Introduction

Chemotherapy is the foundation of lung cancer treatment. However, many patients who will not benefit from chemotherapy—whether cytotoxic agents or newer targeted therapies—are still exposed to the toxic effects of these drugs. In addition, chemotherapy resistance may develop in patients who receive neoadjuvant chemotherapy, and resistance may contribute to treatment failure in such patients. Establishing prognostic or predictive biomarkers in tissue samples from NSCLC patients treated with neoadjuvant chemotherapy would lead to more accurate prognoses and better identification of patients who may benefit from antitumor therapy. To date, several molecular markers have been proposed as candidate predictors of therapeutic response in patients with NSCLC undergoing neoadjuvant chemotherapy. For example, high ERCC1 expression in resected NSCLC tumors correlated with cisplatin resistance [1].
We previously reported that major pathologic response (MPR) criteria applied to resected tumor specimens of patients who received neoadjuvant chemotherapy can predict survival and be used for assessment of tumor response [2–7]. Our previous study demonstrated that MPR as assessed by the percentage of viable tumor cells in the resected specimen correlated with overall survival (OS) in NSCLC patients who were treated with neoadjuvant chemotherapy [4, 5]. We and others also suggested that MPR can be used as a surrogate endpoint for survival, thereby shortening the period needed to evaluate novel chemotherapeutic and biologic therapies in clinical trials [2–4, 6, 8]. The ability to predict tumor response to neoadjuvant chemotherapy using biomarkers will be very helpful for the effective management of NSCLC and for avoiding the development of chemoresistance.

The purpose of this study was to identify biomarkers that predict prognosis or therapeutic response in NSCLC patients treated with neoadjuvant chemotherapy. In this study, we used pyrosequencing to detect mutations in two candidate biomarker genes, KRAS proto-oncogene (KRAS) and epidermal growth factor receptor (EGFR). We also used immunohistochemical analysis to examine expression of five candidate protein biomarkers previously identified in the literature: vascular endothelial growth factor receptor 2 (VEGFR2) [9, 10], histone-lysine-N-methyltransferase EZH2 (EZH2) [11, 12], DNA excision repair protein ERCC1 (ERCC1) [1, 13], DNA repair protein RAD51 homolog 1 (RAD51) [14, 15], and PKR[16, 17] in resected tumor specimens from 98 NSCLC patients who were treated with neoadjuvant chemotherapy. Increased VEGFR-2 gene copy was associated with chemoresistance and shorter survival in patients with non-small-cell lung carcinoma who receive adjuvant chemotherapy [9]. Researcher suggests that EZH2 may be a predictive and prognostic factor for cisplatin-based therapy response and disease survival in advanced NSCLC [11, 12]. ERCC1 plays a major role in repair of cisplatin-induced DNA damage in vitro and in vivo [13]. Preclinical data suggest that Rad51 might play a role in lung cancer resistance to platinums and etoposide, although this has not been confirmed clinically [18]. In NSCLC cell lines, cisplatin exposure increased Rad51 protein induction, and reduction in Rad51 by siRNA significantly increased cisplatin-mediated cell kill by cisplatin [15, 18–20]. We previously demonstrated that PKR plays a critical role in chemoresistant and radioresistance [16, 17, 21]. In this study, we found that cytoplasmic RAD51 expression was associated with MPR (higher percentage of viable tumor cells) and shorter OS time in patients with NSCLC receiving neoadjuvant chemotherapy. Combination of MPR with RAD51 is a significant predictor of prognosis in patients with NSCLC who received neoadjuvant chemotherapy.

**Material and Methods**

**Patient population**

We collected paraffin-embedded hematoxylin- and eosin-stained slides and blocks from tumors resected from 98 patients with NSCLC. The patients had been treated with neoadjuvant chemotherapy followed by complete surgical resection at The University of Texas MD Anderson Cancer Center from 2008 to 2011. All patients already signed an informed consent form for the use of their clinical data and tumor tissue for molecular research. Detailed clinical and pathologic information, including demographic data, smoking history (never- or ever-smoker), pathologic tumor-node-metastasis (TNM) stage, and OS, was available for all patients.

