Aus der Medizinischen Klinik V
Klinik der Universität München

Vorstand: Prof. Dr. Jürgen Behr

Hedgehog pathway activation might mediate pemetrexed resistance in NSCLC cells

Dissertation

zum Erwerb des Doktorgrades der Medizin
an der Medizinischen Fakultät der
Ludwig-Maximilians-Universität München

vorgelegt von
Yichao Liu

aus
Jinan

Jahr
2022

Mit Genehmigung der Medizinischen Fakultät
der Universität München

Berichterstatter:	Prof. Dr. Rudolf M. Huber
Mitberichterstatter:	PD Dr. Svetlana Sirko Prof. Dr. Udo Löhrs
Mitbetreuung durch die promovierten Mitarbeiter:	Dr. Tian Fei, Dr. D. Kauffmann
Dekan:	Prof. Dr. med. Thomas Gudermann
Tag der mündlichen Prüfung:	19.05.2022

Table of contents

Zusammenfassung

Hintergrund: Lungenkrebs ist bekannt für eine hohe Morbidität und Mortalität bei allen Malignomen. Als eine häufige Art von Lungenkrebs teilt der nicht-kleinzellige Lungenkrebs (NSCLC) die beiden Merkmale mit Lungenkrebs. Ungefähr 80%-85% der Patienten mit Lungenkrebs werden als NSCLC diagnostiziert. Eine chemotherapeutische Behandlung ist insbesondere für Patienten mit fortgeschrittener oder metastasierter Erkrankung unerlässlich. Resistenzen gegen Chemotherapeutika sind häufige und fatale Hindernisse für die Verbesserung des Gesamtüberlebens während der Chemotherapie des NSCLC, da die Morbidität von NSCLC hoch ist und Chemotherapeutika umfassend eingesetzt werden. Die Verringerung oder Umkehrung der Chemoresistenz von NSCLC kann der Schlüssel des Problems sein. Der Hedgehog (Hh)-Signalweg zeichnet sich durch eine hohe Konservierung in der Entwicklung aus. Der Hh-Signalweg hat große Auswirkungen auf die Embryonalentwicklung, Zellproliferation und Differenzierung. Frühere Studien legten nahe, dass der Hh-Signalweg nicht nur mit der Entstehung und Entwicklung von Tumoren verbunden ist, sondern auch die Chemoresistenz von Tumoren verursacht und aufrechterhält. Ein aktivierter Hh-Signalweg bei mehreren Krebsarten könnte eine wirksame Umgehnung bei der Milderung der Chemoresistenz von Tumoren sein.

Methoden: Um Pemetrexed-Resistenz zu induzieren und aufrechtzuerhalten, wurden HCC827-Zellen mit Kulturmedium inkubiert, das 130 nM Pemetrexed enthält. Der MTT-Assay wurde angewendet, um die Lebensfähigkeit naiver und Pemetrexed-resistenter Zellen unter dem Einfluss des Hh-Signalweg-Inhibitors Vismodegib und Gant61 zu testen. Die mRNA-Expressionsniveaus von SHH, PTCH1, HHIP, SMO, Gli1, Gli2 und Gli3 wurden durch qPCR nachgewiesen, um die Beteiligung des Hh-Signalwegs in naiven und resistenten Zellen zu beurteilen. Der CellTiter-Glo-Zelllebensfähigkeitsassay wurde angewendet, um die Sensitivität von naiven Zellen und Pemetrexed-resistenten Zellen gegenüber Pemetrexed zu beurteilen, indem der Hh-Signalweg mit Gant61 blockiert wurde.

Ergebnisse: Nach den Ergebnissen der MTT-Proben zeigte Gant61 bei steigenden Konzentrationen eine bessere Hemmwirkung auf Pemetrexed-resistente HCC827-Zellen als Vismodegib. Die meisten verwandten Proteine des Hh-Signalwegs wurden in Pemetrexed-resistenten Zellen überexprimiert. Gli1, Gli2 und Gli3 wurden in Pemetrexed-resistenten Zellen im Vergleich zu naiven Zellen 3,7-, 4,3- bzw. 3,5-fach überexprimiert. Der Ligand SSH wurde in Pemetrexed-resistenten Zellen 5,9-fach überexprimiert. Die Lebensfähigkeit naiver Zellen, die mit Pemetrexed, Gant61 und der Kombination von Pemetrexed bzw. Gant61 behandelt wurden, unterschied sich nicht deutlich. Die Lebensfähigkeit von Pemetrexed-resistenten Zellen hingegen war insbesondere unter dem Einfluss von Gant61 bzw. Pemetrexed plus Gant61 sichtbar vermindert. Pemetrexed-resistente Zellen waren im Vergleich Pemetrexed nicht empfindlich, aber im Gegensatz zu naiven Zellen gegenüber Gant61 anfälliger.

Schlussfolgerung: Der Hh-Signalweg ist in Pemetrexed-resistenten NSCLC-Zellen hyperaktiviert, was bedeutet, dass der Hh-Signalweg auch den Prozess der Chemoresistenz bei NSCLC vermittelt. Die Anfälligkeit von Pemetrexed-resistenten NSCLC-Zellen gegenüber Pemetrexed kann durch eine Blockierung des Hh-Signalwegs verbessert oder wiederhergestellt werden. Der Hh-Signalweg könnte in Zukunft ein potenzielles therapeutisches Ziel für Patienten mit NSCLC sein, insbesondere mit Chemoresistenz.

Abstract

Parts of the abstract were already published in Liu et al., 2020 [1]

Background: Lung cancer is well known for high morbidity and mortality rate among all malignancies. As one common type of lung cancer, non-small cell lung cancer (NSCLC) shares the two features with lung cancer. Approximately 80%-85% of patients with lung cancer are diagnosed as NSCLC. Chemotherapeutic treatment is essential especially for the patients with advanced or metastatic disease. Resistance to chemotherapeutic agents are common and fatal obstacles of improving overall survival during the chemotherapy for NSCLC since high morbidity rate of NSCLC and extensive application of chemotherapeutic agents. Reducing or reversing the chemoresistance of NSCLC may be the key towards the problem. Hedgehog (Hh) signaling pathway is characterized by high conservation in evolution. Hh signaling pathway has great effects on embryonic development, cell proliferation and differentiation. Previous studies suggested that Hh signaling pathway was not only connected with genesis and development of tumors, but also gave rise to and maintain chemoresistance of tumors. Activated Hh signaling pathway in several types of cancers might be a potent bypass in mediating chemoresistance of tumors.

Methods: To induce and maintain pemetrexed resistance, HCC827 cells were incubated with culture medium which contains 130nM pemetrexed. MTT assay was applied to test the viability of naïve and pemetrexed-resistant cells under the influence of Hh signaling pathway inhibitor vismodegib and Gant61. The mRNA expression levels of SHH, PTCH1, HHIP, SMO, Gli1, Gli2 and Gli3 were detected by qPCR to assess the participation of Hh signaling pathway in naïve and resistant cells. The CellTiter-Glo cell viability assay was applied to assess the sensitivity of naïve cells and pemetrexed-resistant cells to pemetrexed by blocking Hh signaling pathway with Gant61.

Results: According to the result of MTT assay, Gant61 appeared better inhibitory efficacy on pemetrexed-resistant HCC827 cells than vismodegib following the increasing concentrations. Most of related Hh signaling pathway proteins were overexpressed in pemetrexed-resistant cells. Gli1, Gli2 and Gli3 were overexpressed by 3.7-, 4.3- and 3.5- fold respectively in pemetrexed-resistant cells compared with that in naïve cells. The ligand SSH was overexpressed by 5.9-fold in pemetrexed-resistant cells. The viability of naïve cells that were treated with pemetrexed, Gant61, and the combination of pemetrexed and Gant61 respectively was not distinctly different. The viability of pemetrexed-resistant cells, by contrast, was visibly decreased especially under the influence of Gant61 or pemetrexed plus Gant61. Pemetrexed-resistant cells were not sensitive to pemetrexed, but more susceptible to Gant61 in contrast to naïve cells.

Conclusion: Hh signaling pathway is hyperactivated in pemetrexed-resistant NSCLC cells, which also means Hh signaling pathway mediates the process of chemoresistance in NSCLC. The susceptibility of pemetrexed-resistant NSCLC cells to pemetrexed can be

improved or restored by obstructing Hh signaling pathway. Hh signaling pathway may be a prospective therapeutic target for patients with NSCLC especially with chemoresistance in future.

List of figures

Figure 1 was adapted from Bray et al., 2018 [2], Figure 2 was adapted from Taciak et al., 2018 [3], Figure 5, 6, 7 and 8 were already published in Liu et al., 2020 [1].

List of tables

Table 1 was adapted from Postmus et al., 2017 [4] and Planchard et al., 2020 [5]. Parts of the contents in the table 2 were published in Liu et al., 2020 [1]

List of abbreviations

Abbreviation	Stands For
ABC	ATP-binding cassette
ALK	anaplastic lymphoma kinase
ALK-TKI	tyrosine kinase inhibitors for ALK
AML	acute myeloid leukemia
AP-1	activator protein-1
APC	adenomatous polyposis coli
AREG	amphiregulin
at-MDR	atypical MDR
ATP	adenosine triphosphate
Bcl-2	B-cell lymphoma-2
BSO	Buthionine Sulfoximine
CK1	casein kinase 1
CK1α	casein kinase 1α
CPTs	camptothecins
CV	cisplatin+vinblastine
DHH	Desert hedgehog
DTCs	disseminated tumor cells
Dvl	dishevelled
EGF	epidermal growth factor
EGFR	Epidermal growth factor receptor
EGFR-TKI	tyrosine kinase inhibitors for epidermal growth factor receptor
EMA	European Medicines Agency
EML4	echinoderm microtubule-associated protein-like 4
EMT	epithelial-mesenchymal transition
ESMO	European Society for Medical Oncology
Fzd	Frizzled
GARFT	glycinamide ribonucleotide formyltransferase
GliR	Gli repressor
Gli2A	Gli2 activator
Gli2R	Gli2 repressor
Gli3A	Gli3 activator
Gli3R	Gli3 repressor
GPX	glutathione peroxidase
GR	glutathione reductase
GRK2	G protein coupled receptor kinase 2
GS	glutathione synthetase
GSH	glutathione
GSK3	glycogen synthase kinase 3
GSTs	glutathione S-transferases

GST-π	glutathione S-transferases Pi
HER	human epidermal growth factor receptor
Hh	hedgehog
IC_{50}	half maximal inhibitory concentration
HHIP	Hh-interacting protein
IgG1κ	human immunoglobulin G1 kappa
IHH	Indian hedgehog
JNK	c-Jun N-terminal kinase
KUM	Klinikum der Universität München
LRP5/6	low-density lipoprotein receptor-related protein 5/6
MDR	multidrug resistance
MEK1	mitogen-activated protein kinase kinase 1
MEK2	mitogen-activated protein kinase kinase 2
MIC	mitomycin C+ifosfamide+cisplatin
MTT	3-[4,5-Dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide
MVP	mitomycin C + vinblastine+cisplatin
NF-κB	nuclear factor κB
NP	vinorelbine+cisplatin
NSCLC	non-small cell lung cancer
PD-1	programmed cell death protein 1
PD-L1	programmed death-ligand 1
PE	cisplatin+ etoposide
P-gp	p-glycoprotein
PKA	protein kinase A
PP2A	protein phosphatase 2A
PTCH	Patched
RhoGTPases	Ras homologue GTPases
ROS1	c-ros oncogene 1 receptor tyrosine kinase
RRM1	ribonucleotide reductase subunit M1
RTK	receptor tyrosine kinase
SCLC	small cell lung cancer
SHH	Sonic hedgehog
SLC19A1	solute carrier family 19 member1
SMO	Smothen
SUFU	suppressor of fused
TNF-α	tumor necrosis factor-α
TOPO	topoisomerase
TS	thymidylate synthase
UNG	uracil–DNA glycosylase
VEGF	vascular endothelial growth factor
VLB	vinblastine
Wg	wingless gene

γ-GT	γ-glutamyltransferase

1. Introduction

Parts of the introduction were already published in Liu et al., 2020 [1]

1.1 Lung cancer

On account of high morbidity and high mortality, lung cancer has been hindering the health as a universal issue all over the world. It is common clinical malignant tumor and also the most lethal malignancy in the world [2]. As figure 1 shows, it is estimated that there were about 2.1 million new patients who were suffering from lung cancer in the world in 2018, accounting for 11.6% of all tumors, and about 1.76 million people died of lung cancer, accounting for 18.4% of all tumor deaths [2].

