KRAS mutation and DNA repair and synthesis genes in non-small-cell lung cancer

VIENNA LUDOVINI[1*], BIAGIO RICCIUTI[1*], FRANCESCA R. TOFANETTI[1], CLELIA MENCARONI[1], DIANA GIANNARELLI[2], ANGELO SIDONI[3], MARIA S. REDA[1], ANNAMARIA SIGGILLINO[1], SARA BAGLIVO[1], LUCIO CRINÒ[4], GUIDO BELLEZZA[3], RITA CHIARI[1] and GIULIO METRO[1]

[1]Department of Medical Oncology, Santa Maria della Misericordia Hospital, AOU di Perugia, I-06129 Perugia; [2]Department of Medical Oncology 1, Regina Elena National Cancer Institute, IRCCS, I-00144 Rome; [3]Department of Experimental Medicine, Division of Pathology and Histology, University of Perugia Medical School, I-06129 Perugia; [4]Department of Medical Oncology, Scientific Institute Romagnolo for The Study and Treatment of Tumors, I-47014 Meldola (FC), Italy

Received May 2, 2018; Accepted September 14, 2018

DOI: 10.3892/mco.2018.1731

Abstract. The aim of the present study was to assess the expression of select DNA repair and synthesis genes in non-small-cell lung cancer (NSCLC) according to *KRAS* mutation status. ERCC1, TS, RRM1, and BRCA1 mRNA expression levels were assessed from either primary or metastatic tumor specimens of patients diagnosed with epidermal growth factor receptor (*EGFR*) wild-type (WT) advanced NSCLC. Total RNA was isolated from paraffin-embedded tumor specimens using the RNeasy FFPE kit and automatically purified using a QiaCube instrument. Quantification levels were analyzed by real-time one-step RT-PCR using QuantiFast technology, and the results were compared considering β-actin as the internal reference gene. One hundred and eighty-four patients with advanced NSCLC were evaluated for the analysis, of which 92 were *KRAS*-mutants. Nearly all patients had adenocarcinoma histology (96.7%). Among *KRAS*-mutants, the majority had a *KRAS* codon 12 mutation (88%), the most common being G12C (44.4% of cases). Mean ERCC1 levels were indicated to be significantly higher in *KRAS*-mutants when compared with *KRAS* WT patients (3,234±6.63 vs. 184±1.24; P=0.05). However, mean TS levels were significantly lower in the *KRAS*-mutant subgroup compared with the *KRAS* WT subgroup (4,481±3.756 vs. 5,941±6.4; P=0.039). *KRAS*-mutant NSCLCs are more likely to express high ERCC1 and low TS levels. This finding may suggest different sensitivity to cytotoxic chemotherapy according to *KRAS* mutation status.

Correspondence to: Dr Giulio Metro, Department of Medical Oncology, Santa Maria della Misericordia Hospital, AOU di Perugia, Piazzale Menghini 1, I-06129 Perugia, Italy
E-mail: giulio.metro@yahoo.com

*Contributed equally

Key words: BRCA1, DNA synthesis and repair genes, ERCC1, *KRAS*, non-small-cell lung cancer, RRM1, TS

Introduction

In the last decade, research in the field of cytotoxic chemotherapy for non-small-cell lung cancer (NSCLC) has considerably slowed down, mainly due to the clinical development of targeted therapies for use in patients whose disease harbors driver genetic alterations, as well as immunotherapy for patients without actionable mutations (1). Nevertheless, the majority of newly diagnosed advanced NSCLCs do not harbor actionable genetic mutations, and only 15-20% derive clinical benefit from immunotherapy, which still makes conventional chemotherapy a fundamental treatment option for a number of patients. In this context, the identification of patients who will benefit from cytotoxic treatment would help physicians to deliver effective treatment to sensitive patients, while preventing others from suffering the side effects of inactive drugs. In recent years, several predictive markers of sensitivity to chemotherapy have been investigated with this purpose, including excision repair cross-complementation 1 (*ERCC1*), thymidylate synthase (*TS*), ribonucleotide reductase M1 (*RRM1*), and breast cancer susceptibility 1 (*BRCA1*), which are DNA synthesis and repair genes that can potentially predict sensitivity to platinum agents, pemetrexed, gemcitabine, and taxanes, respectively (2,3).

