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Optimizing Treatment Response in Lung Squamous Cell Carcinoma by Cell-Based Functional Sensitivity Screening

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Abstract

Traditional chemotherapy is the standard of care for lung squamous cell carcinoma (LSCC). However, recent studies have suggested that precision medicine platforms could help identify better treatment options. Our approach involves testing various chemotherapeutics in cells isolated from LSCC patients' biopsies to make more informed treatment decisions. Five primary LSCC cultures from patient-derived cells were established and exposed to 8 different chemotherapeutics and erlotinib 72 h after culturing, with treatments lasting for 7 days. An immunofluorescence assay with a CK8/18 antibody mixture was utilized to distinguish cancer cells from stromal cells, while a scoring method involving 11 weighted parameters was applied to calculate the drug response score for LSCC patients' cell responses. Our results suggest that erlotinib could be an effective treatment for LSCC patients and that the dosage of erlotinib might be reduced without compromising its effectiveness. Our findings show that gemcitabine, in addition to platinum drugs, could be beneficial for LSCC patients. The results underscore the potential of functional screening methods to identify effective treatments for LSCC.

Introduction

Lung cancer is the primary cause of cancer-related deaths in Serbia, accounting for 16.4% of all cancer diagnoses and around 24.4% of all cancer-related deaths (GLOBOCAN Cancer Today (iarc.fr)). The morbidity and mortality rates of lung cancer in Serbia are among the highest globally. According to the GLOBOCAN estimates for 2022, Serbia ranks in Europe for both incidence and mortality rates, right after Hungary, Poland, and Romania. In comparison to Western Europe and the USA, where a decline in mortality rates has been observed in recent decades, especially for males, mortality rates in Serbia have been continuously rising in both genders, particularly in females.

Non-small-cell lung cancer (NSCLC) accounts for 80%–85% of all lung cancer cases and consists of two major subtypes: lung adenocarcinoma (LADC) and lung squamous cell carcinoma (LSCC) (1). LADC and LSCC have distinct histologies and patterns of occurrence within the lung. Multiple significant oncogenic drivers in LADC have been recognized, such as activating mutations in KRAS, EML4-ALK gene fusions, and activating mutations and/or amplification of the EGFR. The most prevalent somatic mutations in LSCC involve the inactivation of the tumor suppressors LKB1, PTEN, and TP53, while oncogenic mutations present in LADC are rare (2).

Although LSCC incidence has dropped over the years due to a decrease in tobacco smoking, the primary causative

agent of LSCC, its prevalence is still high. Unlike LADC, the majority of patients diagnosed with LSCC are former or current heavy smokers. Tobacco smoking leads to a high somatic mutational burden in LSCC (3). However, the DNA mutations targeted for LADC therapy are essentially absent in LSCC. Therefore, the standard of care for metastatic LSCC is chemotherapy, traditionally using a dual platinum/taxane or platinum/etoposide approach (4).

The FDA-approved non-traditional chemotherapy compounds for LSCC include the antiangiogenic VEGFR inhibitor ramucirumab (for second-line treatment), necitumumab (a monoclonal antibody inhibitor of EGFR for first-line treatment when combined with chemotherapy), afatinib (an EGFR/HER2 inhibitor for second-line treatment), and immune checkpoint inhibitors such as nivolumab, pembrolizumab, atezolizumab, and durvalumab (3). While there have been some advancements in the development of targeted therapies for LSCC, the response rate to these treatments has been limited. Only 7% and 17% of patients have responded positively to targeted therapies or immune checkpoint inhibitors, respectively (5).

Recent studies indicate that precision medicine platforms could potentially act as surrogate biomarkers for cancer types lacking druggable targets. These platforms encompass diverse patient-derived cellular models and screening techniques. Notably, a significant proportion of clinical trials across various cancer types, including lung cancer, have demonstrated correlations with clinicopathological parameters, pointing to the most efficacious therapies for cancer patients (6). Our approach includes screening for different chemotherapeutics in patient-derived cells to discern optimal treatment options for patients with LSCC and address the scarcity of easily targetable treatments. Through this method, we can categorize responses of LSCC patient cells based on anticancer activity, selectivity towards cancer cells, and the nature of the response, whether it is dose-dependent or biphasic. These evaluations are carried out using short-term primary cell cultures comprising cancer and stromal cells (7). In addition, our recent findings indicate an excellent response of both LADC and LSCC patient-derived cells to erlotinib (8). Therefore, this study compared the responses of LSCC primary cell cultures among 8 chemotherapeutics and erlotinib.