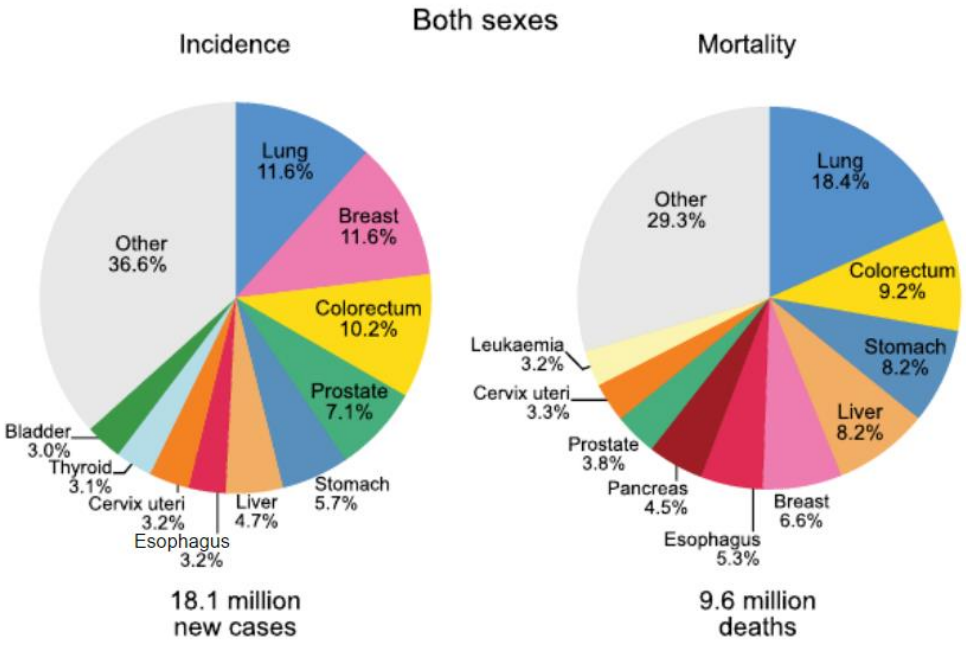


Figure 1. The rates of morbidity of mortality for cancers in 2018. Adapted from Bray et al., 2018 [2]

In general, on the basis of histopathological classification, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) are classified as lung cancer. And squamous cell carcinoma, adenocarcinoma, and large cell carcinoma are classified as NSCLC, which is accumulated up to 85% of the entire lung cancer cases [6]. As a highly malignant type and an undifferentiated lung cancer, SCLC can be characterized by expression of neuroendocrine markers, it is generally thought to originate from Kulchitzky cells in bronchial mucosal epithelium or glandular epithelium [7]. Some researchers believe that it is derived from stem cells that can differentiate into neuroendocrine cells [8]. Lung adenocarcinoma, however, is attracting more and more attention with highest morbidity, high aggressiveness and fatality in most countries of the world [9, 10]. Uncontrolled

proliferation, early metastasis, and anti-apoptosis are typical of Lung adenocarcinoma [11-13]. It is also one of the most frequent reasons behind tumor recurrence and patient death due to its metastasis and chemoresistance. Due to the lack of clear and effective tumor markers and reliable early screening methods, the onset of NSCLC has no symptoms and obvious signs, patients are often at the advanced stage when they are diagnosed. Although medical treatment technology is improving with the advances of molecular biology research, the prognosis of lung cancer and the five-year survival rate of patients with lung cancer are still not as optimistic as we expect [14, 15].

1.2 Treatment of NSCLC

At present, the treatment of NSCLC mainly includes surgical treatment, targeted therapy, immunotherapy, chemotherapy.

1.2.1 Surgical treatment

Generally speaking, patients with stage I or II of lung cancer may receive surgical intervention, which gives rise to not only advantageous prognosis but also 5-year survival rates of 70–90% especially for patients with stage I lung cancer [16]. A study, however, described postoperative locally recurrent rates of 24% following surgery, even up to 19% for patients with stage I lung cancer [17]. Although lung cancer patients at early stage often receive surgery as the main therapy, not only risks of mortality, but also metastasis should be taken into account [18]. Adjuvant chemotherapy after radical surgery has gradually been applied to the patients with early-stage cancers, especially NSCLC, who can undergo surgery as a standard therapy mode. Adjuvant chemotherapy, to a certain extent, can eliminate residual micrometastasis, reduce the chance of local recurrence and distant metastasis [19]. Douillard found that adjuvant chemotherapy after radical resection could prolong the postoperative survival of patients with early-stage NSCLC who can undergo surgery [20]. European Society for Medical Oncology (ESMO) has recommended that adjuvant chemotherapy that is based on cisplatin is applied to patients with resected stage II and III NSCLC or resected stage IB with a primary tumor larger than 4cm [4]. Common adjuvant chemotherapy based on cisplatin for patients with NSCLC includes “mitomycin C+ifosfamide+cisplatin (MIC), mitomycin C + vinblastine+cisplatin (MVP), cisplatin+vinblastine (CV), vinorelbine+cisplatin (NP), cisplatin+ etoposide (PE)” [21] and so on. Among them, NP is the most frequently studied until now. With the emergence of better chemotherapeutic agents, adjuvant chemotherapy will exert an enormous function on further improving the survival rate of NSCLC.

1.2.2 Radiotherapy

Radiotherapy is that killing tumor cells directly or indirectly through the effect of ionizing radiation, so as to reach the goal of disease control. Radiotherapy has a wide range of applications in tumor treatment, almost all malignant solid tumors can be treated with radiation. Radiotherapy is also one of the important treatments for NSCLC. About 64.3%

of patients with NSCLC need radiation therapy, which is also recommended to 45.9% of patients with NSCLC in their initial treatment [22]. In order to obtain better therapeutic effect, radiotherapy is usually combined with surgical treatment or chemotherapy. Preoperative radiotherapy can reduce tumor volume, narrow the operative region, increase the success rate of surgery, and reduce the possibility of the spread cancer cells during surgery [23]. Postoperative radiotherapy can reduce the local recurrence rate of cancer and contribute to the high survival rates of patients with cancer [24]. Chemotherapy can treat the lesions in situ and metastatic lesions, but the lesions in situ are prone to resistance to chemotherapy or recurrence after treatment, while radiotherapy can focus on local lesions, a reasonable combined application of chemotherapy and radiotherapy has complementary advantages. With the development of medical technology, three-dimensional and four-dimensional radiotherapy techniques are gradually being used in clinic, three-dimensional radiotherapy adjusts the radiation field shape so that the dose distribution can be consistent with the shape of targeted area in tumor, and target area can be exposed to a larger dose, at the same time, the normal tissues around targeted area are less exposed to radiation [25], four-dimensional radiotherapy is based on three-dimensional radiotherapy, can more accurately locate and treat the lesion through dynamic analysis of the lesion within a certain period of time [26]. Compared with traditional two-dimensional radiotherapy, they have many advantages such as small side effects, high curative effects, personalization and so on [25, 26].

1.2.3 Targeted therapy

With the continuous exploration of basic research about cancer, the mechanism of tumorigenesis and development is also better known than before. The significant progress in targeted therapy is one of the momentous results of continuous progress in cancer research. Targeted therapy which has been validated in clinical treatment plays a very good role in NSCLC with EGFR, ALK or ROS1 alterations.

As a member of human epidermal growth factor receptor (HER) family, Epidermal growth factor receptor (EGFR) is a kind of glycoprotein transmembrane receptor with tyrosine kinase that has a strong effect on cell proliferation, migration and angiogenesis [27]. EGFR is commonly detected in many kinds of tumors and is activated by various ligands such as amphiregulin (AREG) and epidermal growth factor [27]. If EGFR is activated by ligands, it will dimerize, which causes the domains in the cell membrane to activate tyrosine kinase, thereby activates downstream signal transduction pathways and ultimately leads to survival of tumor cells and continuous proliferation [27]. Tyrosine kinase inhibitors for epidermal growth factor receptor (EGFR-TKI) can inhibit downstream signals by competing with adenosine triphosphate (ATP) which can connect to the intracellular domains of EGFR, preventing autophosphorylation and receptor activation, and inhibits the growth and metastasis of tumor [28]. ESMO recommend that Gefitinib, afatinib, osimertinib, erlotinib and dacomitinib were used as the first-line chemotherapeutic agents for metastatic NSCLC patients with EGFR mutations [5], At present, EGFR-TKI can effectively contribute

to the high survival rate of patients with NSCLC, enhance the quality of patients' life, and has lower side effects than traditional chemotherapy, furthermore it is also easier to be tolerated by patients [5].

As a kind of receptor tyrosine kinase (RTK), anaplastic lymphoma kinase (ALK) is responsible for the signal transduction of the cell, and transmits the signal received on the cell surface to the cell [29]. Common pathogenic mutations in ALK gene are gene fusion, the EML4-ALK fusion formed by the tyrosine kinase region of ALK protein and echinoderm microtubule-associated protein-like 4 (EML4) has been widely seen in NSCLC [30]. Fused EML4-ALK exhibits a strong carcinogenic effect, it can activate the tyrosine kinase activity of the corresponding proteins in the cell, give rise to the activation of downstream cell signaling pathways related to cell proliferation in turn, and eventually cause malignancy [30]. Soda et al. also showed that EML4-ALK induced the occurrence of lung cancer in mouse models, and after application of tyrosine kinase inhibitors for ALK (ALK-TKI), rapid regression of tumor lesions can be observed [31]. As ALK-TKI, crizotinib, ceritinib, brigatinib, lorlatinib and alectinib are considered as first-line chemotherapeutic agents for ALK-positive patients with NSCLC [5], and the clinical effectiveness of ALK-TKI has been confirmed by clinical trials, which is significantly stronger than traditional chemotherapy [32, 33].