Kirsten rat sarcoma viral oncogene homolog (*KRAS*) mutation represents the most common genetic alteration in NSCLC, being found in approximately 20-30% of patients (4). Although *KRAS* acts as a driver mutation in NSCLC, it is not yet an actionable target, since clinical trials with targeted therapies aimed at blocking the RAS pathway have invariably led to disappointing results. On the other hand, recent data have unveiled a negative predictive role for *KRAS* mutation with regard to cytotoxic treatment, particularly platinum-based chemotherapy, which still represents the standard of care for a few *KRAS*-mutant advanced NSCLCs (5,6).

Against this background, we investigated the mRNA expression levels of ERCC1, TS, RRM1, and BRCA1 in *KRAS*-mutant advanced NSCLC patients in order to provide a plausible explanation to the clinical observation that has linked *KRAS* mutation to poor sensitivity to cytotoxic chemotherapy.

Materials and methods

Study design and patients. Patients diagnosed with epidermal growth factor receptor (*EGFR*) wild type (WT) advanced NSCLC at the Medical Oncology of Perugia Hospital from January 2006 and November 2016 were eligible for this study. *EGFR* and *KRAS* mutation tests were performed on tumor tissue (either primary or metastic, if both tissues were available metastatic cancer specimen was preferred) following physician's request in patients who were eligible to receive cytotoxic treatment for advanced disease. The mRNA expression levels of ERCC1, TS, RRM1, and BRCA1 were assessed through reverse transcription-polymerase chain reaction (RT-PCR) in patients with available tumor tissue. If there was not enough tissue for the evaluation of all markers, the analysis was sequentially conducted according to the following order: ERCC1, TS, RRM1, and BRCA1. In case further tissue was available, anaplastic lymphoma kinase (*ALK*) gene status was performed.

This retrospective study was approved by local Ethics Committee (*Comitato Etico Aziende Sanitarie Umbria*), waiving patient consent.

Assessment of EGFR and KRAS mutation status. Formalin-fixed paraffin-embedded (FFPE) tumor blocks were reviewed for quality and tumor content. Tumor cells (≥70%) were macrodissected, and genomic DNA was isolated using QIAmp DNA extraction kit and automatically purified by BioRobot EZ1 instrument (Qiagen S.p.A., Milan, Italy) according to the manufacturer's instructions. Nested polymerase chain reactions (PCRs) were carried out using primers to amplify exons 18 to 21 of *EGFR* and exons 2 to 3 of *KRAS*. To facilitate sequencing, internal primers incorporated an M13Tag. PCR products were purified with Exonuclease 1 and Shrimp Alkaline Phosphatase (ExoSAP-IT) a 37˚C for 15 min followed by heating at 80˚C for 15 min to stop the enzymatic reaction. After purification, the PCR products were sequenced with forward and reverse M13 primers and Big Dye Terminator v1.1 Cycle Sequencing Kit. Sequencing fragments were detected by capillary electrophoresis using 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Electropherograms were analyzed for the presence of mutations using SeqScape v2.7 Software. In all cases, samples harboring mutations were reamplified and resequenced using the same experimental conditions.