Materials and methods

Establishment of LSCC primary cell cultures

Tissue samples from LSCC patients were obtained from the Clinic for Thoracic Surgery at the University Clinical Center of Serbia after informed consent from the patients and approval from the Ethics Committee of the University Clinical Center of Serbia (approval reference number 623/4). After surgical removal, the samples were placed in a sterile tube containing an antibiotic-antimycotic solution (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) and immediately transferred to the research laboratory for further processing.

To establish primary cultures, the tissue was manually minced with a surgical blade in a sterile Petri dish upon arrival at the laboratory. The samples were cut into 3 – 5 mm pieces and dissociated using the Tumor Dissociation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The tissue pieces were incubated in an orbital shaker (KS 4000 ic control, IKA, Königswinter, Germany) at 37 °C and 300 rpm for 90 minutes. After incubation, the dissociated tissue was placed in DMEM/Ham's F12 (1:3) growth medium supplemented with 5% fetal bovine serum (Corning, NY, USA), antibiotic-antimycotic solution, 4 µg/mL hydrocortisone (Sig-ma-Aldrich Chemie GmbH, Taufkirchen, Germany), 1 µg/mL insulin (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany), 10 ng/mL epidermal growth factor (BioLegend, San Diego, CA, USA), and 24 µg/mL adenine (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). The DMEM and Ham's F12 growth media were purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). The dissociated tissue was cultured in T-25 cell culture flasks until cell attachment was observed before the medium was replaced. Five successfully established primary LSCC cultures (stage II: TR36 and TR87, and stage III: TR33, TR34, and TR107) were maintained at 37 °C in a humidified atmosphere with 5% CO2 and grown to confluence before further experiments.

Treatments with chemotherapeutics and erlotinib

Vinorelbine and pemetrexed were purchased from Selleckchem (Houston, TX, USA). Carboplatin, cisplatin, docetaxel, etoposide, gemcitabine, and paclitaxel were purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). Erlotinib was purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). Cisplatin, carboplatin, and gemcitabine were dissolved in sterile water, while etoposide, docetaxel, vinorelbine, paclitaxel, pemetrexed, and erlotinib were dissolved in DMSO and stored at −20 °C. Before treatment, all stock solutions were freshly diluted in sterile water. The following concentrations were used for the treatments: vinorelbine (100, 250, 500, 750, and 1000 nM); pemetrexed (50, 75, 100, 200, and 300 µM); carboplatin (10, 25, 50, 75, and 100 µM); cisplatin (5, 7.5, 10, 12.5, and 15 µM); docetaxel (1, 2, 3, 4, and 5 µM); etoposide (10, 15, 20, 25, and 30 µM); gemcitabine (10, 25, 50, 75, and 100 µM); paclitaxel (1, 2, 3, 4, and 5 µM); and erlotinib (0.5, 1, 2, 3, and 4 µM).

Patient-derived cells were seeded in black, clear-bottom 384-well cell culture microplates (Thermo Fisher Scientific, Waltham, MA, USA) at a density of 1000 cells per well in 50 µL of cell growth medium. The drugs were administered 72 h after seeding, and the treatment lasted for 7 days.