C-ros oncogene 1 receptor tyrosine kinase (ROS1) is very similar to ALK in structure and function [34]. ROS1 gene can be fused with multiple genes, and activates the intracellular tyrosine kinase domain, followed by activating multiple downstream signal pathways, further promoting cell proliferation [34]. When ROS1 is fused with other genes, it generally retains the kinase domain [34]. As one of ALK-TKI, crizotinib is not only applied in ALK gene-positive NSCLC patients, but also effective in NSCLC patients with ROS1 fusion genes [35]. Some Researchers believed that this might be due to the 49% amino acid sequence homology between ROS1 and ALK in the kinase domain [36]. European Medicines Agency (EMA) recommended crizotinib for the treatment to ROS1-positive advanced NSCLC patients [37].

BRAF gene is responsible for encoding B-RAF protein that is serine/threonine-protein kinase and transmits cell signals [38, 39]. Mitogen-activated protein kinase kinase 1 (MEK1) and mitogen-activated protein kinase kinase 2 (MEK2) are the substrates of B-Raf, which is the main activated kinase of MEK [39]. MEK1 and MEK2 can activate extracellular signal-regulated protein kinase (ERK) which has great effects on proliferation, apoptosis, differentiation of cells [39]. In other words, BRAF can activate Ras-Raf-MEK-ERK signaling pathway that is also known as MAPK/ERK signaling pathway, and acts as a bridge between RAS and MEK in the MAPK signaling pathway. Therefore, when the BRAF gene mutates, MAPK/ERK pathway is continuously activated, which leads to the occurrence and development of tumors [40]. There are many types of BRAF gene mutations currently discovered. the most common one is the mutation from T to A in 1799^{th} nucleotide of exon 15 of BRAF gene, which causes the coded valine to be replaced by glutamine, namely BRAFV600E [41]. The incidence of BRAF gene mutations is 3.5%-4% in patients with NSCLC, in which, BRAFV600E mutations accounted for 50% [42], so targeted therapy for BRAF mutations especially BRAFV600E mutations in NSCLC is important. As the B-Raf

inhibitor and MEK1/2 inhibitor respectively, the higher combined efficacy of dabrafenib and trametinib has been proved compared with independent application of dabrafenib [43], their combination has been recommended by EMA as the chemotherapy to BRAFV600E-positive patients with advanced NSCLC [44].

1.2.4 Immunotherapy

Immunotherapy is also an important breakthrough as the therapy for lung cancer with the momentous progress of cancer research, it refers to the method of treating tumors by regulating the body's own immune cells, it represented by programmed cell death protein 1 (PD-1) inhibitors has also obtained very good results in clinic treatment of tumors such as melanoma [45]. PD-1 is an important protein that prevents the activation of immune cells, cancer cells often take advantage of this mechanism to escape the killing of immune cells to themselves [46]. As PD-1 inhibitors, the significance of nivolumab and pembrolizumab in the treatment of NSCLC has been proved in several clinical trials and has been incorporated into clinical applications [47, 48]. When programmed death-ligand 1 (PD-L1) combines with PD-1 on the surface of T cells, the ability of T cells that attack and kill tumor cells is suppressed through the PD-1/PD-L1 pathway [46], therefore, the reduction of PD-L1 may be related to tumor immune response and immune escape of tumor cells in NSCLC. Mu et al. reported that PD-L1 might has a negative impact on NSCLC patient survivability and be considered as a poor prognostic signal [49]. Compared with traditional chemotherapy, atezolizumab, as antibody targeting PD-L1, has not only been used to treat NSCLC with good anti-tumor effects in clinical trials, but also improved the survival rates of patients with NSCLC [50]. It can be seen that PD-L1 still plays a very significant regulatory role in promoting the genesis and progression of tumors, and targeted PD-L1 therapy can also effectively inhibit tumor growth. As the inhibitor that targets PD-L1, durvalumab is a human immunoglobulin G1 kappa (IgG1κ) monoclonal antibody [51], and can block PD-L1 interaction with T cells to relieve immune suppression mediated by PD-1/PD-L1 and promote T cells to attack tumor cells by attaching PD-L1. Some clinic research reported durvalumab, with its manageable safety, had better efficacy for advanced NSCLC patients than placebo [51]. In 2018, EMA approved durvalumab as a treatment for unresectable stage III NSCLC patients with PD-L1-positive tumor when their disease had not progressed after concurrent platinum-based, radiotherapy and chemotherapy [52]. Although some excellent results have been achieved in immunotherapy, and more and more researchers pay attention to immunotherapy in recent years, it is still in its infancy, more rigorous large-scale clinical research is needed for further exploration.

1.2.5 Chemotherapy

Chemotherapy, a traditional tumor treatment, has great effects on the treatment of lung cancer, and could positively impact the patients regardless of cancer stages and the patients who cannot tolerate surgery. Preoperative or postoperative chemotherapy is known as adjuvant chemotherapy, evidence has indicated the advantages of adjuvant

chemotherapy for patients with early stage of NSCLC after surgery, which boosted 5-year survival rates by 4% [53]. Chemotherapy in combination with definitive radiation treatments are highly recommended for patients with stage III of NSCLC, especially with good performance scores and minimal weight loss [54], In advanced stage IV of NSCLC, chemotherapy is typically the main treatment [55]. Cisplatin, carboplatin, docetaxel, gemcitabine, pemetrexed, paclitaxel and vinorelbine are the most commonly used in clinic as chemotherapeutic drugs. In general, two of them are combined to expand the therapeutic effects on NSCLC while minimizing side effects. Platinum-based chemotherapy, as the first-line treatment, has not only been recommended for advanced NSCLC [56], but also applied in adjuvant chemotherapy [57]. According to ESMO guidelines for NSCLC [4, 5], approved treatment options have been listed as follows (table 1).

Table 1. ESMO clinical practice guidelines for non-small cell lung cancer. Adapted from Postmus et al., 2017 [4] and Planchard et al., 2020 [5]

<table>
<tr><th colspan="6">Chemotherapy for Non-Small Cell Lung Cancer</th></tr>
<tr><td>Patients with resected stage II and III NSCLC or resected stage IB with a primary tumor larger than 4 cm</td><td colspan="5">Cisplatin-based ChT:
Cisplatin+vinorelbine
Cisplatin+gemcitabine
Cisplatin+docetaxel
Cisplatin+pemetrexed</td></tr>
<tr><td>Patients with unresectable locally advanced NSCLC (stage III)</td><td colspan="5">Cisplatin-based ChT:
Cisplatin+etoposide
Cisplatin+vinorelbine
Cisplatin+pemetrexed</td></tr>
<tr><td rowspan="4"></td><td colspan="5">PD-L1 expression and molecular tests negative (ALK/BRAF/EGFR/ROS1)</td></tr>
<tr><td colspan="4">Any expression of PD-L1</td><td>PD-L1≥50%</td></tr>
<tr><td colspan="3">PS 0-2</td><td>PS 3-4</td><td>PS 0-1</td></tr>
<tr><td>Nivolumab/ipilimumab+platinum/pemetrexed followed by nivolumab/ipilimumab</td><td>PD-L1-positive</td><td>PD-L1-negative</td><td>BSC</td><td>Pembrolizumab</td></tr>
</table>

<table>
<tr><td rowspan="11">Patients with stage IV NSCLC</td><td colspan="2">Atezolizumab/bevacizumab+carboplatin/paclitaxel followed by atezolizumab/bevacizumab</td><td rowspan="6">Nivolumab /ipilimumab</td><td rowspan="6">Nivolumab /ipilimumab</td><td rowspan="6"></td><td rowspan="6">Atezolizumab</td></tr>
<tr><td colspan="2">Atezolizumab+carboplatin/nab-P followed by atezolizumab</td></tr>
<tr><td colspan="2">Pembrolizumab+platinum/pemetrexed ChT followed by pembrolizumab/pemetrexed</td></tr>
<tr><td colspan="2">Disease progression</td></tr>
<tr><td>PS 0-2</td><td>PS 3-4</td></tr>
<tr><td>Nivolumab
Atezolizumab
Pembrolizumab if PD-L1 ＞1%
Docetaxel
Pemetrexed
Ramucirumab/docetaxel
Nintedanib/docetaxel
Erlotinib</td><td>BSC</td></tr>
<tr><td></td><td colspan="5">Molecular tests positive (ALK/BRAK/EGRF/ROS1)</td></tr>
<tr><td>ALK translocation</td><td>BRAF V600 mutation</td><td colspan="3">EGFR mutation</td><td>ROS1 translocation</td></tr>
<tr><td>Alectinib
Brigatinib
Crizotinib
Ceritinib
Ensartinib</td><td>Dabrafenib/trametinib</td><td colspan="3">Osimertinib
Gefitinib
Erlotinib
+/- bevacizumab
+/- ramucirumab
Afatinib
Dacomitinib
Gefitinib/carboplatin/pemetrexed</td><td>Crizotinib
Lorlatinib
Entrectinib
Repotrectinib</td></tr>
</table>

	Note: BSC, best supportive care; ChT, chemotherapy; nab-P, albumin-bound paclitaxel; PS, performance status.

1.3 Common chemotherapeutic agents

1.3.1 Cisplatin

After more than 40 years of clinical application, cisplatin has made significant achievements in treating diverse types of cancer, such as ovarian cancer, NSCLC [58, 59], especially the cure rate of cisplatin for testicular cancer has been over 90% [60].

Cisplatin is a neutral inorganic compound with a flat square geometry, and contains a Pt center, two stable ammonia ligands and two unstable chlorine ligands [61]. In general, extracellular concentration of chloride in plasma and other physiological fluids is higher than intracellular concentration of chloride, which makes it difficult to hydrolyze cisplatin compounds in extracellular fluid , but easy in the cytoplasm [61]. After cisplatin enters the cytoplasm through passive diffusion, transporter-mediated facilitated diffusion and active absorption [62], it dissociates and loses chloride ions, while binds water molecules to form a positively charged and aquated complex, which selectively binds to the guanine and N7 atoms on the adenine in the DNA molecule to form three complexes with different structures, namely, monoadducts, intrastrand crosslinks, and interstrand crosslinks. All crosslinks will destroy the structure of the DNA and inhibit its function of replication, which bring about apoptosis of cells [63]. However, cisplatin combines with other biological macromolecules in the cytoplasm, especially react promptly with thiol-containing compounds such as glutathione, which results in inefficacy of cisplatin [63]. In spite of the side effects of cisplatin, such as anemia, nausea, vomiting, nephrotoxicity, neutropenia and neurotoxicity, in contrary to non-platinum-based doublet regimens, previous research indicated that cisplatin-based doublet regimens contributed to the patients' survival rates more effectively [64]. As a second-generation platinum compound, carboplatin increases the occurrence of thrombocytopenia, but with lower risk of vomiting, therefore, the patient's own condition and toxic effects of drugs should be taken into account [64].

1.3.2 Pemetrexed

For patients with NSCLC, especially with advanced NSCLC, continuous use of cisplatin chemotherapy alone is difficult to effectively prolong the patient's survival time, and the cumulative toxicity of the drug is likely to aggravate NSCLC, resulting in the inability of patients to receive second-line treatment. Therefore, cisplatin-based adjuvant chemotherapy is commonly combined with other chemotherapeutic agents in clinic.