Assessment of the mRNA expression levels of ERCC1, TS, RRM1 and BRCA1. RNA was extracted and purified from five consecutive 8-μM slides of microdissected FFPE tumor tissue, using RNeasy FFPE Kit on QIAcube instrument (Qiagen, Milan, Italy). Expression levels of ERCC1, TS, RRM1, and BRCA1 were evaluated on 100 ng RNA of each tumor sample and compared to synthetic healthy lung RNA, using QuantiFast Probe Duplex Assays and QuantiFast Probe RT-PCR Plus Kit (Qiagen, Milan, Italy). This kit offers an integrated genomic DNA removal step to avoid false-positive signals. Reverse transcription PCR and RT-PCR took place in the same tube (One Step RT-PCR). QuantiFast Probe Assays were predesigned to enable amplification and detection of specific gene targets (ERCC1 cat. no. QF00270641; TS cat. no. QF00102375; RRM1 cat. no. QF00452382; BRCA1 cat. no. QF0043126 by Qiagen). These assays were based on dual-labeled hydrolysis probe detection using two different dyes, FAM binds the specific gene of interest and MAX binds selected reference gene (duplex PCR). The reaction probe mix was aliquoted into specific PCR tubes for Rotor-Gene Q Instrument and the cDNA samples were then added. Cycling conditions for one-step RT-PCR included: denaturation at 95˚C for 20 min and the PCR conditions included an initial denaturation at 95˚C for 5 min followed by 45 cycles of denaturation at 95˚C for 15 sec and annealing at 60˚C for 30 sec.

All runs included a calibrator sample and a one no-template control, and all samples were measured in triplicate. Comparative Cq method was used for gene expression quantification using β-actin as internal reference gene and commercial RNA control (mRNA from lung, Stratagene, La Jolla, CA, USA) as calibrator. Final expression values were determined as follows: $2^{-(\Delta Ct\ sample-\Delta Ct\ calibrator)}$, where ΔCt values of the sample and the calibrator are estimated by subtracting the Cq value of the target gene from the median of the reference genes values as reported by Livak and Schmittgen (7).

Assessment of ALK gene rearrangement. *ALK* immunohistochemistry based on a 4-tiered score (clone D5-F3, cat. no. 3633, Cell Signaling Technology, dilution 1:250, incubation time: 30 min/room temperature) according to a laboratory developed test was used as screening method for the assessment of *ALK* status. For each stain positive controls, represented by inflammatory myofibroblastic tumour, were used. Based on the mutual exclusiveness of driver mutations, only patients who were *KRAS* WT were tested for *ALK*. In case of any *ALK* IHC positivity, specimens underwent confirmatory FISH (Break Apart Vysis Probe kit). *ALK* FISH-positive patients were excluded from the present analysis.

Statistical analysis. Statistical analysis was conducted using the SPSS statistical software package (version 24; SPSS, Inc., Chicago, IL, USA). Chi-square tests or Fisher exact tests were used to analyze correlations between *EGFR* mutation or *ALK* rearrangement status and clinico-pathologic variables. Data related to mRNA expression levels of ERCC1, TS, RRM1 and BRCA1 were presented as mean and standard deviation. Student's t test was used for comparison of two groups. All statistical tests were conducted at a 2-sided level of significance of $P<0.05$.

Results

Patients characteristics. From January 2006 and November 2016 184 *EGFR* WT advanced NSCLC patients were evaluable for the analysis of at least one DNA repair gene, of which 92 were *KRAS*-mutants. Table I lists patients characteristics. Overall, the median age was 62 years, 60.3% of patients were male, and 89.7% of patients were current/former smokers. Virtually all patients had adenocarcinoma histology. Among *KRAS*-mutants, the majority had a *KRAS* codon 12 mutation (88%), the most common

Table I. Patients characteristics.