Immunofluorescence assay

The immunofluorescence assay was used to distinguish cancer cells from stromal cells using a cytokeratin 8/18 (CK8/18) antibody mixture as previously described (7). CK8/18-positive cells were considered cancer cells, while CK8/18-negative cells were considered stromal cells. Cells were fixed in 4% paraformaldehyde for 20 min at RT and washed using the Wellwash™ Versa microplate washer (Thermo Fisher Scientific, Waltham, MA, USA). Cells were then blocked with 2% bovine serum albumin (BSA) in PBS for 1 h at RT. Cells were then incubated overnight at 4 °C with a primary rabbit CK8/18 antibody cocktail (clone SU0338, #MA5-32118, Thermo Fisher Scientific, Waltham, MA, USA). Cells were then incubated with secondary Alexa Fluor 488 goat anti-rabbit antibody (#A-11008, Thermo Fisher Scientific, Waltham, MA, USA) at RT for 2 h under photoprotective conditions. Cell nuclei were counterstained with 1 µg/mL Hoechst 33342 at RT for 2 h. Cells were stored at 4 °C in the dark before imaging.

Fluorescently labeled cells were imaged using the ImageXpress® Pico Automated Cell Imaging System (Molecular Devices®, San Jose, CA, USA) with a 4x objective after determining the appropriate exposure time for each illumination filter. The obtained images were analysed using CellReporterXpress® software v. 2.8.2.669 (Molecular Devices®, San Jose, CA, USA). The Cell Scoring Analysis Protocol was used to assess the cytotoxicity of drugs as previously described (7).

Scoring of chemotherapeutics and erlotinib responses

LSCC patients' cell responses were scored using a modified scoring method (9). We generated an overall drug response score by considering various weighted parameters. This score was determined through the comparison of drug effects on cancer and stromal cells using our immunoassay.

1) The inhibitory effect (inhibition percentage) at the maximum dose is multiplied by 0.1. If the inhibitory effect is greater with stromal cells, it is multiplied by -0.1.
2) The dose required for 10% inhibition is multiplied by 0.05. If the dose is higher than that for stromal cells, it is multiplied by -0.05.
3) The dose required for 25% inhibition is multiplied by 0.05. If the dose is higher than that for stromal cells, it is multiplied by -0.05.
4) The dose required for 50% inhibition is multiplied by 0.1 (if this dose is above 100 µM, it is multiplied by 0). If the dose is higher than that for stromal cells, it is multiplied by -0.1 (if this dose is above 100 µM, it is multiplied by -10).
5) The dose required for 75% inhibition is multiplied by 0.05 (if this dose is above 100 µM, it is multiplied by 0). If the dose is higher than that for stromal cells, it is multiplied by -0.05 (if this dose is above 100 µM, it is multiplied by -5).
6) The dose required for 90% inhibition is multiplied by 0.05 (if this dose is above 100 µM, it is multiplied by 0). If the dose is higher than that for stromal cells, it is multiplied by -0.05 (if this dose is above 100 µM, it is multiplied by -5).
7) Slope
8) The area under the curve (AUC) is multiplied by 0.35
9) If none of the applied doses stimulates the growth of cancer cells, the lowest dose is multiplied by 0.05, and if the dose stimulates the growth of cancer cells up to 125%, it is also multiplied by 0.05. If the dose stimulates the growth of cancer cells from 125% to 150%, it is multiplied by 0; if it stimulates over 150%, it is multiplied by -0.05.
10) If the drug acts rapidly at lower doses and has a limited effect at higher doses, the breakthrough dose is multiplied by -0.05. If there is a dose-dependent effect, the dose that inhibits 50% is multiplied by 0.05.
11) If the drug incompletely inhibits tumor growth at the highest dose*: if more than 25% of cancer cells remain, the highest dose is multiplied by -0.05, if 10% – 25% of cancer cells remain, the highest dose is multiplied by 0, and if less than 10% of cancer cells remain, the highest dose is multiplied by 0.05.

All individually weighed parameters were combined to calculate the drug response score. A score from 0 to 60 indicates that the treatment is more effective on cancer cells, while a score from 0 to -60 suggests that the treatment has a greater impact on stromal cells. The R package PharmacoGx was utilized to determine dose-response values, slope, and area under the curve (AUC). Specifically, we used the computeAUC function from the PharmacoGx package to calculate the AUC for the drug dose viability curve. This function takes drug concentration and cell viability as inputs and normalizes the AUC by the concentration range. The AUC, representing the response area (1 - Viability), is calculated as the area under the curve on a log10 concentration scale. A higher AUC indicates greater sensitivity to the drug.