Pemetrexed is an antifolate with a pyrrole-pyrimidine group at its core, which is “a novel, multitargeted antifolate that inhibits at least three enzymes involved in the folate pathway. These enzymes are thymidylate synthase, dihydrofolate reductase, and glycinamide ribonucleotide formyl transferase” [65]. The enzymes are critical ingredients for the synthesis of folic acid and also in associated with the biosynthesis of thymine nucleotides and purine nucleotides [65]. When pemetrexed enters the cell, it is converted to polyglutamic acid by the action of folate polyglutamic acid synthase [66]. Polyglutamic acid remains in the cell and becomes an inhibitor of thymidylate synthase (TS) and glycinamide ribonucleotide formyl transferase (GARFT). Polyglutamation shows it has the characteristic of time-dependence and concentration in tumor cells, while in normal tissues, the concentration is very low, the half-life of polyglutamate metabolites in tumor cells extends, thereby the action time of drugs in tumor cells is prolonged [66]. Therefore, pemetrexed mainly inhibits the growth of tumors by disrupting the normal folate-dependent metabolic processes and inhibiting the replication of cells.

In 2004, Hanna et al. assessed the effectiveness and safety of pemetrexed and docetaxel from 571 random patients with advanced NSCLC [67]. The results of treatment with pemetrexed and docetaxel exhibited a 9.1% and 8.8% overall response rate, with 8.3 months and 7.9 months median survival time, respectively, and a similar 1-year survival rate 29.7% [67]. All indicates that pemetrexed and docetaxel are equally effective, furthermore pemetrexed has a lower incidence of adverse reactions in patients with NSCLC. Approximately 90% of pemetrexed can be excreted in the urine within 24 hours after intravenous injection, the risk of adverse reactions is low, and the safety is distinctly high [68].

1.3.3 Vinorelbine

In the 1950s, Noble et al. found vinca alkaloids in Catharanthus roseus [69]. Vinca alkaloids such as vinblastine and vincristine have significant anti-tumor activity and very similar molecular structure, however their toxicological characteristics are very different [70]. This phenomenon has inspired a large number of scientific research groups and pharmaceutical companies to discover new vinca alkaloids with high efficiency and low toxicity through chemical structure modification. Vinorelbine is a kind of vinblastine drug produced by structural modification and has been approved for marketing.

Vinorelbine is a highly effective anti-tumor drug, which mainly specifically acts on the mitotic tube of the cell, inhibits the process of tubulin double microsomes forming into microtubules by binding to tubulin, and further induces the disaggregation of microtubules to inhibit the process of mitosis. Meanwhile the chromosome cannot be attached to the spindle, so that the division and proliferation of tumor cells stops in the middle of mitosis, which achieves the purpose of inhibiting the growth of tumor cells and killing tumor cells [71]. Compared with other vinblastine drugs, vinorelbine has less activity on axons, so it has less neurotoxicity, and it is characterized with the advantages of strong chemical stability, broad anti-tumor spectrum, and little toxicity and side-effects [72]. At present, it is

a clinical first-line chemotherapeutic drug for the treatment of NSCLC, breast cancer, ovarian cancer and so on [72]. The combined application of vinorelbine and various drugs such as cisplatin, etoposide, mitomycin, etc. has enhanced efficacy and unchanged toxicity. In the randomized study treatment, Chevalier et al. divided 612 cases of newly-treated NSCLC into three groups, who were respectively treated with vinorelbine and cisplatin, vindesine and cisplatin, and vinorelbine alone, the effective rates were respectively 30%, 19% and 14%, the median survival time was 40 weeks, 32 weeks and 31 weeks respectively [73], which suggested that the combination of vinorelbine and cisplatin was obviously advantageous in the treatment of NSCLC.

1.4 Obstacles of chemotherapy in NSCLC

Chemotherapy is also crucial as a kind of common therapy for cancer patients, but at present, patients with cancer become resistant to almost all drugs due to increased applications of chemotherapeutic drugs with the passage of time, so that the efficacies of chemotherapeutic drugs on cancer cells may be decreased. Chemoresistance of cancer cells is one of the principal causes for cancer treatment failure, which may cause the cancer to relapse and eventually lead to the death of patients with cancer. When cancer cells resist the effects of chemotherapeutic drugs, they can grow and encourage new tumor regions to form, the process is known as tumor recurrence. Not only chemoresistance but also tumor recurrence is the clinical obstacle to the treatment of NSCLC. It is the challenging problem faced by cancer patients and their families, medical staffs and cancer researchers.

1.4.1 Chemoresistance

The chemoresistance of tumor cells refers to the phenomenon that tumor cells are resistant or insensitive to chemotherapeutic drugs during tumor chemotherapy, which can be divided into innate chemoresistance and acquired chemoresistance [74]. Innate resistance is a phenomenon that tumor cells are inherently insensitive to chemotherapy drugs before treatment to patients with cancer [74]. Tumor cells, however, are sensitive to chemotherapeutic drugs at the beginning. After several courses of treatment, the efficacy of chemotherapeutic drugs gradually decreases, and tumor cells become resistant or insensitive. This phenomenon is known as acquired drug resistance of tumor cells [74].

The most prominent and common chemoresistance is multidrug resistance (MDR). When it occurs, the tumor cells develop resistance to other anti-tumor drugs with different structures and mechanisms [75]. MDR is the common and primary factor that leads to the chemotherapeutic failure, which is severely and negatively impact the treatments for tumors. The mechanisms of MDR tend to be more complicated, such as regulating the apoptosis of tumor cells and the concentrations of chemotherapeutic drugs in tumor cells [75]. MDR can influence tumor cells by the known or unknown mechanisms. According to the mechanism of chemoresistance of tumor cells, it can be divided into chemoresistance

occurring in the cell membrane, in the intracellular metabolic process, and in the nucleus of tumor cells.

1.4.2 Chemoresistance occurring in the cell membrane

The typical representative of this type is P-glycoprotein (p-gp). In 1976, Juliano et al. discovered that a transmembrane phosphoglycoprotein (molecular weight 170 KD) was expressed on the membrane of Chinese hamster ovary cells resistant to colchicine, and named p-glycoprotein [76]. This protein is a kind of pump in lipid membrane, which can actively pump various compound from the inside of the tumor cells to the outside of the cells, and is also an ATP-dependent drug transport protein, namely ATP-binding cassette (ABC) transporter. In other words, P-gp can not only be combined with drugs but also ATP, actively transport anti-tumor drugs and hydrophobic lipophilic compounds outside tumor cells by energy through ATP hydrolysis, which results in the development of resistance to chemotherapeutic drugs in tumor cells until the intracellular concentration of drugs is lower than the effective concentration [77]. P-gp is encoded by the MDR1 gene, which is located on human chromosome 7q21 [77]. Some Study has found that various stimuli such as DNA damage, UV irradiation and serum starvation can activate MDR1 [78]. Related report also showed that various activation signals such as epidermal growth factor (EGF) tumor necrosis factor-α (TNF-α), doxorubicin, nuclear factor κB (NF-κB) can stimulate the expression of MDR1 [79]. NF-κB can induce p-gp expression in addition to the anti-apoptotic gene expression and anti-apoptotic effect [80]. Bentires-Alj et al. confirmed that there was a NF-κB binding sequence in the first intron of the MDR1 gene promoter region, and NF-κB can stimulate the transcription of the gene connected to the MDR1 promoter [81]. Therefore, the current research believes that p–gp mainly leads to the resistance to anti-tumor drug through two ways: one is the role of drug delivery, and the other is raising the threshold of apoptosis. How to inhibit the function of p–gp has been the focus of research to solve the problem of multidrug resistance.

P-gp that is made up of 1280 amino acid residues possesses two homologous symmetric domains, each homologous domain contains 6 transmembrane peptide chains and 1 ATP binding region. The 6 transmembrane peptide chains of p-gp are hydrophobic regions, which have the function of binding and transporting drugs. The ATP binding region is a hydrophilic region, which can bind to ATP, hydrolyze it to ADP, and release energy [82]. Therefore, p–gp can transport many drugs and endogenous substances. Cationic or amphoteric hydrophobic molecules that can passively diffuse through the lipid membrane are the substrates of p-gp [83]. Some reports have demonstrated that the expression of p-gp was in connection with unresponsiveness to chemotherapy in certain tumors, including sarcoma, leukemia, lymphoma and multiple myelomas [84, 85]. The relationship among MDR1, the chemotherapy resistance and the cancer patients' prognosis has been the focus of research. Studies have shown that high expression of MDR1 is not only connected with acquired multidrug resistance of patients with lung cancer, but also closely related to their prognosis [86, 87]. Even some researchers have discovered that p-gp not only exhibited high expression in recurrent carcinoma, but also significantly and negatively influenced patients' survival time and prognosis [88]. Therefore, detecting the expression

of p-gp in tissues of NSCLC before chemotherapy can predict the sensitivity of tumors to chemotherapeutics and estimate the prognosis of patients with cancer, which is significant for the options of clinical treatment, if high expression of MDR1 is found before chemotherapy, it indicates that the patient has innate multidrug resistance. Drugs that reverse MDR would be considered to be applied to increase the susceptibility of tumor cells to chemotherapeutic drugs. Detecting the expression of MDR1 in tumor chemotherapy can predict tumor response to treatment, recurrence, and metastasis. A study reported that p-gp mediated doxorubicin resistance of NSCLC cells, and the chemoresistance was reduced through inhibiting p-gp by verapamil [89]. According to Chan's research, he believed that the expression of p-gp was related to the mutation of P-53, and their pathological indicators might be correlated with the biological characteristics of liver cancer cells [90]. However, some experts reported that the expression of P-gp did not participate in the resistance to chemotherapy of lung cancer or did not have great effects on the mechanism of resistance, for example, some researchers have found that the P-gp expression within certain lung cancer cell line which was resistant to chemotherapeutic drugs was low, furthermore did not exhibit correlation with intrinsic chemoresistance of lung cancer cells [91], it is possible that the occurrence, development and resistance of tumor cells are not caused by a single mechanism.

1.4.3 Chemoresistance occurring in the intracellular metabolic process

Glutathione (GSH) and its enzyme system in cells are not only related to MDR, but also its effect of detoxification on chemotherapeutic drugs may exert an enormous function on the resistance of tumor cells to chemotherapeutic drugs. Its system is composed of GSH and various enzymes, including γ-glutamyl transferase (γ-GT), glutathione synthetase (GS), glutathione peroxidase (GPX), glutathione reductase (GR), glutathione S-transferases (GSTs) [92]. And GSTs that is most important for GSH system is closely related to tumor multidrug resistance.

As a tripeptide, glutathione is made up of glutamate, cysteine and glycine [93]. In general, glutathione that widely exists in mammalian cells can combine with peroxides and free radicals under the action of GSTs to protect the proteins which contain sulfhydryl group from being destroyed by oxidants, and also protect the important organs from free radicals [93]. Jamali et al. have found that intracellular GSH was correlated with cisplatin resistance of cancer cells, and its concentration is in a time-dependent manner [94]. This means intracellular GSH may be an indicator that reveal the level of cisplatin resistance in cancer cells, on the basis of its characteristic, cisplatin resistance may be better dealt with. Yu et al. also found that renal cancer cell line RCC8701/ADM800 which were obviously more resistant to adriamycin and epirubicin than the parent cells (RCC8701) was cross-resistant to cisplatin and 5-fluorouracil, GSH and glucose-6-phosphate dehydrogenase in cytoplasm increased significantly, but the expression of P-gp did not increase [95]. One research had also suggested high level of GR might mediate chemoresistance of tumor cells by regulating redox homeostasis and GSH [96]. All of the results indicate that intracellular

GSH may tend to increase when tumor cells develop MDR, in other words, GSH is involved in the process of MDR of tumor cells.