Variable	All patients	*KRAS*-mutant	*KRAS* wild type	P-value
Number of patients, n	184	92	92	-
Median age, year (range)	62 (23-85)	63 (42-82)	62 (23-85)	0.589
Sex, n (%)				
Male	111 (60.3)	63 (68.5)	48 (52.2)	**0.023**
Female	73 (39.7)	29 (31.5)	44 (47.8)	
Performace status, n (%)				
0-1	174 (94.5)	88 (95.7)	86 (93.5)	0.474
≥2	10 (5.5)	4 (4.3)	6 (6.5)	
Stage IV				
De novo	129 (70.1)	65 (70.7)	64 (69.6)	0.871
Recurrent	55 (29.9)	27(29.3)	28 (30.4)	
Smoking history, n (%)				
Never[a]	19 (10.3)	4 (4.3)	15 (16.3)	**0.003**
Current/former	165 (89.7)	88 (95.7)	77 (83.7)	
Histology, n (%)				
Adenocarcinoma	178 (96.7)	91 (98.9)	87 (94.6)	0.090
Squamous cell carcinoma	6 (3.3)	1 (1.1)	5 (5.4)	
ALK gene status				
Negative	57 (31.0)	-	57 (62.0)	-
Not assessed	127 (69.0)	92 (100.0)[b]	35 (38.0)[c]	
KRAS mutations, n (%)				
Codon 12		**81 (88.0)**		
G12C		36 (44.5)		
G12V		18 (22.2)		
G12D		10 (12.3)		
G12A		8 (9.9)		
G12F	-	6 (7.4)	-	-
G12R		3 (3.7)		
Codon 13		**6 (6.6)**		
G13C		3 (50.0)		
G13D		3 (50.0)		
Codon 59		**1 (1.1)**		
Codon 61		**4 (4.3)**		

[a]<100 cigarettes in a lifetime. [b]Not assessed because of mutual exclusiveness of driver mutations. [c]Not assessed due to unavailable tissues for *ALK* gene assessment. Statistically significant P-values are provided in bold. *KRAS*, Kirsten rat sarcoma viral oncogene homolog; *ALK*, anaplastic lymphoma kinase.

being G12C (44.4% of cases). When *KRAS*-mutant patients were compared with those who were *KRAS* WT, significantly more individuals in the *KRAS*-mutant group were current/former smokers (P=0.003) and males (P=0.023) (Table I). No other statistically significant differences were observed.

ERCC1, TS, RRM1 and BRCA1. Overall, TS and RRM1 expression levels were significantly lower in patients with non-squamous NSCLC as compared with squamous patients (P=0.032 and P=0.017, respectively) (Table II). Similarly, never smokers had significantly lower levels of TS expression vs. smokers (P=0.021). No significant differences were noted for any characteristics according to the type of *KRAS* mutation (data not shown). Likewise, no significant differences were noted with regard to the distribution of ERCC1, TS, RRM1, and BRCA1 in relation to the type of *KRAS* mutation (G12C vs. other) (Table III).

Table IV lists the mRNA expression levels of ERCC1, TS, RRM1, and BRCA1 in all patients and according to *KRAS* mutation status. ERCC1 levels ranged from 0.07 to 55.02, with a mean of 2.54 and median of 1.55; as for TS, the levels ranged from 0.04 to 35.0, with a mean of 5.21 and median of 3.78. RRM1 levels ranged from 0.001 to 93.9, with a mean of 12.82 and a median of 6.86; BRCA1 levels ranged from 0.01 to 64.3, with a mean of 11.11 and median of 7.62. When analyzed according to *KRAS* mutation status, *KRAS*-mutant

Table II. Patients characteristics and expression levels of ERCC1, TS, RRM1, and BRCA1.

Variable	ERCC1 (mean)	TS (mean)	RRM1 (mean)	BRCA1 (mean)
Sex, n (%)				
Male	2.41	5.42	12.15	11.07
Female	2.73	4.9	13.82	11.17
P-value	NS	NS	NS	NS
Performace status, n (%)				
0-1	2.59	5.31	12.72	11.07
≥2	1.16	2.6	13.73	10.3
P-value	NS	NS	NS	NS
Stage IV				
De novo	2.12	4.88	11.85	11.16
Recurrent	3.37	5.87	14.78	10.99
P-value	NS	NS	NS	NS
Smoking history, n (%)				
Never	2.66	5.4	13.02	11.55
Current/former	1.36	2.88	10.75	7.14
P-value	NS	**P=0.021**	NS	NS
Histology, n (%)				
Adenocarcinoma	2.53	5.07	12.39	10.87
Squamous cell carcinoma	2.68	9.28	24.98	17.65
P-value	NS	**P=0.032**[a]	**P=0.017**[a]	NS

