Abstract: Background: Several studies suggest that patients with KRAS-mutant NSCLC fail to benefit from standard systemic therapies and do not respond to EGFR inhibitors. Most recently, KRAS 12c data suggest specific treatment for improving ORR and OS. There is a clear need for therapies specifically developed for these patients. Moreover, data that might be suggestive of a response to specific therapies, such as BRCA1, are needed, and two mutations that were studied in other malignancies show more response to PARP inhibitors. Molecular profiling has the potential to identify other potential targets that may provide better treatment and novel targeted therapy for KRAS-mutated NSCLC.

Methods: We purified RNA from archived tissues of patients with stage I and II NSCLC with wild-type (wt) and mutant (mt) KRAS tumors; paired normal tissue adjacent to the tumor from 20 and 17 patients, respectively, and assessed, using real-time reverse transcriptase–polymerase chain reaction (RT-PCR), the expression of four genes involved in DNA synthesis and repair, including thymidylate synthase (TS), BRCA1, ECCR1, RAP80, and the proto-oncogene SRC. Additionally, we assessed the expression of PD-L1 in mt KRAS tumors with immunohistochemistry using an antibody against PD-L1.

Results: Our results show that in mt KRAS tumors, the level of expression of ERCC1, TS, and SRC was significantly increased in comparison to paired normal lung tissue (p ≤ 0.04). The expression of BRCA1 and RAP80 was similar in both mt KRAS tumors and paired normal tissue. Furthermore, the expression of BRCA1, TS, and SRC was significantly increased in wt KRAS tumors relative to their expression in the normal lung tissue (p < 0.044). The expression of ERCC1 and RAP80 was similar in wt KRAS tumors and paired normal tissue. Interestingly, SRC expression in mt KRAS tumors was decreased in comparison to wt KRAS tumors. Notably, there was an expression of PD-L1 in the tumor and stromal cells in a few (5 out of 20) mt KRAS tumors. Our results suggest that a greater ERCC1 expression in mt KRAS tumors might increase platinum resistance in this group of patients, whereas the greater expression of BRCA1 in wt KRAS tumor might be suggestive of the sensitivity of taxanes. Our data also suggest that the combination of an SRC inhibitor with a TS inhibitor, such as pemetrexed, might improve the outcome of patients with NSCLC and in particular, patients with wt KRAS tumors. PD-L1 expression in tumors, and especially stromal cells, suggests a better outcome. Conclusion: mt KRAS NSCLC patients might benefit from a treatment strategy that targets KRAS in combination with therapeutic agents based on pharmacogenomic markers, such as SRC and BRCA1. mt KRAS tumors are likely to be platinum-, taxane-, and pemetrexed-resistant, as well as having a low level of PD-L1 expression; thus, they are less likely to receive single-agent immunotherapy, such as pembrolizumab, as the first-line therapy. wt KRAS tumors with BRCA1 positivity tend to be sensitive to taxane therapy and, potentially, platinum. Our results suggest the
need to develop targeted therapies for KRAS-mutant NSCLC or combine the targeting of oncogenic KRAS in addition to other therapeutic agents specific to the molecular profile of the tumor.

Keywords: systemic therapy; mutations; molecular profiling

1. Introduction

The cloning and studies of the normal human genes homologs of the rat Harvey-(HRAS) reveal that the mutation in oncogenic alleles is a result of a single-point mutation in KRAS and neuroblastoma-RAS (NRAS). This discovery laid the foundations for the study by Santos et al. who first reported mt KRAS in lung cancer, which was later confirmed [1–3]. While the link between lung cancer and KRAS mutation (mt KRAS) is a widely discussed topic in research, attempts to develop targeted therapy are reported to be elusive [4,5]. The failure to yield positive results may be, in part, because the KRAS gene belongs to the large RAS gene family, which was originally discovered during the study of retroviruses causing cancer in animals in the early 1960s [6] and encodes a family of membrane-bound, 21-Kd, GTP-binding proteins involved in the regulation of key processes, such as cell growth, differentiation, and apoptosis. This activity is achieved through interacting with multiple effectors, including those in the MAPK, STAT, and PI3K signaling cascades [7,8]. After the single-point mutation, KRAS protein produces defective activity from the GTPase; targeting KRAS by itself is challenging; therefore, targeting its effector pathways may be a better option.