However, Chemotherapeutic drugs can bind GSH and lose their toxic effects through the catalysis of GSTs. GSTs can not only catalyze the binding of glutathione to electrophilic substances, but also itself can be combined with lipophilic drugs to increase their water solubility, which promotes drug efflux, reduces the cytotoxicity of anti-tumor drugs and protect tumor cells [93]. GSTs are a superfamily of isozymes, which can be divided into some subfamilies: α, μ, π et al. according to their sequence homology and location of chromosome [97]. GST-π which is encoded by glutathione S-transferases Pi (GSTP) gene is connected with the chemotherapeutic sensitivity of malignant tumors, and have been widely studied in recent years. The GST-π gene is located on the 11q13 fragment of the autosome, its total length is about 3kb, and it contains 7 exons and 6 introns [98]. GST-π is mainly present in the placenta and lung, breast and urinary bladder [99]. GST-π can catalyze electrophilic hydrophobic compounds such as carcinogens and chemotherapeutic drugs to combine with GSH, then form a substance that is easy to be excreted from cells. It also can prevent the damage of lipid peroxidation, conjugate and transport hydrophobic compounds such as hormones, drugs, carcinogens and so on. Therefore, while GST-π protects cells, it also causes the resistant to chemotherapeutic drugs of tumor cells. Cheng et al. reported that GST‐π was commonly detected after chemotherapy in patients with ovarian carcinoma who did not respond to chemotherapy, and compared with responders, the ratio of GST‐π density after chemotherapy to GST‐π density before chemotherapy distinctly increased in non-responders [100]. According to the analysis of 135 NSCLC tissue samples, Rybárová found that the expression rate of GSTP1 was as high as 70%, furthermore 77% in adenocarcinoma samples, and was correlated with prognosis of patients [101]. All of the findings suggest that GST-π may be essential in chemotherapeutic resistance of tumor cells.

1.4.4 Chemoresistance occurring in the nucleus of tumor cells

Topoisomerase (TOPO) which includes TOPO I and TOPO II has the function of adjusting the topology of DNA, and is closely related to genome replication, DNA recombination and gene transcription [102]. TOPO I can catalyze the break of a single strand of DNA [102]. TOPO II can noncovalently binds to the substrate DNA to cut the duplex and unwind the helix, then TOPO II switches its configuration under the action of Mg^{2+}, Mn^{2+} and ATP, and exhibits the activity of ligase, which will reconnect the broken double-stranded DNA, whereafter, TOPO II exhibits the original activity again [103,104].

MDR gene is not overexpressed, and chemotherapeutic drugs does not accumulate in tumor cells which are resistant to chemotherapy, this phenomenon that is mediated by TOPO II is known as atypical MDR (at-MDR) [105], and at-MDR is cross-resistant to anthracyclines and epipodophyllotoxins but not vinca alkaloids [106]. Now research showed that many chemotherapeutic agents including non-intercalating agents such as etoposide and teniposide [107] and intercalating agents such as Adriamycin might target TOPO II. Intercalating agents can be inserted between the base pairs to form a reversible

combination with the double helix of DNA, which promotes the combination of DNA and TOPO II to form a stable complex, and eventually breaks the DNA [108]. The expression of TOPO II is inversely proportional to tumor resistance, the reason is chemotherapeutic agents' cytotoxicity that targets TOPO II would depends on cleavable complexes' formation of DNA, TOPO II and the subsequent effects of DNA damage. De Jong S et al. had demonstrated that TOPO II levels in SCLC cells which were resistant to TOPO II inhibitors may be reduced [109]. Matsumoto et al. have also found that the mutation and deletion of TOPO II gene occurred and were closely related to MDR in tumor cells which were resistant to TOPO II inhibitors [110]. The phosphorylation levels of TOPO II are inversely proportional to its catalytic activity and ability to the dissociation of complex, the research of Takano et al. has suggested that phosphorylation level of topo II increased in human cancer KB cells which were resistant to etoposide, and might reduce etoposide -induced cleavages of DNA-TOPO II complexes [111]. All the results demonstrate that if the level of TOPO II in tumor cells drops or the level of phosphorylation of TOPO II increases, they can lead the barriers to the accumulation of intracellular agents and cross-resistance of tumor cells. Camptothecins (CPTs) which specifically target TOPO I such as topotecan and irinotecan are cytotoxic quinoline alkaloid [112], As similar to TOPO II inhibitors, CPTs can bind to the TOPO I–DNA complex to inhibit the religation of DNA. Some reports described that the mechanism of tumor cell resistance to CPTs might mainly consist of insufficient accumulation of drugs in tumors, variations in TOPO I resistance, or variations in cellular responses to TOPO I-CPTs interactions. [113]. Lee et al. also reported that the resistance of ovarian cancer cells to CPTs was reduced by inhibiting the activity of TOPO I, in other words, the expression of TOPO I was involved in the sensitivity of ovarian cancer cells to CPTs [114]. Jensen et al. reported that the mutant TOPO I gene was expressed in resistant human colon cancer cells, and may also refer to the resistance of tumor cells to CPTs [115].

1.4.5 Blocking of apoptosis pathway

B-cell lymphoma-2 (Bcl-2) gene that has significant effects on inhibiting apoptosis of cells is an oncogene, this gene was first cloned from the breakpoint of the chromosome translocation t (14: 18) in patients with follicular non-Hodgkin lymphoma in 1985 [116]. Bcl-2 protein is a membrane integrin which is mainly expressed on several membranes, including the outer mitochondrial, nuclear and endoplasmic reticulum membranes [117]. Overexpression of Bcl-2 can reduce the decrease of transmembrane potential and the apoptosis of cells which is caused by multiple stimuli such as chemotherapeutic agents, radiation, deficiency of growth factor and apoptotic genes [117, 118], it is currently believed that Bcl-2 protein can stabilize mitochondrial membrane voltage, prevent mitochondrial release and other cytokines, inhibit apoptosis induced by various factors, and have great effects on the regulation of mitochondrial apoptosis pathway. Studies have shown that Bcl-2 may be antagonistic to the apoptotic gene Bax, prevent cytochrome C from releasing into the cytoplasm and prevents cytochrome C from activating caspase [119], which is the key of apoptosis, various factors that cause apoptosis need to activate caspase before apoptosis of cells. Bcl-2 can inhibit apoptosis by promoting glutathione to enter the nucleus of cells, change the redox reaction in the nucleus, and prevent the activation of caspase

and other nuclear changes [120]. Many chemotherapeutic drugs exert anticancer effects by inducing the apoptosis of tumor cells, therefore, the overexpression of Bcl-2 and loss of activated apoptotic genes will relatively make tumor cells resistant to the toxic effects of chemotherapeutic agents. Sartorius et al. have demonstrated that 3 sensitive human SCLC cell lines cultured in subtherapeutic concentrations of etoposide were cross-resistant to cisplatin and doxorubicin, which was closely related to the overexpression of Bcl-2 in SCLC [121]. In addition, Bcl-2 could promote cells to resist the influence of oxidizing agents, and protected cells from oxidative stress by upregulating redox capacity, so that tumor cells could survive for a long time [122]. Restoring normal apoptosis and capacity to oxidative stress of tumor cells by inhibiting the expression of Bcl-2 may receive good treatment effect in some tumors. Some report has showed that the growth and development of melanoma cells were inhibited by antisense oligonucleotides which was against Bcl-2 gene [123]. Therefore, Bcl-2 is beneficial to prolonging the survival of tumor cells and inducing their resistance to stressful microenvironment and chemotherapeutic agents. In addition, the normal apoptotic pathway is blocked, the damaged cells do not undergo apoptosis or maintain the damaged state, which eventually leads to the occurrence and development of tumor.

1.4.6 Wnt signaling pathway

Wnt gene is a proto-oncogene cloned from mouse breast cancer induced by murine breast cancer virus, which was discovered and reported by Nusse in 1982, it was called Int1 gene at that time [124]. Subsequent studies found that Int1 gene and Wingless gene (Wg) of Drosophila were homologous genes, hence these genes were universally addressed as Wnt. Wnt signaling pathway is very conservative in evolution [3]. Wnt has 3 pathways in cells: the canonical Wnt/β-catenin signaling pathway, non-canonical Wnt/Ca^{2+} signaling pathway, and non-canonical Wnt/planar cell polarity signaling pathway [3]. In the canonical Wnt/β-catenin signaling pathway, a kind of destruction complex that includes “adenomatous polyposis coli (APC), axin, glycogen synthase kinase 3 (GSK3), casein kinase 1α (CK1α) and Dishevelled (Dvl) proteins” [3] could degrade cytoplasmic β-catenin, if Wnt signaling is absent (Figure 2), in other word, β-catenin may not accumulate in the cytoplasm without Wnt signaling [3]. As figure 2 shows, however, when extracellular Wnt proteins attach to Frizzled (Fzd) and low-density lipoprotein receptor-related protein 5/6 (LRP5/6), they could form a complex, which cause dephosphorylation and instability of Axin and deactivation of GSK3, so that destruction complex loses the function of degrading β-catenin, β-catenin piles up in the nucleus of cells and regulates the transcription of target genes [3].

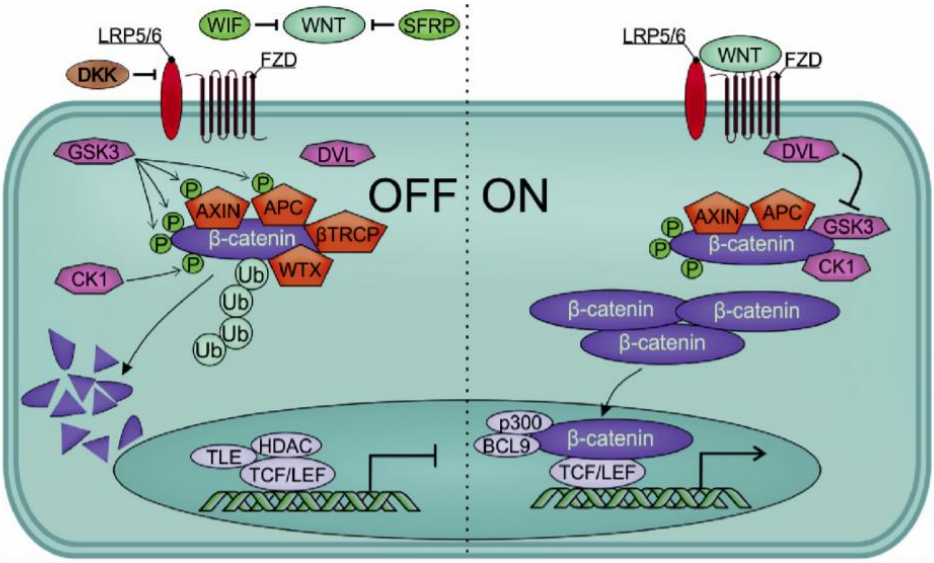


Figure 2. The canonical Wnt signaling pathway. Due to lack of ligands, the activity of complex Fzd-LRP5/6 is inhibited, destruction complex that consists of APC, axin, GSK3 and CK1α promotes proteolytic cleavage of β-catenin in the cytoplasm. In contrast, when complex Fzd-LRP5/6 combined with ligands, Dvl binds to Fzd, then promotes axin to combine with β-catenin and inactivate GSK3, which ultimately leads to the accumulation of β-catenin in the nucleus of cells and the expression of target genes. Adapted from Taciak et al., 2018 [3]

Wnt1 protein is a signal protein that is involved in the Wnt signaling pathway. Some studies suggested the expression of Wnt1 protein in NSCLC tissues was related to prognosis, and can be used as a distinct prognostic factor, as well as the expression of Wnt1 is connected with its downstream genes such as Ki-67, c-Myc, Cylin D1, MMP-7, VEGF-A [125,126]. This indicates that the Wnt signaling pathway may be correlated with the occurrence and development of NSCLC. In addition, Gao et al. have reported that inhibition of the activity of GSK-3β in the cytoplasm of A549/DDP cells can enhance its resistance to cisplatin in NSCLC cells [127], which means canonical Wnt/β-catenin signaling pathway was in relation to cisplatin resistance of NSCLC cells. Zhang et al. also demonstrated the level of β-catenin protein in the cytoplasm and nucleus and MDR1/P-gp was reduced by suppressing Fzd1, resulting in improving sensitivity of breast cancer cells to chemotherapeutic drugs [128]. In brief, Wnt signaling pathway also has great effects on chemoresistance of tumors.