Statistically significant P-values are provided in bold. [a]Although statistically significant, this comparison should be interpreted with caution due to the low number of squamous cell carcinoma. ERCC1, excision repair cross-complementation 1; TS, thymidylate synthase; RRM1, ribonucleotide reductase M1; BRCA1, breast cancer susceptibility 1; n, number; NS, not significant.

patients had significantly higher mean levels of ERCC1 as compared with *KRAS* WT patients (3.23±6.63 vs. 1.84±1.24; P=0.052, Table IV, Fig. 1A). On the other hand, mean expression levels of TS were significantly lower in patients who were *KRAS*-mutant as compared with *KRAS* WT patients (4.48±3.75 vs. 5.94±6.4; P=0.039, Table IV, Fig. 1B). No statistically significant differences were noted for the median expression levels of ERCC1 and TS according to *KRAS* mutation status (Table IV). Similarly, no significant differences were observed neither for mean nor for median levels of RRM1 and BRCA1 according to *KRAS* mutation status.

Discussion

The aim of this study was to associate the mRNA expression levels of ERCC1, TS, RRM1 and BRCA1 with *KRAS* mutation status in patients with advanced NSCLC. In light of the mutual exclusiveness existing between *EGFR* mutation and *KRAS* mutation, and the evidence that *EGFR*-mutant patients belong to a biologically distinct subset of patients, we considered it was important to exclude from the analysis *EGFR*-mutant patients, thus focusing only on individuals whose tumor had a documented *EGFR* WT status.

Interestingly, we showed for both ERCC1 and TS a similar range of expression levels and median values as compared with a previous report from Maus *et al*, which analyzed the distribution of ERCC1, TS, and RRM1 in >2,000 NSCLC specimens (8). Likewise, similarly to the Maus *et al* study, we found that these markers were expressed at a lower level in adenocarcinoma histology as compared with squamous cell carcinoma, which was statistically significant only for TS and RRM1 in our study (Table II). However, it should be noted that the present study was not powered for addressing differences according to histology owing to the small number of squamous cell carcinomas that were included. Therefore, these results should be interpreted very cautiously.

Importantly, we observed that *KRAS*-mutant patients had significantly higher mean ERCC1 expression levels as compared with *KRAS* WT patients (P=0.052, Table IV, Fig. 1A). Accordingly, our group and others have previously reported that *KRAS*-mutant advanced NSCLCs perform poorly on platinum-based chemotherapy (9,10). This finding has been further corroborated by two metanalyses, in which *KRAS*-mutant advanced NSCLCs treated with platinum-based chemotherapy appeared to experience significantly lower response rates and progression-free survival as compared with the *KRAS* WT counterpart (5,6). Therefore, the higher mean levels of ERCC1 that have been found in *KRAS*-mutant patients provide a molecularly plausible explanation for the poor sensitivity to platinum-based chemotherapy observed in patients whose tumor harbors a *KRAS* mutation.

On the other hand, we reported significantly lower mean levels of TS expression in *KRAS*-mutant patients as compared with *KRAS* WT patients (P=0.039, Table IV,

Table III. Median and mean expression levels of TS, ERCC1, RRM1, and BRCA1 according to *KRAS* codon 12 mutations.