**DNA extraction and mutation analysis**

To extract DNA from the formalin-fixed, paraffin-embedded (FFPE) tumor specimens, we first placed two to four slices of tumor tissue (10 μm thick) in 1.5-mL labeled tubes. DNA was purified using a SPRI-TE Nucleic Acid Extractor (Beckman Coulter, Brea, CA), which uses solid-phase reversible immobilization technology. For each tumor DNA sample, both the concentration and the quality of the samples were assessed.

To detect gene mutations in the tumor samples, we used pyrosequencing confirmed by direct sequencing. For pyrosequencing, polymerase chain reaction (PCR) amplification was carried out in a 50-μL reaction tube containing 2 μL of bisulfite-treated DNA, 5 μL of 10× PCR buffer (Applied Biosystems, Foster City, CA), 2 mmol/L MgCl₂, 10% dimethyl sulfoxide, 0.2 mmol/L dNTP, 0.25 U of AmpliTaq Gold (Applied Biosystems), 0.1 μmol/L primers for p16, DAPK, RASSF1A, and GSTP1 promoters, 0.01 μmol/L 5′-tailed, unlabeled forward universal primer or reverse universal primer, and 0.09 μmol/L biotinylated universal primer. PCR products with a 5′-biotinylated strand were captured on streptavidin-coated beads (Amershams Bionsciences, Uppsala, Sweden). Subsequently, the biotinylated PCR products were purified and made into single-stranded DNA to which a sequencing primer was annealed using a vacuum prep tool (Pyrosequencing, Inc., Westborough, MA). Pyrosequencing reactions were performed according to the manufacturer’s specifications on a PSQHS system (Pyrosequencing AB, Uppsala, Sweden). The KRAS exon 1 and exon 2 primers used were as follows: exon 1: forward: 5′-TCTTAAGCGTCCGATGGAG GAG-3′; reverse: 5′-TGACATCCCTCAGAAAAGTAAA G-3′; exon 2: forward: 5′-ATGCCCATTGGTGGACATC TCAT-3′; reverse: 5′-AAGTACTCCACTGCCTAAT CCC-3′. The EGFR primers used were as follows:
Histopathologic evaluation

Immunohistochemical staining for biomarkers was performed as described previously [22]. Briefly, FFPE tissue sections (5 μm thick) were deparaffinized, hydrated, and heated in a steamer for 10 min with 10 mmol/L of sodium citrate (pH 6.0) for antigen retrieval. The slides were blocked with 3% H₂O₂ in methanol at room temperature for 15 min and then in 10% bovine serum albumin in Tris-buffered saline with Tween-20 for 30 min. The slides were then incubated with a primary antibody at 1:400 dilution for 65 min at room temperature. Next, the slides were washed with phosphate-buffered saline and then incubated with a biotin-labeled secondary antibody for 30 min. Finally, the samples were incubated with a 1:40 solution of streptavidin-peroxidase for 30 min. The staining was developed with 0.05% 3′3-diaminobenzidinetetrahydrochloride prepared in 0.05 mol/L of Tris buffer at pH 7.6 containing 0.024% H₂O₂. The slides were then counterstained with hematoxylin. An anti-ERCC1 (8F1) antibody was obtained from Thermo Fisher (Waltham, MA; catalog# MS-671P). An anti-EZH2 antibody was obtained from Leica Biosystems (Novocastra Reagents, Buffalo Grove, IL; catalog #NCL-L-EZH2). Anti-FLK-1 (KDR or VEGFR2, catalog # SC-6251), anti-RAD51 (catalog #sc-8349), and anti-PKR (SC-707) antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX).

Immunohistochemical protein expression was quantified using a 4-value intensity score (0 for negative, 1 for weak, 2 for moderate, and 3 for strong), and the percentage of tumor cells within each category was estimated [23]. A final score was obtained by multiplying intensity and extension values (0× % negative tumor cells + 1× % weakly stained tumor cells + 2× % moderately stained tumor cells + 3× % strongly stained tumor cells). The final scores ranged from a minimum of 0 to a maximum of 300.