1.4.7 Tumor recurrence

Tumor recurrence is a common and intractable problem in the clinical treatment of cancer, there are many reasons for the tumor recurrence, but among which the cancer stem cells (CSCs) theory is accepted by the majority of researchers. Further study has confirmed that stem cells can self-renew, differentiate, produce mature and differentiated progeny cells, and also can construct tissues and organs in a state of differentiation [129]. On the basis of the theory of stem cells, the concept of CSCs is proposed on the basis of the stem cell theory. Tumor stem cells theory believes that tumor-derived cells are able to form tumors, which is different from other tumor cells, these potential tumor-derived cells possess the capacity of infinite proliferation, self-renewal and multi-directional differentiation, and are the seed of tumor formation and recurrence [130]. Bonnet et al. isolated a kind of cells whose surface antigen markers is $CD34^{++}$ $CD38^{-}$ in human acute myeloid leukemia (AML) cells, the amount of this kind of cells accounts for about 0.2% of the total leukemic cells. it will not only cause the occurrence of AML after transplanting these cells into immunodeficient mice (NOD/SCID), but also possessed the strong capacity of self-renewal and differentiation [131]. The research team believed that these cells with $CD34^{++}$ $CD38^{-}$ might be leukemia stem cells [131]. Since tumor stem cells are the same as normal stem cells, they are in a relatively static state [132]. Many chemotherapeutic agents, however, target the tumor cells which are in cell cycle [133], CSCs may escape the killing effect of chemotherapeutic agents. The most of normal tumor cells can be eliminated after sufficient chemotherapy, tumor stem cells which survive may be the origin of tumor recurrence, tumor may recur in the near future.

Tumor dormancy is a state in which tumor cells persist in body without obvious proliferation or metastasis, and is also the result of the proliferation and apoptosis of tumor cells tending to balance [134]. In general, there are two viewpoints on tumor dormancy: firstly, when the level of tumor cells proliferation and apoptosis achieve equilibrium, they may appear to be dormant; Secondly, the tumor cell cycle is temporarily stopped when the tumor cells are in quiescent state, tumor cells would be dormant [134]. Tumor dormancy can occur in the process of tumor occurrence and development, including: (1) may occur in the early stage of the primary tumor; (2) may appear in the residual lesion after treatment of the primary tumor, and become the source of clinical recurrence in the future; (3) may appear in disseminated tumor cells (DTCs) or micrometastasis [134]. After a period of incubation, these dormant tumor cells are reactivated and progress to metastatic tumors. The long-term existence of tumor dormant cells is an important cause of tumor recurrence, metastasis and chemotherapy resistance, which makes it difficult to cure the tumors [134].

1.5 Pathway involved in NSCLC

Signaling pathway is a group of enzymatic reactions that transmit molecular signals inwards through the cell membrane [135]. These extracellular molecular signals are called ligands, consisting of cytokines, growth factors, hormones, neurotransmitters and other small molecule compounds [135]. When the ligand specifically binds to the receptor on the

cell membrane, a series of proteins in the cell will sequentially regulate the activity of downstream proteins, including activation or inhibition, thereby external signals gradually are amplified and transmitted, which eventually induces a series of comprehensive cellular responses [135]. Signaling pathways have been proved to the decisive factors to tumors [135]. Hh signaling pathway is especially abnormally activated during the occurrence and development of NSCLC.

1.5.1 Hedgehog signaling pathway

The Hh signaling pathway was detected in drosophilae by Christiane Nuesslein-Volhard and Weischaus in 1980 [136]. Due to the cuspate and short cuticle on the skin of the mutant drosophila larvae, it is named as hedgehog signaling pathway [137]. From drosophilae to humans, although there are some molecular differences, the secretion of Hh proteins and processes of signal transduction are similar [137]. Hh signaling pathway is the decisive factor to embryonic development, regulate cell proliferation and differentiation, and coordinate key steps in the progress of tissues and organs such as skin and brain [137]. Hh signaling pathway regulates the proliferation of stem cells [137]. Recent studies had suggested that hyperactive Hh signaling pathway was correlated with the occurrence and development of basal cell carcinoma, pancreatic cancer, lung cancer and other tumors [137].

Hh signaling pathway of mammals is mainly made up of three ligands: “Sonic hedgehog (SHH), Indian hedgehog (IHH) and Desert hedgehog (DHH)” [137], two transmembrane receptors: Patched (PTCH) and Smothen (SMO), three transcription factors: Gli1, Gli2 and Gli3 and downstream target genes [137]. SHH, DHH and IHH can be modified with cholesterol and palmitate to form fully functional proteins, these proteins are secreted from the cell by the transmembrane transporter dispatched which can promote the release of Hh proteins from secretory cells and binds to the receptor PTCH on the responding cells [137, 138]. PTCH family consists of PTCH1 and PTCH2 [139], PTCH1 is a 12-pass transmembrane receptor with three large loops, two of them are located in extracellular region, one of them is located in intracellular region [140]. PTCH1 primarily binds to SHH [139], and inhibit the activity of SMO [139]. SMO is a 7-pass transmembrane protein, which belongs to the G protein-coupled receptor superfamily and has great effects on the signal transduction process of the pathway [141].

Primary cilia have great effects on Hh signaling pathway in vertebrates [142]. PTCH1 is highly expressed in primary cilia, SMO and Gli proteins are commonly detected in primary cilia after the activation of SHH signaling [142]. In other word, SHH signaling pathway mainly takes place in primary cilia. Due to the lack of SHH, PTCH1 that is located in primary cilia can catalytically inhibit the activity of SMO in case that SMO locates and accumulates in primary cilia [142]. At this time the signaling pathway is deactivated (Figure 3A). As figure 3B shows, PTCH1 is activated by combining with SHH, so that it loses the inhibitory effect on SMO, then SMO localizes and accumulates in primary cilia, and deliver the signal to the zinc finger transcription factors Gli family in the cytoplasm [142]. In order to ensure the proper activity of the entire signal pathway, Hh ligands that bind to PTCH is

tightly regulated by some Hh binding proteins. For example, there is a close affinity between Hh-interacting protein (HHIP) and Hh ligands, so HHIP may compete with PTCH for the combination with Hh ligands. Because of its special characteristic, HHIP prevents excessive activation of Hh signaling pathway and regulates negatively it [143].

The Gli family consists of three homologs: Gli1, Gli2 and Gli3. Gli1 is composed of zinc finger domain and C-terminal activator domain. Gli2 and Gli3 are composed of N-terminal suppressor domain, zinc finger domain and C-terminal activator domain [144]. Gli1 is generally considered as activator, Gli2 and Gli3 as activator and repressor. Protein kinase A (PKA), GSK3, and casein kinase 1 (CK1), suppressor of fused (SUFU), in general way, inhibits the activity of Gli1 by binding to transcription factors Gli family, followed by promoting the process of Gli repressor (GliR) and inhibiting the combination of full-length Gli and DNA [144]. As figure 3A shows, in the absence of Hh signaling activity, Gli2 and Gli3 undergo phosphorylation under the influence of PKA, CK1 and GSK3 and truncation via proteasome, eventually transformed into Gli2 repressor (Gli2R) and Gli3 (Gli3R), which break away from SUFU and attach to target genes for transcription inhibition [145].

Figure 3B shows that SMO gets rid of the inhibition from PTCH1 when Hh signal is sufficient, and assembles in primary cilia, followed by phosphorylation under the influence of CK1α and G protein coupled receptor kinase 2 (GRK2) [146], activated SMO induce the release of full-length Gli2 and Gli3 from SUFU as Gli2 activator (Gli2A) and Gli3 activator (Gli3A) in primary cilia, as well as Gli1, which promote transcription by binding to target genes in nucleus [145, 147]. Gli1 is not only a transcription factor but also a downstream target gene. Its expression is highly dependent on the activity of Hh signaling pathway, so it is often regarded as a sign of whether the Hh signaling pathway is activated [145]. HHIP is also one of the Hh signaling pathway target genes, which maintain the balance and stability of signaling pathway [145].

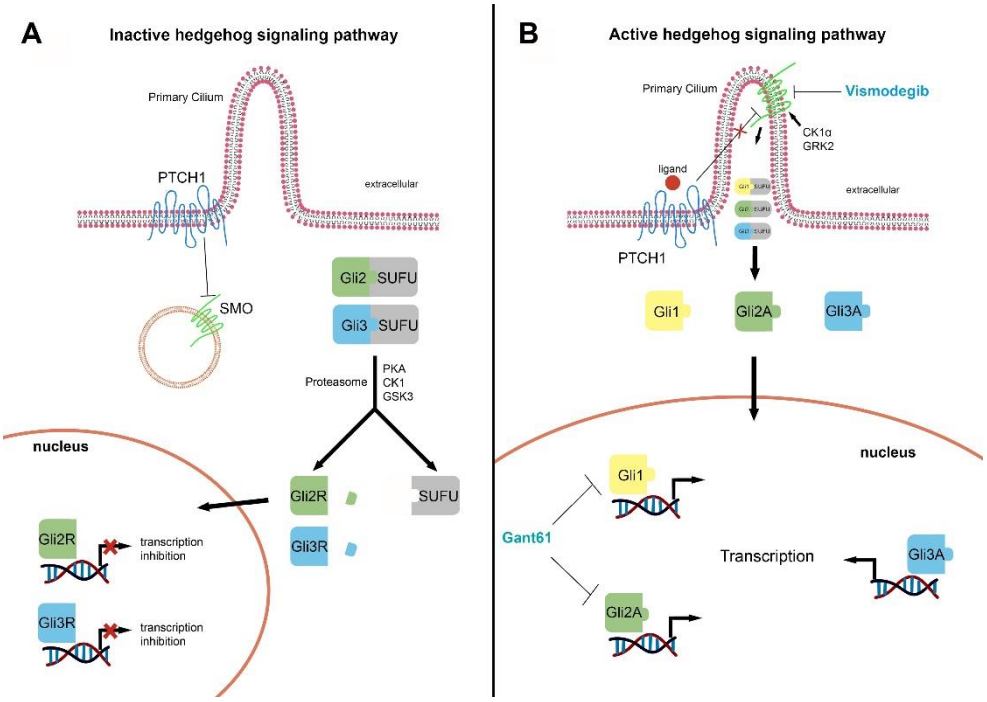


Figure 3. Hedgehog signaling pathway. (A) Due to lack of SHH, PTCH1 prevents SMO from moving in primary cilia, phosphorylated Gli2 and Gli3 by PKA, CK1 and GSK3 undergo proteolytic cleavage by proteasome to ultimately form Gli2R and Gli3R, which combine with target genes to inhibit transcription after break away from SUFU. (B) The combination of SHH and PTCH1 makes SMO move in primary cilia, phosphorylated SMO by CK1α and GRK2 promotes Gli1, Gli2 and Gli3 to separate from SUFU, so full-length of Gli family, Gli1, Gli2A and Gli3A combine with target genes and facilitate transcription. As the inhibitors, vismodegib targets SMO, and Gant61 targets Gli1 and Gli2 to inhibit the activation of Hh signaling pathway.

