3) had significantly shorter survival duration than patients with wild type KRAS (n = 42). Tumour expression levels of BRCA1 and TYMS, but not SRC, were significantly lower in patients with, than in those without, KRAS mutations. Tumour expression level of BRCA1 was positively correlated with survival duration.

Conclusions: KRAS mutation status and BRCA1 tumour expression are potential biomarkers for tailoring chemotherapy and predicting clinical outcome.
Introduction

Non-small cell lung cancer (NSCLC) accounts for ~80% of lung cancer cases and has a 5-year survival rate of 16%.

A major obstacle to increasing overall survival is the poor response to available treatments.

Research into the genetics and molecular biology of lung cancer has identified multiple mutations and molecular pathways that are involved in tumour pathogenesis and drug resistance, allowing the design of tailored treatment plans and the prediction of treatment outcomes in selected lung cancer patients, based on the results of molecular screening.

These include EGFR mutations associated with improved overall response rate to EGFR tyrosine kinase inhibitors such as gefitinib and erlotinib, and ALK fusion gene mutations that improve response to ALK inhibitors such as crizotinib and ceritinib.

Despite these advances, predicting treatment outcome and overall survival remains difficult in the majority of patients, and it is therefore important to identify additional relevant genetic mutations.

Oncogenes in the RAS family (HRAS, KRAS, and NRAS) encode small GTPases that act as intracellular transducer proteins and transmit extracellular signals through their receptors, such as EGFR, to downstream signalling transduction pathways.

Proteins encoded by mutated RAS genes cannot hydrolyze GTP to GDP, leading to aberrant activities mediated by downstream signalling pathways.

About 30% of patients with lung adenocarcinoma carry mutant KRAS, but it is unclear whether this mutation is a causative factor in poor survival and treatment resistance.

Platinum-based chemotherapy remains popular for treating cancers, especially in patients with genetic or pathological profiles that respond poorly to targeted therapies. The platinum compound binds to DNA and forms platinum–DNA adducts that in turn cause the formation of crosslinks among DNA strands and the distortion of DNA conformation, leading to inhibition of DNA replication. DNA adduct formation and nucleotide excision repair (NER) of platinum–DNA adducts may influence clinical response and therapeutic outcome.

The BRCA1 (breast cancer 1) and TYMS (thymidylate synthetase) genes encode proteins involved in adduct formation and NER, and have been evaluated as biomarkers for prognostic purposes and treatment selection. It is not clear whether the expression of these genes is affected by KRAS mutation status, however.

The aim of the present retrospective study was to evaluate the associations between KRAS mutation status and tumour expression levels of BRCA1, TYMS and SRC (a gene that is aberrantly overexpressed in lung cancer) in a cohort of patients with NSCLC treated exclusively with conjunctive platinum-based doublet chemotherapy. The association between survival and BRCA1, TYMS and SRC expression or KRAS mutation status was investigated, in an attempt to identify additional molecular biomarkers for drug selection and outcome prediction.

Patients and methods

Study population

The study retrospectively recruited consecutive patients diagnosed with NSCLC at Liyang People’s Hospital, Liyang, Jiangsu,
China, between February 2009 and December 2013. There were no age or sex restrictions. Tumour tissue samples were collected by surgical section or computed tomography-guided biopsy, processed immediately and preserved in formalin-fixed paraffin wax-embedded (FFPE) tissue blocks. Tumour staging was undertaken using the American Joint Committee on Cancer Staging Manual, 7th edition. Histology was determined according to World Health Organization criteria. Histopathological characteristics, including tumour subtype, were independently evaluated by two pathologists (including Y.D.), with disagreements resolved by a third pathologist.

The institutional ethics committee of Liyang People’s Hospital, Liyang, Jiangsu, China, approved the study, and all patients provided written informed consent.