Variable	All patients	G12C	Other codon 12 mutation	P-value
ERCC				
N	81	36	45	-
Mean ± SD	3.37±6.93	4.98±10	2.08±2.03	0.062
Median (range)	1.69 (0.43-55.02)	1.91 (0.43-11.4)	1.38 (0.53-55.02)	0.074
TS				
N	79	35	44	-
Mean ± SD	4.25±3.38	3.92±3.05	4.52±3.45	0.423
Median (range)	1.73 (0.04-14.7)	3.03 (0.04-11.8)	3.48 (0.25-14.7)	0.415
RRM1				
N	77	34	43	-
Mean ± SD	12.87±14.83	14.64±19.2	11.47±8.99	0.343
Median (range)	8.84 (1.04-93.98)	8.89 (1.04-93.98)	8.02 (1.35-38.29)	0.516
BRCA1				
N	72	33	39	-
Mean ± SD	9.54±8.45	9.74±8.87	9.9±8.27	0.863
Median (range)	6.66 (0.01-35.21)	5.73 (0.01-32.18)	7.05 (0.45-35.21)	0.921

ERCC1, excision repair cross-complementation 1; TS, thymidylate synthase; RRM1, ribonucleotide reductase M1; BRCA1, breast cancer susceptibility 1; SD, standard deviation; N, number.

Table IV. Median and mean expression levels of TS, ERCC1, RRM1, and BRCA1.

Variable	All patients	*KRAS*-mutant	*KRAS* WT	P-value
ERCC1				
N	184	92	92	-
Mean ± SD	2.54±4.82	3.23±6.63	1.84±1.24	**0.052**
95% CI	1.83-3.24	1.86-4.6	1.58-2.1	
Median (range)	1.55 (0.07-55.02)	1.61 (0.1-55.02)	1.54 (0.07-7.12)	0.623
TS				
N	180	89	91	-
Mean ± SD	5.21±4.75	4.48±3.75	5.94±6.4	**0.039**
95% CI	4.51-5.91	3.79-5.35	4.87-7.51	
Median (range)	3.78 (0.04-35.0)	3.34 (0.05-17.6)	3.91 (0.04-35.3)	0.133
RRM1				
N	176	87	89	-
Mean ± SD	12.82±12.05	12.82±14.02	12.82±9.82	0.991
95% CI	11.03-14.61	9.83-15.81	10.75-14.89	
Median (range)	6.86 (0.001-93.9)	8.84 (1.04-93.9)	9.1 (0.001-56.8)	0.332
BRCA1				
N	171	82	89	-
Mean ± SD	11.11±10.03	10.16±8.98	11.99±10.88	0.234
95% CI	9.6-12.63	8.18-12.13	9.7-14.29	
Median (range)	7.62 (0.01-64.3)	7.58 (0.01-44.5)	7.99 (0.1-64.3)	0.223

Statistically significant P-values are provided in bold. ERCC1, excision repair cross-complementation 1; TS, thymidylate synthase; RRM1, ribonucleotide reductase M1; BRCA1, breast cancer susceptibility 1; SD, standard deviation; CI, confidence interval; N, number.

Fig. 1B). This finding might imply an increased sensitivity to pemetrexed-based chemotherapy in *KRAS*-mutant advanced NSCLCs, which would be of clinical relevance owing to the fact that *KRAS* mutation are mainly found