* The concentrations used were chosen based on clinically relevant levels of anticancer drugs, with the upper limit concentration (the highest dose) set as the maximum concentration reached in human plasma during therapy (Cmax) (10).

Results

Two LSCC primary cell cultures (TR36 and TR87) were derived from patients with stage II disease, while three (TR33, TR34, and TR107) were derived from patients with stage III. A month after surgical resection, three LSCC patients (TR87, TR33, and TR107) were scheduled to undergo three cycles of cisplatin-etoposide chemotherapy, but TR36 did not attend the follow-up examination after surgery, and TR34 experienced disease progression immediately after surgery

and passed away three months later. The LSCC patients who were supposed to receive chemotherapy did not receive it at the hospital where they were enrolled in this study.
Five primary cell cultures derived from LSCC patients were exposed to eight chemotherapeutics (cisplatin, carboplatin, etoposide, paclitaxel, docetaxel, gemcitabine, pemetrexed, and vinorelbine) and an EGFR inhibitor (erlotinib).
Stage II LSCC samples (TR36 and TR87) exhibited a similar selectivity pattern towards cancer cells, with only pemetrexed showing a more pronounced effect on cancer cells than on stromal cells (Figure 1). TR87 demonstrated a superior response to all chemotherapeutics compared to TR36 (Figure 1). TR36 displayed significant resistance to all chemotherapeutics, with none of them reaching 50% inhibition in the case of cancer cells within the given concentration range (Figure 1). On the other hand, carboplatin, cisplatin, and gemcitabine exhibited noteworthy anticancer activity against TR87 but with a similar effect on stromal cells (Figure 1).
Stage III LSCC samples (TR33, TR34, and TR107) exhibited varied responses without following a consistent pattern (Figure 2). TR33 exhibited a positive response only to cisplatin, whereas TR34 responded well to cisplatin and gemcitabine

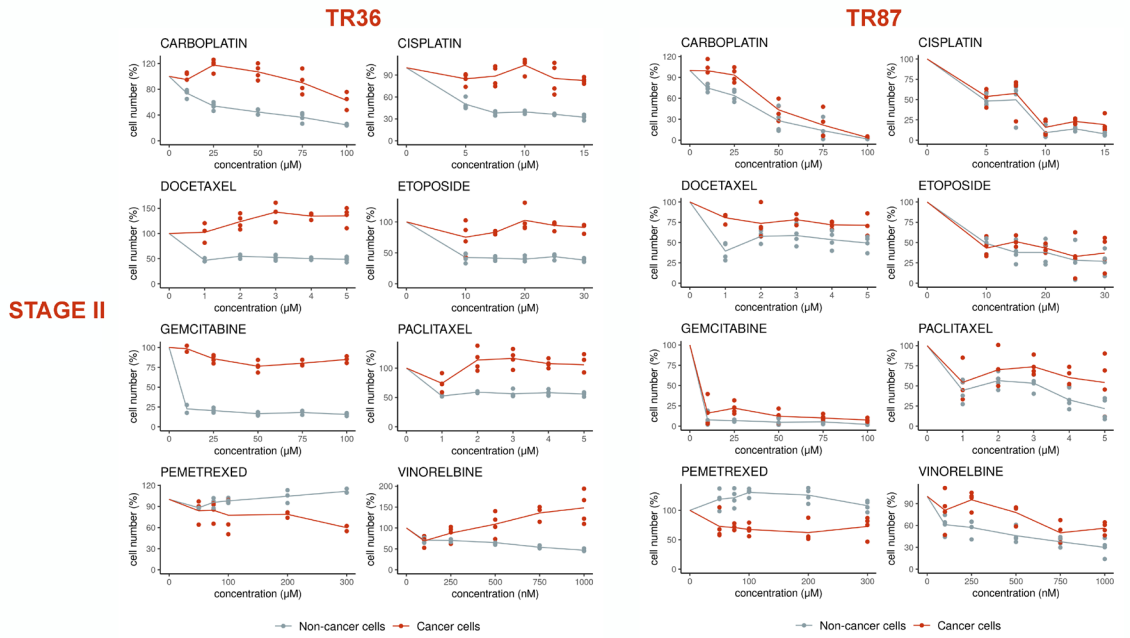

Figure 1. Cytotoxicity of 8 chemotherapeutics evaluated in two patient-derived lung squamous cell carcinoma (LSCC) cultures stage II. The CK8/18 antibody was used to discriminate between cancer and non-cancer (stromal) cells in a mixed culture. Treatments with chemotherapeutics lasted for 7 days. Values are expressed as mean ± standard error of the mean (SEM) (n = 4).