Subsequently, when targeted therapy was investigated in a specific subset of KRAS mutations, promising results were achieved. Recently, Govindan et al. reported an ORR of 54% (all partial responses), with 46% of patients achieving stable disease, and a DCR of 100% when targeting KRAS G12C (AMG 510) [9].

The Kirsten rat sarcoma viral oncogene homolog (KRAS) is the most common mutated oncogene in non-small cell lung carcinoma. In Western countries, the KRAS mutations are found in approximately 25% of NSCLCs [10] and are most frequently observed in exon 2 (codons 12 and 13) and, less commonly, in exon 13 (codon 61) [11]. KRAS mutations are typically clonal, occur in early carcinogenesis, and are mutually exclusive with other mutations in lung cancer such as EGFR and ALK [12]. The most frequent KRAS amino acid mutations in NSCLC are guanine to thymine or guanine to cytosine. The glycine 12 to cysteine (G12C) accounts for 41% of patients, followed by glycine 12 to valine (G12V); both mutations are associated with a tobacco-smoking history. Whereas mutations involving guanine to adenine nucleotide changes, such as glycine 12 to aspartic acid (G12D), are more common in non-smokers [11]. It has also been noted that about 50% of mt KRAS has co-mutations in NSCLC. The most frequent non-oncogenic co-mutations are TP53 (40%), serine/threonine kinase 11 (STK11) (20%), and Kelch-like ECH-associated protein 1 (KEAP1) (13%) [13]. The understanding of the ultrastructure of oncogenic mutations in KRAS pathways led to the devolvement of allele-specific inhibitors for mt KRAS in NSCLC [10].

BRCA1 and BRCA2 are tumor suppressor genes involved in cell cycle regulation, including replication, mitotic spindle assembly, and apoptosis. Their regulatory role also affects transcription and DNA damage response (DDR) [14,15]. BRCA-mutated individuals have an impaired ability to repair DNA. Thus carriers of mutated BRCA are more sensitive to therapies that induce DNA damage, such as platinum-based chemotherapy and radiotherapy [16]. Poly (ADP-ribose) polymerase (PARP) inhibitors (PARPi) are the first approved agents to target DNA repair in cancer. PARP inhibitors changed therapeutic strategies by targeting DNA repair in various cancers, including breast, pancreatic, ovarian, and prostate cancers [17–19]. BRCA1 and BRCA2 mutations occur in 5–10% of NSCLC. These mutations lead to the inactivation of homologous recombination (HR)-mediated DNA repair [20]. Normal lung tissue does not harbor germline BRCA1/2 mutations; how-
2. Materials and Methods

2.1. Study Population

Patients with known or suspected lung cancer that were enrolled in the Thoracic Tumor Registry at the University of Cincinnati were studied. The Institutional Review Board (IRB) approved the protocol, and informed consent was obtained from all patients. Tumors, normal lung tissue, clinical and outcome data were prospectively collected. All patients with stage I and II non-small cell lung cancer (adenocarcinomas) who underwent curative resection by a dedicated thoracic surgical oncologist between 1 March 2006 and 30 April 2012 were included. Patients who received neoadjuvant chemotherapy or radiation were excluded. A portion of resected tumor specimens along with normal lung tissue. The results of their study show that patients who received platinum-based adjuvant chemotherapy in combination with paclitaxel or gemcitabine had shorter disease-free intervals and overall survival rates than patients with a high expression of TS in NSCLC [24].