Treatment

All patients received conjunctive platinum-based doublet chemotherapy after surgery as first-line treatment, using cisplatin, carboplatin or nedaplatin in combination with gemcitabine, pemetrexed or docetaxel for between 4 and 6 weeks. Treatment was terminated if patients exhibited unacceptable toxic adverse events or progressive disease. All patients were followed up until September 2014 or death. Overall survival was calculated from the date of first chemotherapy to date of death or last visit.

Detection of KRAS mutations

Genomic DNA was extracted from tumour tissue via QIAamp® DNA FFPE kit (Qiagen, Valencia, CA, USA), according to the manufacturer’s instructions. KRAS mutations were detected using an amplification refractory mutation system and multiple quantitative polymerase chain reaction (PCR) analysis (ARMS-multi-qPCR) with a Human KRAS and BRAF Mutation Detection Kit (YuanQi Bio-Pharmaceutical Co., Ltd., Shanghai, China). PCR cycling conditions were 94°C for 3 min followed by 40 cycles of 94°C for 15 s and 60°C for 1 min, using an ABI 7500 Real Time PCR System (ThermoFisher Scientific, Waltham, MA, USA). Primer sequences were: forward 5′-TTTGATTAAAAAGGTACTGGTGG-3′, and reverse 5′-CCTCTATTTGGATCATATTGC-3′. Mutations were verified via direct sequencing using the following primer: 5′-TGTATTAAAAAGGTACTGGTGGAGG-3′.

RNA isolation and gene expression analysis

Total RNA was extracted from tumour tissue using an RNeasy FFPE Kit (Qiagen), as described. RNA was treated with DNase I (DNA-free; ThermoFisher Scientific) to remove any potential DNA contamination, quantified and quality-checked, and first strand cDNA was synthesized using a SuperScript® III First-Strand Synthesis System (ThermoFisher Scientific). The cDNA was used in real time quantitative PCR for BRCA1, SRC and TYMS, using the appropriate Tumour Gene Expression Analysis Kit (YuanQi Bio-Pharmaceutical Co., Ltd., Shanghai, China), according to the manufacturer’s instructions. The housekeeping gene ABL was used as an internal control. Cycling conditions for all genes were 94°C for 5 min followed by 40 cycles of 94°C for 15 s and 60°C for 1 min, using an ABI 7500 Real Time PCR System (ThermoFisher Scientific).

Statistical analyses

Data were presented as mean ± SEM or n (%). Pairwise comparisons were performed using Student’s t test for normally distributed data and nonparametric tests for non-normally distributed data. Categorical data were analysed using χ²-test or Fisher’s exact
test. Overall survival was plotted using Kaplan–Meier survival curves, and differences between patients with or without \textit{KRAS} mutations were evaluated using log rank test with \( \chi^2 \). Pearson’s correlation analysis was performed to determine correlations among \textit{BRCA1}, \textit{TYMS} and \textit{SRC} expression levels, and between overall survival and expression level of each gene. Statistical analyses were performed using GraphPad Prism version 6 (GraphPad Software, Inc., San Diego, CA, USA). \( P \)-values < 0.05 were considered statistically significant.

### Results

The study enrolled 46 patients, one of whom was excluded from the final analysis due to lack of clinical data. The final study population included 45 patients (29 male/16 female; median age 62.4 years; age range 40 – 90 years). Demographic and clinical characteristics of the study population are given in Table 1. Of patients with advanced stage cancer (total \( n = 19 \); stage IIIb, \( n = 4 \); stage IV, \( n = 15 \)), 14 had adenocarcinoma (\( n = 3 \) at stage IIIb; \( n = 11 \) at stage IV).

A total of three patients were found to have \textit{KRAS} mutations Table 2, and all had adenocarcinoma. Patients with \textit{KRAS} mutations were significantly more likely to have stage IV disease than patients without mutations (\( P < 0.03 \)). There was no significant between-group difference in the presence of metastasis. The prevalence of \textit{KRAS} mutation was 8.8% in adenocarcinoma (three of 34) and 21.4% (three of 14) in advanced stage adenocarcinoma (IIIb and IV). None of the patients carrying \textit{KRAS} mutations survived to the end of the investigation (mean survival 4.5 \( \pm \) 1.5 months).