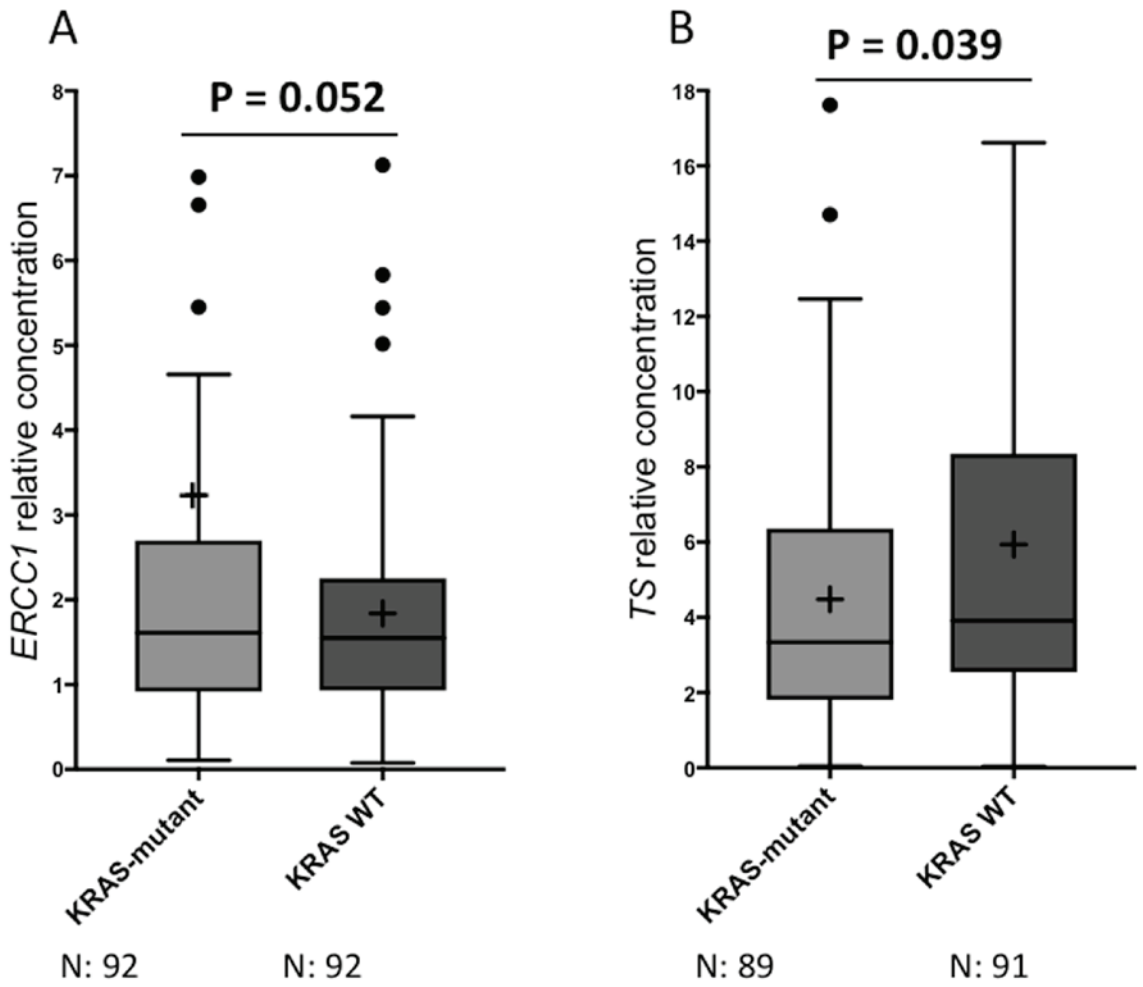

Figure 1. (A) Box-plot graph of the distribution of ERCC1 expression levels. A significant difference in the mean (+) ERCC1 level was observed between *KRAS*-mutant patients and those who were *KRAS* WT (two-sided t test P-values are provided). (B) Box-plot graph of the distribution of TS expression levels. A significant difference in the mean (+) TS level was observed between *KRAS*-mutant patients and those who were *KRAS* WT (two-sided t test P-values are provided). WT<wild-type.

in adenocarcinoma patients, and pemetrexed is approved for clinical use in this histological subset only (4,11). Previously, Moran and colleagues have already reported that *KRAS*-mutant cell line depend on enhanced folate metabolism in functional experiments (12). Accordingly, a small retrospective analysis suggested that *KRAS* mutation might predict sensitivity to pemetrexed (13). Moreover, several clinical studies have already reported that TS levels may represent a predictive biomarker for antifolate agents in NSCLC (2). Against this well-established background, we found that patients with *KRAS*-mutant NSCLC had lower levels of TS, which might explain the enhanced sensitivity to pemetrexed reported in literature. Although preliminary, these findings suggest that the *KRAS*-mutant subset of patients, for whom no targeted therapies are available, may exhibit a particular sensitivity to pemetrexed, which could represent the basis for the design of future clinical studies aimed to further address this issue.