Thymidylate synthase (TS) is the rate-limiting enzyme in DNA synthesis. TS expression is more pronounced in metabolically active sites such as the nucleus and mitochondria. The inhibition of TS activity within cancer cells makes TS a target for anticancer therapies [23]. Studies have shown conflicting data on TS expression and prognosis. A study by Kulda et al. revealed that TS is highly expressed in NSCLC tumor tissue in comparison to normal lung tissue. The results of their study show that patients who received platinum-based adjuvant chemotherapy in combination with paclitaxel or gemcitabine had shorter disease-free intervals and overall survival rates than patients with a high expression of TS in NSCLC [24].

Figure 1. The study protocol for patient selection, tissue extraction for molecular analysis, and PD-L1 expression.
2.2. DNA Extraction from Paraffin Tissue Blocks

Serial 10 µm sections were cut from formalin-fixed, paraffin-embedded lung tissue (tumor or normal lung tissue) onto a glass slide. A simultaneous section was hematoxylin-eosin (H&E)-stained to determine tumor and normal tissue. The tumor was micro-dissected into an Eppendorf tube, deparaffinized in xylene, and further washed with 100% ethanol. The supernatant was removed after centrifugation, and the residual tissue (tumor or normal) was incubated in tissue lysis buffer with 20 µg of Proteinase K (Qiagen, Germantown, MD, USA). DNA was extracted using a Qiagen Purification kit as per the manufacturer’s protocol. The concentration of the DNA was determined using a Nanodrop spectrophotometer ND-1000.

2.3. KRAS Mutation at Codons 12 and 13

PCR was performed on 90 µL containing 6 µL DNA, 20 µM forward and reverse primer mixed with True Allele Premix (Applied Biosystems) at an annealing temperature of 55 °C. The primers set for codon 12 and 13 of the KRAS gene were (forward) 5′ GGT ACT GGT GGA GTA TTT GAT AGT G 3′ and (Reverse) 5′ AAA GAA TGG TCC TGC ACC 3′. The PCR product was separated on 2% agarose gel to confirm the product size. The PCR product was further purified using ExoSAP-IT (USB Corporation, Cleveland, OH, USA) to detect KRAS mutation using an automatic DNA sequencer (Applied Biosystems, Foster City, CA, USA).

2.4. Immunohistochemistry

The formalin-fixed, paraffin-embedded tissues were sectioned to 4 µm and deparaffinized. Antigen retrieval was carried out using a 10 mM citrate buffer maintained at pH 6. The sections were then incubated in 0.5% of hydrogen peroxide in methanol for 15 min to block endogenous peroxidase activity. The sections were incubated with a PD-L1 rabbit monoclonal antibody (Cell Signaling, Danvers, MA, USA) at 4 °C overnight. The slides were washed in 1X PBS and incubated at room temperature for 1 h with 1:200 dilution of biotinylated anti-rabbit secondary antibody (Vector, Burlingame, CA, USA). The slides were washed in 1X PBS and incubated in ABC (Vector Laboratories, Burlingame, CA, USA) for 30 min. The sections were then developed for color using 3-3′-diaminobenzidine (Vector Laboratories, Burlingame, CA, USA).

2.5. To Evaluate the Level of PD-L1 Expression in the Stroma, the Percentage of Positively

Stained stromal cells were identified, and the staining intensity was graded on a scale of 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (intense staining).

For each sample, the staining percentage and staining intensity scores were multiplied together to give the stromal staining index.

2.6. RNA Extraction from Tissue Blocks

An H&E stain from each specimen was obtained and reviewed by a pathologist to accurately select blocks with a high percentage of tumor cells and normal tissue. Only tumor samples containing more than 60% of tumor tissue using the H&E-stained section were analyzed. Three to five adjacent unstained slides of 10 µm were obtained from the corresponding block for the extraction of total RNA. Total RNA was extracted from FFPE tissue sections with a Qiagen RNeasy FFPE Kit and from OCT tissues sections with an Agilent Absolutely RNA FFPE Kit, following the manufacturers’ protocols. The concentration of RNA was assessed.