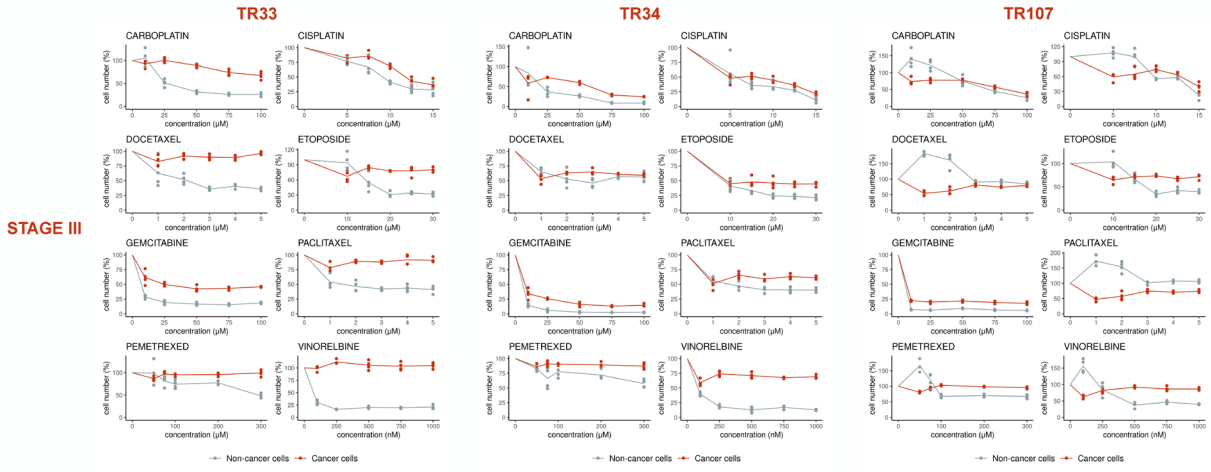

Figure 2. Cytotoxicity of 8 chemotherapeutics evaluated in three patient-derived lung squamous cell carcinoma (LSCC) cultures stage III. The CK8/18 antibody was used to discriminate between cancer and non-cancer (stromal) cells in a mixed culture. Treatments with chemotherapeutics lasted for 7 days. Values are expressed as mean ± standard error of the mean (SEM) (n = 4).

(Figure 2). TR107 displayed the most favorable selectivity pattern, demonstrating a preference for cancer cells, particularly at lower concentrations of carboplatin, cisplatin, docetaxel, and paclitaxel. Nevertheless, only carboplatin and gemcitabine showed significant anticancer effects in the TR107 sample (Figure 2).
All LSCC samples exhibited a consistent response to erlotinib, as shown in Figure 3. Erlotinib demonstrated selectivity