Statistical analysis

In the univariate analysis, continuous and categorical variables were analyzed using an independent-samples t-test or chi-square test, respectively. The Kaplan–Meier method was used to estimate survival probability as a function of time. Protein expression levels were categorized as either low or high based on a cutoff point set at the median score. A log-rank test was used to measure between-group differences in patient survival time. The influence of biomarker expression on survival time was calculated using a multivariate Cox proportional hazards model with adjustment for demographic, clinical, and histopathologic parameters (age, sex, smoking status, and tumor histologic subgroup). A two-sided t-test was used to test equal proportions between groups in two-way contingency tables. The generalized estimating equation approach was used to estimate differences in means between groups. Statistical significance was set at P < 0.05.

Results

Patient characteristics

Table 1 shows the demographic and clinical characteristics of the 98 NSCLC patients treated with neoadjuvant chemotherapy included in this study. The study population included 54 (55%) men and 44 (45%) women; the patients’ median
age was 62 years (range, 41–85 years). The histologic tumor types were adenocarcinoma (n = 49), squamous cell carcinoma (n = 26), and others (n = 23). Most of the patients (n = 90, 92%) had received platinum-based neoadjuvant chemotherapy. The majority of the patients (79 patients, 81%) received a combination platinum- and taxane-based neoadjuvant chemotherapy regimen. The median number of treatment cycles was 3 (range, 2–7 cycles).

Table 2. KRAS and EGFR mutations in NSCLC tumors after neoadjuvant chemotherapy.

| Patients | Histology | %Viable tumor cells | KRAS mutation | EGFR mutation |
|----------|-----------|---------------------|---------------|---------------|
| 1        | ADQ       | 32                  |               |               |
| 2        | ACC       | 33                  | Codon 13 (GCC>GGT) |               |
| 3        | NSCLC-NOS | 37                  |               | Exon 21 (GCC>ACC, A859T) |
| 4        | ADQ       | 45                  |               | Exon 21 (GCC>ACC, A859T) |
| 5        | NSCLC-NOS | 47                  |               | Exon 19 (Deletion, E746-A750) |
| 6        | NSCLC-NOS | 47                  |               | Exon 21 (CTG>CGG, L858R) |
| 7        | ACC       | 50                  | Codon 12 (GGT>TGT) | Exon 21 (CTG>CGG, L858R) |
| 8        | ACC       | 56                  | Codon 12 (GGT>TAT) |               |
| 9        | ADQ       | 60                  |               |               |
| 10       | ACC       | 60                  | Codon 12 (GGT>GTT) | Exon 19 (Deletion, L747-A750) |
| 11       | ACC       | 61                  | Codon 13 (GGC>GAT) |               |
| 12       | ACC       | 62                  |               |               |
| 13       | ACC       | 63                  | Codon 12 (GGT>GTT) |               |
| 14       | ACC       | 68                  | Codon 12 (GGT>GTT) |               |
| 15       | ACC       | 70                  | Codon 12 (GGT>GTT) |               |
| 16       | ACC       | 74                  |               | Exon 21 (GCC>ACC, A859T) |
| 17       | ACC       | 75                  |               | Exon 19 (Deletion, E746-A750) |
| 18       | ACC       | 75                  | Codon 12 (GGT>GTT) | Exon 21 (GCC>ACC, A859T) |
| 19       | ACC       | 81                  | Codon 12 (GGT>GTT) |               |

ACC, adenocarcinoma; ADQ, adenosquamous; NSCLC-NOS, NSCLC-not otherwise specified.