The mean duration of survival was significantly shorter in patients with \textit{KRAS} mutations than in those without (\( P < 0.001 \); Figure 1).

Tumour expression levels of \textit{BRCA1} and \textit{TYMS} were significantly lower in patients with \textit{KRAS} mutations compared with those without (\( P < 0.05 \); Figure 2a and b). There was no significant between-group difference in \textit{SRC} expression (Figure 2c).

In the total study population, there were significant positive correlations between overall survival duration and \textit{BRCA1} expression level (\( r = 0.3015 \); \( P < 0.02 \)), and between \textit{BRCA1} and \textit{TYMS} expression

### Table 1. Demographic and clinical characteristics of patients with nonsmall cell lung cancer included in a study to evaluate the associations between \textit{KRAS} mutation status and tumour expression levels of \textit{BRCA1}, \textit{TYMS} and \textit{SRC} \((n = 45)\).

| Characteristic                  | \( n \) (%)     |
|---------------------------------|-----------------|
| Sex, male/female                | 29/17 (64.4/37.8) |
| Age, years                      |                 |
| 40–49                           | 4 (8.9)         |
| 50–59                           | 10 (22.2)       |
| 60–69                           | 21 (46.7)       |
| \( \geq 70 \)                    | 10 (22.2)       |
| Smoking history                 |                 |
| Nonsmoker                       | 16 (35.6)       |
| Smoker                          | 28 (62.2)       |
| Unknown                         | 1 (2.2)         |
| Family history of cancer        |                 |
| No                              | 35 (77.8)       |
| Yes                             | 6 (13.3)        |
| Unknown                         | 4 (8.9)         |
| Metastasis                      |                 |
| No                              | 15 (33.3)       |
| Yes                             | 30 (66.7)       |
| Tumour stage                    |                 |
| I/II                            | 20 (44.4)       |
| IIIa                            | 6 (13.3)        |
| IIIb                            | 4 (8.9)         |
| IV                              | 15 (33.3)       |
| Histology                       |                 |
| Adenocarcinoma                  | 34 (75.6)       |
| Squamous                        | 9 (20.0)        |
| Other                           | 2 (4.4)         |
| Response*                       |                 |
| CR/PR                           | 16 (41.0)       |
| SD                              | 9 (23.1)        |
| PD                              | 14 (35.9)       |

Data presented as \( n \) (\%) of patients.

*Excluding six uncharacterized cases; \( n = 39 \).
levels ($r = 0.4749$; $P < 0.001$). There were no significant correlations between survival duration and $TYMS$ or $SRC$ expression level.

**Discussion**

Resistance to platinum-based chemotherapy is a major impediment to successful treatment of lung cancer. In our cohort of 46 patients with NSCLC treated with platinum-based chemotherapy, we found that $KRAS$ mutations were associated with shorter survival and thus poor treatment response. In addition, patients with $KRAS$ mutations had significantly lower tumour expression of both $BRCA1$ and $TYMS$, but not $SRC$, compared with patients without such mutations. Our finding that $BRCA1$ levels alone (not $TYMS$ or $SRC$) were correlated with overall survival duration underscores the complex pathology of NSCLC.