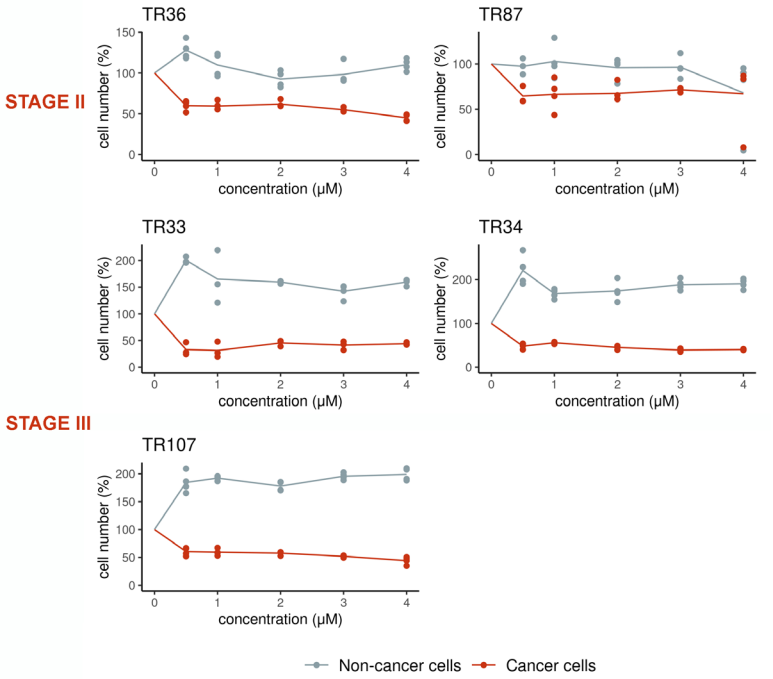

Figure 3. Cytotoxicity of erlotinib evaluated in five patient-derived lung squamous cell carcinoma (LSCC) cultures. The CK8/18 antibody was used to discriminate between cancer and non-cancer (stromal) cells in a mixed culture. Treatments with chemotherapeutics lasted for 7 days. Values are expressed as mean ± standard error of the mean (SEM) (n = 4).

towards cancer cells across all five LSCC patients' samples and exhibited biphasic activity, achieving 50% inhibition of cancer cell growth at the lowest concentration of 0.5 μM (Figure 3). However, the anticancer effect of erlotinib was not further enhanced with higher concentrations (Figure 3).

Scoring results have validated these findings and have provided a comprehensive overview of the responses observed in LSCC patients (Figure 4). A score above 10 is indicative of a good response. Notably, TR36 and TR33 exhibited positive responses exclusively to erlotinib, TR87 responded well to erlotinib, cisplatin, and carboplatin, with the most significant response to gemcitabine, TR34 responded favorably to erlotinib, cisplatin, and gemcitabine, while TR107 showed positive responses to erlotinib, carboplatin, gemcitabine, paclitaxel, and docetaxel (Figure 4).

Discussion

The treatment landscape for NSCLC is rapidly evolving, especially in EGFR inhibitors and immunotherapy, with many ongoing clinical trials. Advances in molecular characterization have led to improved treatment options and increased survival rates for LADC. However, targeted molecular therapies still need to make significant progress for LSCC (11). An earlier study demonstrated the benefits of erlotinib as a second-line therapy for advanced LSCC (BR.21 trial). It showed increased median overall survival and reduced tumor-related symptoms such as dyspnea, pain, and cough compared to the placebo group (12).

In LSCC, identifying predictive markers for responsiveness to EGFR inhibitors like erlotinib is a critical area of research. High levels of EGFR protein expression may correlate with a better response to erlotinib (13). While gene amplification tends to be more common in LADC, it can also play a role in a subset of LSCC. Although activating mutations in the EGFR gene are less frequent in LSCC than in LADC, their presence can significantly influence sensitivity to erlotinib. Mutations such as L858R in exon 21 or deletions in exon 19 enhance the response to erlotinib (14).

Erlotinib explicitly targets the ATP-binding site of the EGFR, leading to multiple downstream effects on signaling pathways that promote cell proliferation and survival, including the MAPK and PI3K/AKT pathways. Altered expression of downstream signaling molecules, such as PI3K/AKT, may change the response to EGFR inhibition. Furthermore, co-alterations in other pathways, such as RAS mutations or loss of PTEN, may influence the response to erlotinib (15).

Treatment with erlotinib can lead to cell cycle arrest, particularly at the G1 phase, and promote apoptosis in cancer cells (16). In vitro studies have shown that NSCLC cell lines exhibit decreased proliferation rates and increased markers of apoptosis (e.g., cleaved PARP and caspases) in response to erlotinib (17).

While the efficacy of EGFR inhibitors in treating LSCC after previous treatment failures (second and further-line therapy) is established, there is ongoing debate regarding their effectiveness in first-line and combination therapies for LSCC. Despite concerns about potential toxicity, EGFR inhibitors continue to be considered a viable option due to their oral administration, tolerability, and positive impact on patients' quality of life (13).