2.7. Quantitative RT-PCR Analysis

Complementary DNA (cDNA) was synthesized using oligo dT and random hexamer primers. Aliquots of cDNA were used for quantitative real-time PCR in triplicates to assess the expression of thymidylate synthase (TS), BRCA1, ECCR1, RAP80, and the proto-oncogene SRC (Figure 2). The relative expression levels of the studied genes were
normalized to the expression of the housekeeping gene b-actin. The data are expressed as a relative unit and as fold change compared to normal tissue.

| Gene   | primer | Sequence                  |
|--------|--------|---------------------------|
| BRCA1  | Forward| GGCTATCCTCTCAGAGTGACATTCTTA |
|        | Reverse| GCTTTATCAGGGTTATGTGTCATGGT |
|        | Probe  | CCACTCAGAGAGGG            |
| ERCC1  | Forward| TCAGACCTCTCTGACCAGATT     |
|        | Reverse| GGGGATAAGGCGAGATCTTCTC    |
|        | Probe  | TCTCTGAAACAGCTCATGCGCCA   |
| RAP80  | Forward| ACATCAAGTCTCACCAAGACAGGAC |
|        | Reverse| TGCGACCTGCTTCTCTCAT       |
|        | Probe  | TCAGGCTGCTTTACCA          |
| TS     | Forward| CCTCGTGCGAGAAGTGAG        |
|        | Reverse| TGGCGTTGCTGAAGTCA         |
|        | Probe  | CACGAGAAGGTGCTACTGCGCTCTC |
| SRC1   | Forward| TGGGATCCAAAGAGATCTTTCCTAG |
|        | Reverse| AGTCGACTTGTCCACCACAGAAGAT |
|        | Probe  | CGCTGCGCTTACATGCGCCCTC   |
| Beta-actin | Forward | CGAGGGCGGCTACGCTTT |
|        | Reverse| TCCTTAATGTACCCAGAGATT    |
|        | Probe  | ACCACACGCGGCGGGGG        |

Figure 2. Primers and probes used in quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR).

2.8. Statistical Analysis

The Student’s t-test was used to determine statistical significance. \( p \leq 0.05 \) was considered statistically significant.

3. Results

In \( mt \) KRAS-positive tumors, the levels of expression of ERCC1, TS, and SRC were significantly increased in comparison to paired normal lung tissue \( (p < 0.04) \). Meanwhile, BRCA1 and RAP80 expressions showed no significant differences in both \( mt \) KRAS tumors and the paired normal tissue. Furthermore, the expression of the BRCA1, TS, and SRC genes was significantly increased in \( wt \) KRAS tumors relative to their expression in the normal lung tissue \( (p < 0.044) \). Expressions of ERCC1 and RAP80 were similar in \( wt \) KRAS tumors and with paired normal tissue. Interestingly, SRC expression was reduced in \( mt \) KRAS tumors compared to \( wt \) KRAS tumors (Table 1, Figures 3 and 4).

Table 1. The table illustrates the mutations found in \( wt \) KRAS, \( mt \) KRAS, and normal lung tissue.

| Oncogene.     | ERCC1 | BRCA1 | TS | SRC | PD-L1 |
|---------------|-------|-------|----|-----|-------|
| \( mt \) KRAS | +     | −     | +  | −   | 25%   |
| \( wt \) KRAS | −     | +     | +  | +   |       |
| Normal lung tissue | − | − | − | − | − |
2.8. Statistical Analysis

The Student's t-test was used to determine statistical significance. \( p \leq 0.05 \) was considered statistically significant.

3. Results

In \textit{mt} KRAS-positive tumors, the levels of expression of ERCC1, TS, and SRC were significantly increased in comparison to paired normal lung tissue (\( p < 0.04 \)). Meanwhile, BRCA1 and RAP80 expression showed no significant differences in both \textit{mt} KRAS tumors and the paired normal tissue.