In the entire pathway signal transduction process, all links are strictly regulated, the up-regulation of Gli1 in Hh signaling pathway forms a positive feedback loop, and PTCH, HHIP, SUFU, etc. form a negative feedback loop [145]. Hh signaling pathway forms a complex signal transduction network, which regulates embryonic development and the homeostasis of adult tissues by stimulating or inhibiting the expression of target genes through positive and negative feedback [148]. Imbalance of Hh signaling pathway may lead to cell proliferation and evasion of apoptosis, tumor invasion, metastasis and resistance to chemotherapeutic agents [148].

The canonical abnormally activated Hh signaling pathway during tumorigenesis and development is characterized by ligand-dependence [149]. Ligand-dependent Hh signaling pathway has autocrine and paracrine types [149]. The autocrine type is that the tumor cells themselves produce ligands that bind to receptors on themselves or nearby tumor cells, which can trigger a cascade of reactions in tumor cells [149]. While paracrine type has two subtypes, one is that the ligands produced by the tumor cells attach to the receptors on the mesenchymal cells, afterwards activate the cascade reaction in the mesenchymal cells. Mesenchymal cells produce growth factors such as vascular endothelial growth (VEGF) to support the growth of tumor cells. The other subtype is that the Hh ligands produced by

mesenchymal cells binds to receptors on cancer cells, resulting in abnormally increased signal pathway activity in tumor cells [149]. The canonical activated Hh signaling pathway is commonly detected in NSCLC [149]. Besides the canonical activation pathway, there is also non-canonical Hh activation pathway, which is not activated by ligands but Gli-dependent [150]. There is crosstalk between non-canonical and other signaling pathways, so that downstream transcription factors are directly upregulated by other pathways without the effects of ligands. For example, pathways, such as RAS and PI3K, etc., may directly upregulate the expression of Gli1 and promote tumor growth [150]. Aberrantly activated SMO in the absence of Hh ligands is also able to activate non-canonical Hh pathway [150].

Studies have shown that Hh signaling pathway was very important for the differentiation and development of trachea and lungs. Exposure of Hh signaling pathway inhibitor to animals in early pregnancy can lead to abnormal development of embryonic lung [151]. The results of studies about epithelial injury of animal lung indicated that the continuous injury of the respiratory tract is an effective stimulating factor for Hh signaling pathway activation to help the proliferation and differentiation of respiratory epithelial stem cells and promote tissue repair [152]. Smoking is the decisive factor to the occurrence of lung cancer. The harmful substances in smoke can make the respiratory epithelium undergo repeated damage and repair. The Hh signal pathway may be continuously activated during this repeated stimulation, which leads to the destruction of homeostasis and uncontrolled cell proliferation and differentiation, and creates conditions for the occurrence and development of malignant tumors. In existing reports, however, there is still controversy about the role of Hh signaling pathway in NSCLC. According to the study of Chi et al., the activation of Hh signaling pathway was detected in less than 10% of lung cancers [153]. Moreover, by functional verification of in vivo and in vitro models, tumor-bearing mice and cell lines without Hh signal pathway activity may be not sensitive to monoclonal antibodies or inhibitors [152]. But other studies have been shown that the Hh signal pathway is generally activated in NSCLC. According to the study of Abe and Tanaka, Gli family can be administered through SMO-independent pathway, which suggests that there is paracrine mechanism of Hh signaling pathway in the progress of NSCLC [154]. Singh et al. found that the tumorigenic properties of NSCLC cells depend on the Hh signaling pathway activity of tumor cells. Inhibition of Hh signaling pathway activity in tumor cells could significantly reduce the expression of Hh target genes, and also there was autocrine mechanism of Hh signaling pathway in the development of NSCLC [155]. Therefore, the mechanism of signaling pathways is very diverse in NSCLC.

Recent research has suggested that the Hh signaling pathway not only takes part in the occurrence and development of tumors, but also in chemoresistance of tumors. Kobune et al. found that the sensitivity of $CD34^+$ leukemia cells to cytarabine was improved after the application of SMO antagonist, which proved that the Hh signaling pathway was correlated with the chemoresistance of leukemia cells [156]. Chen et al. also found the correlation between the activation of Hh signaling pathway and chemoresistance in breast cancer cells, and Hh signaling pathway may regulate chemoresistance of human breast cancer through MDR-1/P-gp [157]. Some studies about Hh signaling pathway showed that Gli1 were correlated with chemoresistance, invasion and metastasis of tumor cells [158,

159]. Therefore, the abnormally activated Hh signaling pathway strongly supports tumor cells to fight or escape chemotherapy, and leads to resistance to chemotherapeutic drugs. In addition, there is also crosstalk between the Hh signaling pathway and EGFR signaling pathway, which facilitates the expression of Gli1 [160]. Under the influence of such interaction, tumors may further get support from multiple factors, which improves the resistance to chemotherapeutic agents. However, the mechanism of Hh signaling pathway involved in chemoresistance of tumor is not very clear, especially in NSCLC.

1.5.2 Hh signaling pathway inhibitors

In the view of their different targets, common Hh signaling pathway inhibitors mainly target ligands of Hh signaling pathway, transmembrane receptor SMO, transcription factor Gli family and so on [149]. In the study, SMO inhibitor and Gli family inhibitor were applied. Cyclopamine can also bind to SMO and inhibit the activity of the Hh pathway [161]. Due to its poor chemical properties, the therapeutic effects of cyclopamine are restrained [162]. Other Hh signaling pathway inhibitors are getting more and more attention. Vismodegib, as Hh inhibitor that targets SMO, was recommended by the EMA in 2013 for the therapeutic application to the patients with locally advanced or metastatic basal cell carcinoma who improperly accept surgery or radiotherapy [163]. Vismodegib brought new hope for tumor treatment. Vismodegib can directly prevent the activation of downstream Gli transcription factors after inhibiting the activity of SMO [164], thereby inhibiting the expression of downstream genes connected with the occurrence and evolvement of tumors. However some researchers have found in the clinic that SMO often mutates such as c.1417G > A and c.1406G > A in patients, which affects the efficacy of vismodegib, and even gives rise to the resistance of tumor cells to it [165].

Gant61 can specifically inhibit the activity of Gli1 and Gli2 as Hh signaling pathway inhibitor [166]. Agyeman et al. explained the binding mode of Gant61 and zinc finger protein Gli1, the combination of Gant61 and Gli1 was located at amino acids E119 and E167 which were in finger 2 and finger 3 of Gli1 instead of DNA-binging domain [166]. Mutations in these two binding sites resulted in significant failure of the combination of Gant61 and Gli1 [166], which means the efficacy of Gant61 is brought down by mutations and chemoresistance of tumor cells may gradually develop. Meanwhile, Gant61 has not only proved to induce apoptosis and inhibit the malignant behaviors of tumor cells [167], but also enhance the sensitivity of tumor cells to chemotherapeutic drug [168]. Therefore, Gant61 may be one of the chemotherapeutic drugs with better prospects.

2. Aims

Parts of the Materials and Methods were already published in Liu et al., 2020 [1].

Despite the fact that enormous progress has been achieved in lung cancer treatments in past decade, the problem of chemoresistance after treatment is still a huge obstacle to the treatment for patients with lung cancer. The in-depth research of tumor microenvironment, however, has also provided new ideas for chemoresistance of tumor. Therefore, the purpose of this study is to discover the impact of the Hh signaling pathway on pemetrexed resistance of NSCLC cells, determine whether inhibiting the Hh signaling pathway can reduce their resistance, and provide a scientific basis about reducing chemoresistance of tumor cells for clinic and basic research.

3.Materials and methods

Parts of the Materials and Methods were already published in Liu et al., 2020 [1]

3.1 Experimental materials

Table 2. Experimental materials. Parts of the contents in the table 2 were published in Liu et al., 2020 [1]

No.	Name	Origin
1	HCC827 NSCLC cells	ATCC, Manassas, VA, USA
2	RPMI1640 medium	Sigma–Aldrich, Deisenhofen, Germany
3	fetal bovine serum	Invitrogen, Carlsbad, CA, USA
4	penicillin	Biochrom GmbH, Berlin, Germany
5	streptomycin	Biochrom GmbH, Berlin, Germany
6	MTT	Sigma–Aldrich, Deisenhofen, Germany
7	Trizol	Sigma–Aldrich, Deisenhofen, Germany
8	hexamers and SuperScriptII	Roche Diagnostics, Penzberg, Germany
9	iTaq SYBR Green Supermix	Bio-Rad Lab-oratories, Hercules, CA, USA

10	Celltiter-Glo reagent	Promega Corporation, Madison, WI, USA
11	DMSO	Sigma–Aldrich, Deisenhofen, Germany
12	Dulbecco's phosphate Buffered Saline	Sigma–Aldrich, Deisenhofen, Germany
13	Pemetrexed	Apotheke KUM, Munich, Germany.
14	Trypan blue stolution	Sigma–Aldrich, Deisenhofen, Germany
15	Gan61	Selleckchem, Munich, Germany
16	vismodegib	Selleckchem, Munich, Germany
17	10 ml Sterile disposable plastic pipettes	Sterilin Ltd, Newport, Uk
18	5 ml Sterile disposable plastic pipettes	Sterilin Ltd, Newport, Uk
19	Pipettes	Eppendorf, Hamburg, Germany
20	50 ml Cellstar centrifuge plastic tubes	Greiner Bio‐One GmbH, Frickenhausen, Germany
21	15 ml centrifuge tubes	Corning, New York, USA
22	96-well plates	Corning, New York, USA
23	75 cm^2 cell culture flask	Corning, New York, USA
24	Life Touch Cycler	Bioer, Hangzhou, China
25	Victor plate reader	Berthold Technologies, Bad Wildbad, Germany
26	SPSS 24 statistical software	IBM Corp., Armonk, NY, USA

3.2 Cell culture

HCC827 NSCLC cells (ATCC, Manassas, VA, USA) were cultured in 75 cm^2 cell culture flask with culture medium which contains RPMI1640 medium (Sigma–Aldrich, Deisenhofen, Germany), 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin (Biochrom GmbH, Berlin, Germany) and 100 μg/ml streptomycin (Biochrom GmbH, Berlin, Germany). All flasks were kept in a 37˚C incubator which contains 5% CO^2 and saturated humidity.

3.3 Method to obtain pemetrexed resistance of cells

Added 130nM pemetrexed to 75 cm^2 cell culture flask with HCC827 cells in the logarithmic phase of growth, changed new culture medium after 24h, and repeated once every week. After 4 weeks, HCC827 cells were always cultured with culture medium which contains 130nM pemetrexed.