Our functional screening approach demonstrated that all five LSCC samples responded favorably to erlotinib, outperforming platinum drugs. Three of the five LSCC patients (TR87, TR34, and TR107) could benefit from platinum-based treatment, the standard of care for LSCC patients. TR36 and TR33 showed insensitivity to cisplatin and carboplatin.

LSCC		erlotinib	cisplatin	carboplatin	etoposide	pemetrexed	gemcitabine	paclitaxel	docetaxel	vinorelbine
Stage II	TR36	20.98	-30.94	-35.06	-31.34	1.57	-35.52	-29.41	-37.56	-35.05
	TR87	17.55	19.02	12.61	5.65	-8.42	35.74	7.01	-19.23	-24.73
Stage III	TR33	27.97	6.30	-34.54	-31.44	-53.11	-2.15	-29.55	-36.14	-40.25
	TR34	28.74	14.13	2.30	3.68	-44.77	20.84	-12.06	9.61	-15.50
	TR107	21.75	6.07	18.26	-18.00	-57.85	21.67	16.06	13.35	-26.88

60	15	-20
50	10	-25
40	5	-30
35	0	-35
30	-5	-40
25	-10	-50
20	-15	-60

Figure 4. Drug sensitivity scoring array for five patient-derived lung squamous cell carcinoma (LSCC) cultures against 8 chemotherapeutics and erlotinib. Scores from 0 to 60 indicate increasing efficacy in toxicity towards cancer cells, while scores from 0 to -60 reflect cases in which non-cancer (stromal) cells are more sensitive than cancer cells. Responses with a score above 10 are considered beneficial.

None of the samples responded to etoposide, and only one LSCC sample (TR107) exhibited sensitivity to paclitaxel and docetaxel, suggesting that the standard approach of combining platinum drugs with either etoposide or paclitaxel may offer little to no benefit to LSCC patients. Notably, three out of five LSCC samples (TR87, TR34, and TR107) positively responded to gemcitabine. Recent findings indicate that platinum-gemcitabine combination therapy is highly effective among platinum doublet regimens and holds promise as a treatment for advanced LSCC (18).
Among five LSCC patients, TR33 has shown the highest tumor mutation burden (TMB) according to our earlier publication (8). TR34, TR36, and TR107 had a considerable TMB, while TR87 had the lowest TMB in the examined NSCLC patients' cohort (8). In a recent study, it was observed that NSCLC patients in the low TMB group demonstrated a significantly longer progression-free survival (PFS) when treated with chemotherapy (19). Conversely, NSCLC patients in the high TMB group exhibited extended PFS when administered immunotherapy subsequent to unsatisfactory chemotherapy (19). In line with these findings is the unresponsiveness of TR33, with the highest TMB (1476 mutations), to 8 tested chemotherapeutics. Mutations in TP53, which are characteristic of LSCC patients, were identified in four out of five samples: in TR36 and TR107 (missense mutations), TR33 (splice site), and TR34 (a nonsense mutation) (8). The additional mutation that can influence drug response identified in TR36 is a PIK3CA missense mutation (8), a common LSCC driver (20). Concomitant TP53 and PIK3CA mutations may cause TR36 resistance to all tested chemotherapeutics. A recent study has shown that both mutated genes are associated with poorer therapeutic responses compared to the TP53 mutation alone in breast cancer (21).

Conclusion

Our research suggests that conducting functional sensitivity screening is essential for determining the optimal treatment strategies for each LSCC patient. Our previous study found that erlotinib was equally effective in patient samples with low and high TMB, regardless of their EGFR mutational status (8). The concern that many LSCC patients may lack well-defined biomarkers to predict their response to anticancer drugs can be addressed by the functional approach we utilized. Our scoring method, which considers selectivity, efficacy, and inhibitory pattern (dose-dependency), is particularly valuable for guiding treatment decisions. Furthermore, we observed that erlotinib exhibited anticancer effects in all patients at the lowest tested concentration, and this effect did not increase with higher concentrations. This suggests that the dose of erlotinib for LSCC patients could be reduced without compromising its efficacy.

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