Importantly, *KRAS*-mutant NSCLC is a heterogeneous disease, as many type of different type amino-acid substitutions result into several types of *KRAS* gene mutations. On this basis, in order to exclude an imbalance in terms of expression levels of either ERCC1 and TS, we performed an analysis in *KRAS*-mutant codon 12 patients according to the type of *KRAS* mutation (other *KRAS* codon 12 mutations excluded due to the low number). However, we were not able to identify any differential mRNA expression levels for any markers (Table III). Therefore, we conclude that ERCC1 and TS expression levels were homogeneously distributed in *KRAS*-mutant codon 12 patients, regardless of the *KRAS* mutation variant.

Of note, our findings further enlarge the evidence indicating that each molecularly defined subgroup of NSCLC is associated with a different of expression of DNA synthesis and repair genes. In fact, some authors have previously reported that *EGFR*-mutant NSCLCs express lower ERCC1 expression levels as compared with *EGFR* WT patients, which might account for the increased sensitivity to platinum-based chemotherapy in *EGFR*-mutant NSCLCs (14-16). Likewise, lower *TS* expression levels were observed in *ALK*-positive NSCLCs, which results into greater benefit from pemetrexed-based chemotherapy in *ALK*-positive patients (17-19). Therefore, we provide evidence that, despite being a heterogeneous disease, also *KRAS*-mutant NSCLC is associated with a peculiar pattern of expression of DNA synthesis and repair genes, mainly ERCC1 and TS, which, in turn, could account for a different sensitivity to platinum agents and pemetrexed, respectively.

Certainly, our study is affected by some limitations, including the retrospective design, the relatively small sample size of patients, the lack of clinical outcome information, and the absence of confirmation by additional functional experiments. In addition, *ALK* status was assessed only in 62% of *KRAS* WT patients. However, as *ALK* rearrangements have been associated with low levels of TS, it is very unlikely that this could have affected the results of this study in terms of TS levels (17). On the other hand, it could be argued that immunohistochemistry (IHC) rather than RT-PCR would provide a direct measure of the acting element, namely the protein. However, there are several arguments against using an IHC-based technique, including the need for highly specific antibodies, the lack of standardized tissue fixation and staining protocols, difficulty in finding a consensual standard for microscopic evaluation, and universal cutoff value (20). For the same reason, some researchers have previously attempted to develop a more reliable and reproducible IHC method based on a fully automated and quantitative immunofluorescence technique (AQUA) (21).

In conclusion, to the best of our knowledge, this is the first study that has evaluated the expression of DNA synthesis and repair genes according to *KRAS* mutation status in advanced NSCLC patients, which suggests significantly higher mRNA expression levels for ERCC1 and lower for TS in *KRAS*-mutant patients, but no difference for either RRM1 and BRCA1. As for ERCC1, these results provide a rationale behind the poor sensitivity to platinum-based chemotherapy of *KRAS*-mutant advanced NSCLCs. On the other hand, whether lower TS expression levels translate into enhanced clinical efficacy of pemetrexed-based chemotherapy in *KRAS*-mutant patients remains to be determined in prospective clinical trials.

Acknowledgements

Not applicable.

Funding

The study was supported by Associazione Italiana per la Ricerca contro il Cancro (AIRC) (grant no. 15713 AIRC 2014).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

VL, LC and GM designed the research. FRT, CM, ASid, ASig, MSR, RC, SB and GB performed the experiments. BR, DG and GM analyzed the data. BR and GM wrote the paper. VL and GM critically revised the manuscript for important intellectual content.

Ethics approval and consent to participate

Comitato Etico Aziende Sanitarie (CEAS) Umbria approved the study (approval no. 4796/15/AV).

Patient consent for publication

CEAS Umbria approval waived patient consent.

Competing interests

The authors declare that they have no competing interests.

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