Furthermore, the expression of the BRCA1, TS, and SRC genes was significantly increased in \textit{wt} KRAS tumors relative to their expression in the normal lung tissue (\( p < 0.044 \)). Expressions of ERCC1 and RAP80 were similar in \textit{wt} KRAS tumors and with paired normal tissue. Interestingly, SRC expression was reduced in \textit{mt} KRAS tumors compared to \textit{wt} KRAS tumors (Table 1, Figures 3 and 4).

Table 1. The table illustrates the mutations found in \textit{wt} KRAS, \textit{mt} KRAS, and normal lung tissue.

| Gene        | wt KRAS | mt KRAS |
|-------------|---------|---------|
| ERCC1       | +       | +       |
| BRCA1       | +       | +       |
| TS          | +       | +       |
| SRC         | -       | -       |
| PD-L1       | 25%     | 25%     |

Figure 3. The expression of SRC, BRCA1, RAP80, ERCC1, and TS in normal tissue and tumor tissue with \textit{mt} KRAS and \textit{wt} KRAS (fold change to normal tissue).

Figure 4. The expression of SRC, BRCA1, RAP80, ERCC1, and TS in normal tissue and tumor tissue with \textit{mt} KRAS and \textit{wt} KRAS (relative unit).
PD-L1 Expression Analysis

Positive PD-L1 protein expression was observed in five patients (25%) in tumor-infiltrating lymphocytes/immune cells. Tumor cells display a cytoplasmic staining pattern. The programmed death ligand inhibitor protein expression is classified into four categories.

1. Negative (−) with staining of less than 1% of tumor cells (Figure 5).
2. Weak positive (+) with staining of 2–10% of tumor cells
3. Moderate positive (++) with staining of 11–50% of tumor cells (Figure 6).
4. Strong positive (+++) with diffused staining in more than 60% of tumor cells (Figure 7).

Figure 5. Immunohistochemistry demonstrates a negative staining pattern for PD-L1 expression (400×).

Figure 6. Immunohistochemistry demonstrates a focal (weak) staining pattern for PD-L1 expression (400×).
4. Discussion

Despite major advances in cancer treatment, platinum-based therapy has remained the cornerstone in the treatment of advanced NSCLC [25–29]. However, *mt KRAS* lung cancer was found to be particularly resistant to such therapies, hence the need to develop a wider molecular profile and tailored therapies for this group of patients. Targeted approaches to treatment can be more successful in terms of survival advantage and improvements in quality of life as it can replace cytotoxic conventional chemotherapy [30,31]. In a retrospective study conducted on 47 NSCLC patients, researchers found that *mt KRAS* NSCLC had a significantly lower expression of BRCA1 and TS proteins. When comparing their results in wild-type NSCLC patients, however, SRC expression showed no significant changes between *mt* and *wt* KRAS tumors [30]. Although BRCA 1 overexpression is associated with treatment resistance in NSCLC [22], our results reveal similar expressions of BRCA1 in both *mt* KRAS tumors and paired normal tissue. Both studies’ results conclude that the poor response of *mt KRAS* NSCLC to treatment is independent of BRAC1 expression. A low expression of receptor-associated protein 80 (RAP80) was found to have a positive impact on the survival of NSCLC patients, and both BRCA1 and RAP80 had low expression levels in patients, showing an increased benefit from platinum therapy [32,33].

Conversely, levels of ERCC1 expression were significantly increased in comparison to paired normal lung tissue (*p* < 0.04). ERCC1 expression was repeatedly linked to resistance to platinum therapy [34,35], which suggests that it can be linked to the poor response of *mt KRAS* tumors to platinum therapy [3].