Figure 1. Gene mutation profiles in NSCLC tumors from 98 patients who underwent neoadjuvant chemotherapy. (A) Representative example of wild-type (WT) and mutated (Mut) KRAS and EGFR. (B and C) Kaplan–Meier curves comparing overall survival by KRAS (B) and EGFR (C) mutation status.
Mutation analysis

We examined KRAS and EGFR mutations in NSCLC tumors from patients who underwent neoadjuvant chemotherapy. We identified mutations in KRAS (codons 12 and 13) and EGFR (exons 19 and 21) via pyrosequencing and confirmed these mutations using direct sequencing. The two methods showed similar results. KRAS and EGFR mutations were detected in samples with a minimum of 32% viable tumor cells (Table 2). In 18 samples with less than 32% viable tumor cells, we detected no mutations, but we found KRAS and EGFR mutations in 10 of 80 (13%) patient samples with 32% or more viable tumor cells. A point mutation in KRAS codon 12 was detected in eight of 80 (10%) samples. All of the KRAS mutations detected were in adenocarcinoma specimens. Of the 10 EGFR mutations identified, three were a deletion in exon 19, and seven were a point mutation in exon 21. Of the point mutations, three were L858R mutations involving an amino acid substitution from leucine (L) to arginine (R) at position 858 in exon 21. The remaining four point mutations were A859T mutations involving an amino acid substitution from alanine (A) to threonine (T) in exon 21 at position 859. One patient tumor had both EGFR and KRAS mutations. Figure 1A shows mutation profiles of four patients. Patient 2 had a KRAS mutation in codon 12, and patient 4 had an EGFR mutation in exon 21. Patients with KRAS mutations tended to have shorter OS durations than did patients with wild-type KRAS, but EGFR mutation did not affect OS duration (Fig. 1B and C).

Expression of candidate protein biomarkers

We next examined the selected protein biomarkers using immunohistochemical analysis. We selected five candidate biomarkers (VEGFR2, EZH2, ERCC1, RAD51, and PKR) on the basis of the literature. Figure 2 shows representative images of VEGFR2, EZH2, ERCC1, RAD51, and PKR staining in NSCLC cells from three patients treated with neoadjuvant chemotherapy. We observed that the VEGFR2
Table 3. Univariate and multivariate analyses for overall survival in 98 NSCLC patients treated with neoadjuvant chemotherapy.

| Characteristics              | No. of patients | HR (95% CI) | P     |
|------------------------------|-----------------|-------------|-------|
| Univariate analyses          |                 |             |       |
| Age (continuous)             | 98              | 1.00 (0.97–1.04) | .830  |
| Gender                       |                 |             |       |
| Female (reference)           | 44              | 1.00        | .070  |
| Male                         | 54              | 0.5 (0.23–1.07) |       |
| Histology                    |                 |             |       |
| Adenocarcinoma (Reference)   | 49              | 1.00        | .220  |
| Squamous cell carcinoma      | 26              | 0.56 (0.26–1.20) |       |
| Other                        | 23              | 0.62 (0.29–1.31) |     |
| Pathological stage           |                 |             |       |
| 0/IA/IB (reference)          | 33              | 1.00        | .008  |
| IIA/IB                       | 29              | 0.73 (0.31–1.72) |       |
| IIA/IB                       | 33              | 2.52 (1.27–5.03) |       |
| IV                           | 3               | 2.78 (0.63–12.35) |      |
| %Viable tumor cells (continuous) | 98          | 1.02 (1.01–1.03) | .004  |
| EZH2 (continuous)            | 98              | 1.00 (0.99–1.01) | .510  |
| VEGFR2 (continuous)          | 98              | 1.00 (0.99–1.01) | .680  |
| ERCC1 (continuous)           | 98              | 0.99 (0.99–1.00) | .650  |
| RAD51 (continuous)           | 98              | 1.01 (1.00–1.01) | .02   |
| PKR (continuous)             | 98              | 1.00 (0.99–1.01) | .980  |
| %Viable tumor cells ≤10% (or <=10%) (MPR+) (reference) | 8 | 1.00 | .030 |
| >10% (MPR−)                  | 90              | 3.05 (1.07–8.72) |       |
| RAD51                        |                 |             |       |
| Low (reference)              | 75              | 1.00        | .005  |
| High                         | 23              | 2.41 (1.31–4.43) |       |
| Multivariate analyses        |                 |             |       |
| Pathological stage           |                 |             |       |
| 0/IA/IB (Reference)          | 33              | 1.00        | .007  |
| IIA/IB                       | 29              | 0.74 (0.31–1.76) |       |
| IIA/IB                       | 33              | 2.63 (0.32–1.76) |       |
| IV                           | 3               | 2.34 (1.32–5.22) |       |
| %Viable tumor cells (continuous) | 98            | 1.01 (1.00–1.03) | .040  |
| RAD51 (continuous)           | 98              | 1.01 (1.00–1.01) | .020  |
| %Viable tumor cells ≤10% (or <=10%) (MPR+) (reference) | 8 | 1.00 |       |
| >10% (MPR−)                  | 90              | 2.91 (1.06–7.65) |       |