Proteins of the KRAS family transduce extracellular signals through membrane-bound receptors, such as from EGFR to downstream intracellular (RAS–RAF–MEK) pathways. $KRAS$ mutations are mutually exclusive with $EGFR$ mutations. Patients with $EGFR$ mutations respond well to EGFR tyrosine kinase inhibitor-based drugs, including gefitinib and erlotinib, but these drugs are no more effective than platinum-based or other traditional therapies in patients carrying $KRAS$ mutations.
Figure 2. Tumour levels of (a) BRCA1, (b) TYMS and (c) SRC mRNA in patients with non-small cell lung cancer, stratified by the presence ($n = 3$) or absence (control; $n = 42$) of KRAS mutations. Data presented as mean ± SEM; Student’s t-test.
This poor response from KRAS mutation-harbouring tumours is likely due to aberrant activation of the RAS–RAF–MEK–ERK signalling pathway, which is downstream to EGFR and independent of EGFR tyrosine kinase activity inhibition.\textsuperscript{11} Although it is the most common mutation in lung adenocarcinoma, KRAS mutation remains an intriguing therapeutic target, as there has been little success in attempts to directly inhibit KRAS activity.\textsuperscript{11}

It has been shown that KRAS mutations are present in up to 30\% of lung adenocarcinomas, but are rarely found in squamous cell lung cancers.\textsuperscript{28} This is in accordance with our current finding that no squamous carcinomas were positive for KRAS mutations. The frequency of KRAS mutation in adenocarcinoma was 8.8\% in the present study, consistent with studies indicating that the prevalence of KRAS mutation in adenocarcinomas is much lower in Asians than in Caucasians.\textsuperscript{29} All KRAS mutations in the present study were found in stage IV metastatic tumours. This is in line with the hypothesis that KRAS mutations play a critical role in NSCLC tumourigenesis and are more common in advanced lung cancers.\textsuperscript{11}

An accumulating pool of evidence suggests that KRAS mutations are associated with poor overall survival,\textsuperscript{30–33} although some studies have reported inconclusive results.\textsuperscript{11,34} Patients with KRAS mutations had a significantly shorter survival time than patients with wild type KRAS in the present study, supporting the notion that KRAS mutation is associated with poor overall survival in NSCLC. Interestingly, we also found that KRAS mutation was associated with low tumour expression of BRCA1, which encodes a tumour suppressor protein involved in repairing damaged DNA.\textsuperscript{35} BRCA1 mutation has been linked to increased risk of breast and ovarian cancers,\textsuperscript{35,36} and its expression level has been identified as a potential biomarker for prognostic purpose in several cancers.\textsuperscript{16,17,37–44}

In support of this hypothesis, we found that low BRCA1 expression was associated with poor survival in our study. It is possible that oncogenic KRAS mutations may lead to decreased BRCA1 expression, which in turn may lead to resistance to platinum-based doublet chemotherapy and poor overall survival.

Mutations in KRAS were associated with low TYMS expression in the present study, and there was a positive correlation between TYMS and BRCA1 expression levels. Others have suggested that TYMS is associated with treatment outcome and survival after fluorouracil (5-FU)/oxaliplatin chemotherapy in metastatic colorectal\textsuperscript{18,19} and gastric cancer,\textsuperscript{20} but there was no association between TYMS expression level and overall survival in the present study. This may reflect the heterogeneity and complexity of NSCLC and the functional differences in DNA repair between TYMS and BRCA1 or other DNA repair enzymes.\textsuperscript{18}

The gene SRC is aberrantly activated and overexpressed in a wide range of human cancers,\textsuperscript{45} including lung cancers.\textsuperscript{21} Aberrant KRAS activation has been shown to increase SRC expression and activity, driving metastatic growth and therapy resistance in pancreatic cancer.\textsuperscript{46} There was no association between KRAS mutations and SRC expression level in the present study.

Our study is limited by the small cohort; the resulting low frequency of KRAS mutations may limit the power of statistical analyses.

In conclusion, KRAS mutations are associated with poor overall survival and resistance to platinum-based doublet chemotherapy in patients with NSCLC. In addition, KRAS mutations are associated with low tumour expression of BRCA1 and TYMS, but not SRC. KRAS mutation status and tumour expression of BRCA1 are potential biomarkers for tailoring chemotherapy and predicting clinical outcome.
Declaration of conflicting interest

The authors declare that there are no conflicts of interest.

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