Although KRAS activation was linked to SRC overexpression in pancreatic carcinoma, the same study failed to establish a link between *mt KRAS* and SRC overexpression [36]. Our patients had a significantly increased SRC expression in *mt KRAS* compared to paired normal tissue (*p* < 0.04). While the number of patients is limited, this finding should be further investigated as SRC is a druggable target that can offer a treatment option in *mt KRAS* patients. TS and SRC overexpression were previously linked to each other, and it was reported that pemetrexed resistance emerges as a result of TS overexpression. Although the link between the TS and SRC pathways is not yet clear, TS overexpression is associated with concomitant SRC overexpression, leading to the hypothesis that using TKIs can block...
SRC and consequently, lead to TS overexpression. The results have been validated in cell lines but not in human subjects thus far [31].

Remarkably, prolonged survival was noted in patients whose samples expressed increased PD-L1 in tumor cells in contrast to patients with decreased or no expression [37]. Our results are consistent with other studies in suggesting that patients with robust PD-L1 protein expression are expected to respond better to immunotherapy [38].

In the phase 2 trial, patients with KRAS-G12C-mutated NSCLC were previously treated with standard therapies such as platinum-based chemotherapy and program death inhibitors (PD-1) or program death-ligand inhibitors (PD-L1). These patients received selective reversible inhibitors of the G12C-activated KRAS oncogene, such as sotorasib. In this multicenter study, the objective response was evaluated by the radiological review of the patients. The results show that 3.2% of patients showed a complete response to sotorasib, and 34% of patients had a partial response to the KRAS G12C inhibitor. The median progression-free survival was 7 months, the median duration of response was 11.1 months, and the overall survival was 12.5 months [39]. The current standard therapies for advanced NSCLC include immune checkpoint inhibitors with combination chemotherapy or immunotherapy alone in patients with newly diagnosed KRAS-mutated NSCLC [40]. Disease progression in patients after immunotherapy treatment is generally treated with single-agent chemotherapy such as docetaxel or pemetrexed; however, the response rate in these patients is less than 10% [41]. In the REVEL and LUME-Lung 1 trials, the survival rate was longer with the addition of ramucirumab, a monoclonal antibody targeting the vascular endothelial growth factor receptor, or nintedanib, a broadly acting tyrosine kinase inhibitor [42]. In this phase 2 trial, patients with advanced NSCLC previously treated with both platinum-based chemotherapy and immune checkpoint inhibitors showed a rapid and durable response to sotorasib. The response to sotorasib was observed in all PD-L1 expression levels [39]. KRAS inhibitors, such as adagrasib and sotorasib, showed promising results in patients with advanced NSCLC. The mechanism of resistance to these therapies is largely unknown. A study by Awad et al. revealed a putative mechanism for resistance to adagrasib. In this study, acquired KRAS alterations included G12D/R/V/W, G13D, G61H, R61H, Y96C, and H95D/Q/R. Amplification in the expression of the KRAS G12C allele was also observed. Other bypass mechanisms for resistance included MET amplification, activating mutations in NRAS, BRAF, MAP2K1, and RET. Several oncogenic fusions were observed involving ALK, RET, BRAF, RAF1, and FGFR3. Loss-of-function mutations were also observed in NF1 and PTEN. Thus, this study reveals diverse genomic mechanisms which impart resistance to KRAS G12C [43].

5. Limitations

The tissue for this study was collected from the biorepository tissue bank of the University of Cincinnati, OH, USA and approved by the IRB committee. However, the clinical data of the patients were provided for this study, which is its main limitation. The tissue used for this study was exhausted; unfortunately, no further molecular testing can be carried out.

6. Conclusions

Our results suggest that mt KRAS NSCLC patients might benefit from a treatment strategy that targets KRAS in combination with therapeutic agents based on pharmacogenomic markers such as SRC and BRCA1 expression levels. SRC expression has been described as a potential resistance mechanism to pemetrexed-based therapy. BRCA1 expression is related to responsiveness to taxane-based therapy. Both pemetrexed- and taxane-based therapies are utilized in the treatment of patients with KRAS mutations. Only, KRAS G12c has an approved targeted therapy. Future studies that focus on the selection of chemotherapeutic agents in combination with KRAS-targeted therapies are needed.
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