CI, confidence interval; HR, hazard ratio.

Correlation of protein biomarker expression with clinicopathologic features and disease outcomes

Next, we determined whether expression of VEGFR2, EZH2, ERCC1, RAD51, and PKR was associated with MPR and OS time. The surgical pathologic stage, the percentage of viable tumor cells (or MPR), and RAD51 expression were associated with OS in both the univariate and multivariate analyses (Table 3). Figure 3 shows Kaplan–Meier survival curves comparing OS durations by percentage of viable tumor cells (MPR+ vs. MPR−) (Fig. 3A) and by RAD51 expression (Fig. 3B). OS was significantly longer in MPR+ patients who had 10% or less viable tumor cells than in MPR− patients with more than 10% viable tumor cells ($P = .02$) (Fig. 3A). We also found that patients with high RAD51 expression levels had a significantly poorer prognosis than did those with low RAD51 expression ($P = .004$) (Fig. 3B). RAD51 expression level was also significantly associated with MPR as indicated by the percentage of viable tumor cells ($P = .01$) (Fig. 3C). However, we found no association between VEGFR2, EZH2, ERCC1, or PKR expression and MPR (data not shown). We also found no significant relationships between VEGFR2, EZH2, ERCC1, or PKR expression and age, sex, tumor status (T status), lymph node status (N status), metastasis status (M status), clinical stage, tumor cell type, or tumor cell differentiation (data not shown). Figure 3D shows representative images of stained tumor tissue from patient 1, with 77% viable tumor cells and high cytoplasmic RAD51 expression, and patient 2, with 9% viable tumor cells and low RAD51 expression in the cytoplasm. We found no associations between the percentage of viable tumor cells or VEGFR2, EZH2, ERCC1, RAD51, or PKR expression and KRAS or EGFR mutation (data not shown).

Prognostic significance of combinations of MPR and RAD51 biomarker

We next further determined whether RAD51 marker provided prognostic information for NSCLC patients treated with neoadjuvant chemotherapy in addition to that provided by MPR. We combined RAD51 and MPR to stratify patients into four groups: MPR+ and RAD51 (Low); MPR+ and RAD51 (High); MPR− and RAD51 (Low); and MPR− and RAD51 (High). Among patients, the 5-year overall survival rate in MPR+/RAD51 (High) patients (27%) was significantly lower than that in MPR+/RAD51 (Low) patients (48%) and MPR−/RAD51 (Low) patients (87%).
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We did not observe any patients in second group: MPR+ and RAD51 (High) (Fig. 3E). Our results also revealed that the MPR/RAD51 was significantly associated with prognosis and was an independent indicator of survival duration in NSCLC patients treated with neoadjuvant chemotherapy.

**Discussion**

The significance of mutations in KRAS, EGFR, ALK, ERBB2/HER2, PI3KCA, and BRAF has been documented in primary NSCLC tumors [24]. However, only a limited number of studies have investigated gene mutations in NSCLC tumors that have been previously treated with neoadjuvant chemotherapy [25]. In this study, we first investigated EGFR and KRAS gene mutations in NSCLC tumors treated with neoadjuvant chemotherapy. We observed no association between EGFR mutation and OS or MPR. However, we observed that KRAS mutation was associated with OS. We further evaluated the ability of five candidate markers (VEGFR2, EZH2, ERCC1, RAD51, and PKR) to predict prognosis and therapeutic response. We demonstrated that cytoplasmic RAD51 expression was associated with both MPR (as indicated by the percentage of viable tumor cells) and OS. We found that patients with high RAD51 expression levels had a poorer prognosis than did those with low RAD51 expression. Our results suggest that RAD51 expression in the cytosol is a useful prognostic biomarker in patients with NSCLC who have undergone neoadjuvant chemotherapy.

Our results indicated that the MPR in the resected specimen may serve as a surrogate endpoint for survival to evaluate novel chemotherapeutic therapies and immunotherapy response in biomarker-driven translational clinical trials. Assessment of biomarker could be combined with MPR to accurately serve as surrogate endpoints for treatment efficacy. One potential limitation of our study is that we did not compare pretherapy and post-therapy tissue specimens from patients whose tumors did not respond to neoadjuvant therapy. Unfortunately, we were...
unable to collect FFPE biopsy specimens from these patients, so we could not compare them with post-therapy tissues from the same patients.

Increased RAD51 expression has been shown to be associated with poorer outcomes in patients with several tumor types treated with chemoradiotherapy [14, 15, 18–20, 26]. Furthermore, a number of reports demonstrated that RAD51 is involved in resistance to anticancer treatments such as radiation and platinum chemotherapy agents in various tumor types, including lung cancer [14, 15, 19, 20, 26]. For instance, silencing the RAD51 gene improved sensitivity to doxorubicin in soft tissue sarcoma cell lines [14]. Downregulation of RAD51 expression by gefitinib (a selective EGFR tyrosine kinase inhibitor) sensitized mitomycin C and gemcitabine-induced cell inhibition in lung cancer cells [26, 27].

RAD51 plays a critical role in a common DNA damage response pathway associated with the activation of homologous recombination and double-strand break repair [14, 15, 26]. In the nucleus, RAD51 binds to single- and double-stranded DNA and exhibits DNA-dependent ATPase activity [15]. In the cytoplasm, RAD51 is involved in maintenance of the mitochondrial genome [18]. Cytoplasmic RAD51 plays important roles in maintaining the integrity of mitochondrial DNA and facilitating its repair [18]. Several studies have indicated that RAD51 protein can translocate between cytoplasmic and nuclear compartments [14, 15, 26]. Several other proteins have recently been found to be involved in mitochondrial DNA repair, including aprataxin [28], tyrosyl-DNA phosphodiesterase 1 (TDP1) [28], and flap endonuclease 1 (FEN1) [29]. Aprataxin is involved in the repair of DNA strand breaks caused by various DNA-damaging agents, including H2O2, methyl methane sulfonate, and the irinotecan-related compound camptothecin [30]. High levels of aprataxin expression are associated with poor response to irinotecan-based chemotherapy [30]. TDP1 has been linked with resistance to camptothecin and a topoisomerase I inhibitor in human lung cancer [31]. Several studies have demonstrated that downregulation of overexpressed FEN1 using a short interfering RNA or an inhibitor increased sensitivity to cisplatin in brain, lung, and gastric cancer cells [32–34]. Further study is needed to explore other candidate markers in existing FFPE tissue from NSCLC patients treated with neoadjuvant chemotherapy. Understanding the mechanisms of interaction of biomarkers will clarify their contribution to chemoresistance and may lead to the recognition and use of these markers in clinical practice.

In conclusion, we demonstrated that high cytoplasmic RAD51 expression was associated with MPR (as indicated by the percentage of viable tumor cells) and shorter OS in patients with NSCLC receiving neoadjuvant chemotherapy. Combination of MPR with RAD51 is a significant predictor of prognosis in patients with NSCLC who received neoadjuvant chemotherapy. Prediction of a patient’s prognosis could be improved by combined assessment of standard clinical variables, MPR, and molecular biomarkers.

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**Conflict of Interest**

The authors declare no conflict of interests.

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