3.4 MTT assay

This research employed Hh signaling pathway inhibitors vismodegib and Gant61. The experiment utilized 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma–Aldrich, Deisenhofen, Germany) as a substrate for whole-cell dehydrogenase activity. HCC827 naïve cells and HCC827 pemetrexed-resistant cells were cultured in 96-well plates at 2×10^3/well cell volume and kept in incubator overnight. Treated naïve and pemetrexed-resistant cells with 0.08μM, 0.8μM, 8μM and 80μM vismodegib or Gant61 respectively. At the same time, naïve and pemetrexed-resistant cells without treatment were considered as control group. This experiment evaluated the amount of formazan product after fully dissolving with isopropanol by a Victor plate reader (Berthold Technologies, Bad Wildbad, Germany).

3.5 Introduction to quantitative real-time reverse transcription polymerase chain reaction (qPCR)

The extraction from cell lines, duplication and purification of total RNA were performed according to the Trizol method (Sigma–Aldrich, Deisenhofen, Germany) and the manufacturer's manual. Random hexamers (Roche Diagnostics, Penzberg, Germany) and SuperScriptII reverse transcriptase (Invitrogen) were applied for reverse transcription of total RNA.

One noted from one's previous work published in [1] that doublets experiments were conducted. Firstly, qPCR amplifications were performed on Life Touch Cycler (Bioer, Hangzhou, China). Their respective genes were replicated using 40ng complementary DNA, 500nM forward and reverse primer, and iTaq SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) in a final volume of 20μl. PCR reactions ran for 40 cycles, each consisted of initial 15 sec' denaturation at 95˚C, then primer annealing for 15 sec at 55˚C and extension for 30 sec at 72˚C [1]. Secondly, amplification of the housekeeping gene TATABox-binding-Protein (TBP) was performed to systematize the amount of RNA samples. Corresponding quantification of gene expression was performed by the ΔΔ-ct method as mentioned above [169].

One quoted the primer pairs (5'->3' orientation) from [1] as the following: "Gli1, AGCTACATCAACTC CGGCCA, GCTGCGGCGTTCAAGAGA; Gli2, TTCTCCAACGC

CTCGGAC, GCCTGGGATCTTGCAGATGT; Gli3, TCAAAGCG GGAAGAATGCC, CTGACCACCAGGGCTTGG; PTCH1, TTGATT GTGGGTGGCACAGT, GCTTGGGAGTCATTAACTGGAAC; SMO, GGAGAGGAGCCATACTGCCC, TCAACCAGCCACAGGTTGG; SHH, CTGGGTGTACTACGAGTCCAAGG, CAGCCTCCCGATTT GGC; HHIP, TGTACATCATTCTTGGTGATGGG, AGCCGT AGCACTGAGCCTGT; TBP, GCCCGAAACGCCGAATAT, CCGT GGTTCGTGGCTCTCT."

3.6 Measurement of cell viability

HCC827 naïve cells and HCC827 pemetrexed-resistant cells were cultured in 96-well plates at 2×10^3/well cell volume and kept in incubator overnight. Pemetrexed (4μM) and/or Gant61 (27.7μM) were applied to cells. At the same time, naïve and pemetrexed-resistant cells without treatment were considered as control group. The luminescence value for each well was measured by a Victor plate reader (Berthold Technologies, Bad Wildbad, Germany) for cell viability on day 1, 3, 5 and 7. Each measurement was completed after treating cells with 100μl Celltiter-Glo reagent (Promega Corporation, Madison, WI, USA).

3.7 Statistical analyses

All analyses were performed by SPSS 24 statistical software (IBM Corp., Armonk, NY, USA). Through Two-Way ANOVA and the Student unpaired t-test, all data were expressed as means ± standard deviation where $p<0.05$ was considered statistically significant.

4. Results

Parts of the results were already published in Liu et al., 2020 [1]

4.1 Effects of vismodegib and Gant61 on naïve cells and pemetrexed-resistant cells

To understand the effects of vismodegib and Gnat61 on NSCLC cells, the inhibition ratios of vismodegib and Gnat61 on HCC827 cells and HCC827 pemetrexed resistance were tested respectively by MTT assay. Figure 4 showed vismodegib and Gnat61 had higher inhibitory effects on pemetrexed-resistant cells with increasing concentrations compared with the inhibitory effects on naïve HCC827 cells. In other words, their inhibitory effects were much more significant, especially at high concentrations. And Gant61 was more effective against pemetrexed-resistant cells than vismodegib ($p < 0.05$). On the basis of the result, a hypothesis that Hh signaling pathway might be connected with chemoresistance of NSCLC, and might regulate it directly or indirectly by itself or activating other signaling pathways was taken into account.

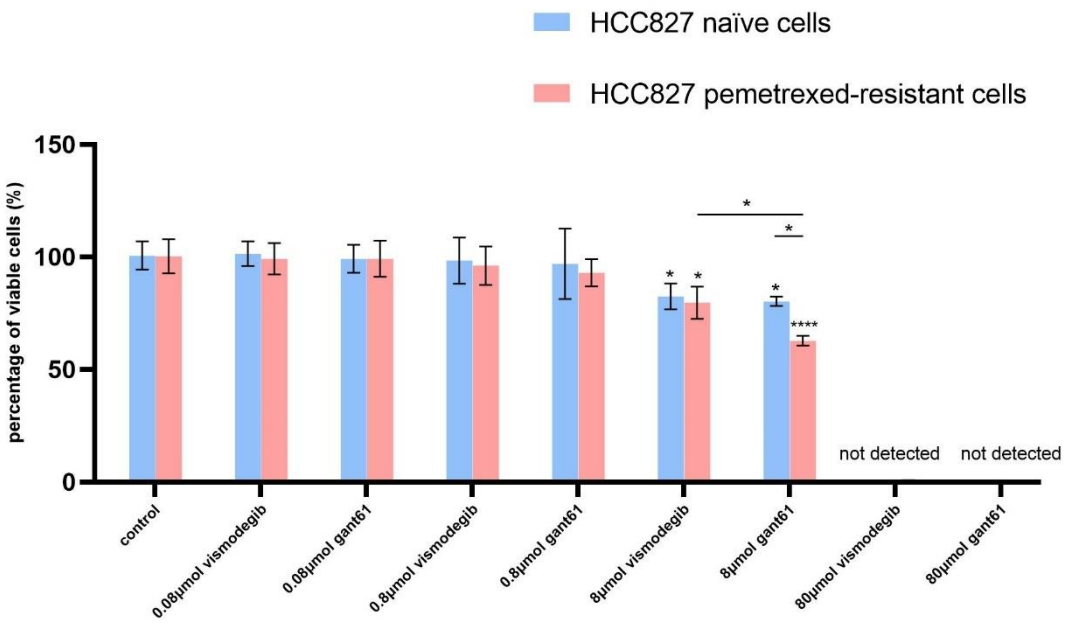


Figure 4. Inhibitory potencies of vismodegib and Gant61 on pemetrexed-resistant and naïve cells detected by MTT assay. Naïve cells and pemetrexed-resistant cells were treated with 0.08μmol, 0.8μmol, 8μmol and 80μmol vismodegib or Gant61 respectively. Both of vismodegib and Gnat61 showed higher inhibitory effects on pemetrexed-resistant cells with increasing concentrations than those on naïve HCC827 cells. Gant61 was more effective than vismodegib in inhibiting pemetrexed-resistant cells. Error bars indicated means ± standard deviation of three independent experiments, $p<0.05$ was considered statistically significant, * meant $p < 0.05$, **** meant $p < 0.0001$. calculated by Two-Way ANOVA / Bonferroni's Multiple Comparison Test.

4.2 Increased expression of genes involved in Hh signaling pathway in pemetrexed-resistant NSCLC cells

After obtaining resistance of cells to pemetrexed by treating cells with sub-lethal doses of pemetrexed, IC_{50} of pemetrexed to pemetrexed was 4μM by testing through preliminary experiments. Three experimental groups were set up: (1) naïve cells and resistant cells without pemetrexed; (2) naïve cells + pemetrexed (4μM); (3) resistant cells + pemetrexed (4μM) to test pemetrexed resistance of HCC827 cells. Group naïve cells and resistant cells without pemetrexed was used as a benchmark to evaluate the effect of pemetrexed on the non-resistant group and the resistant group. The percentage of viable cells in all the groups was tested through CellTiter-Glo cell viability assay. With the passage of time, as demonstrated in figure 5, the cell viability of naïve HCC827cells showed an obvious linear decline under the influence of pemetrexed. The cell viability of pemetrexed-resistant HCC827 cells, however, showed a relatively slow decline. Comparing with naïve HCC827, resistant HCC827 cells showed their optimum resistance to pemetrexed. The viability of naive HCC827 cells was dramatically low via the treatment with IC_{50} of pemetrexed compared with pemetrexed-resistant cells.

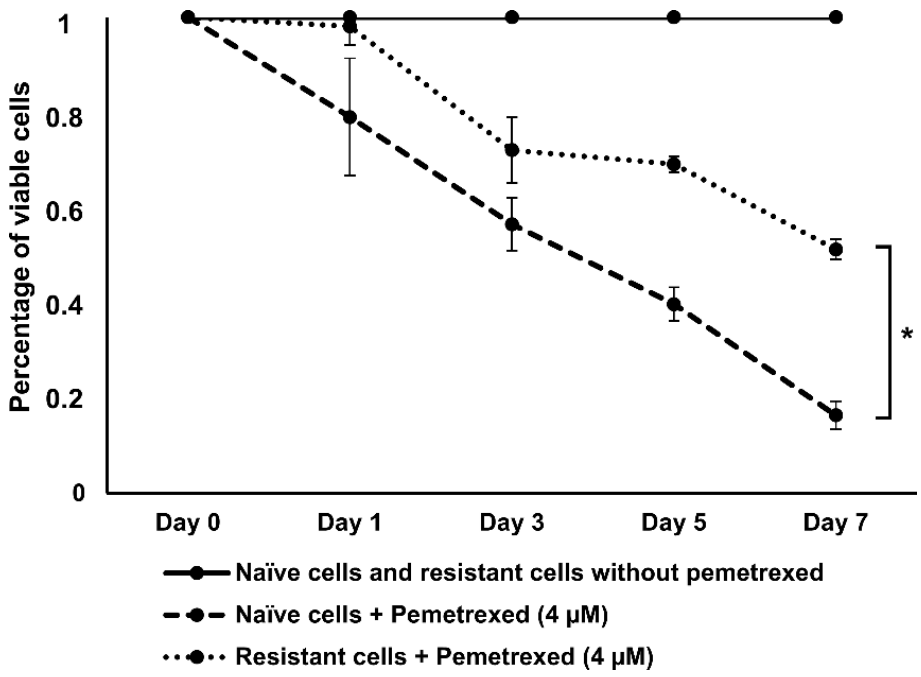


Figure 5. Inhibitory potency of pemetrexed on pemetrexed-resistant and naïve cells detected by CellTiter-Glo cell viability assay. The result showed the percentages of viable cells from three different groups at different time: group naïve cells and resistant cells without pemetrexed (solid line), group naïve cells + pemetrexed (4μM) (dashed line), group resistant cells + pemetrexed (4μM) (dotted line). Pemetrexed-resistant HCC827 cells showed stable chemoresistance to pemetrexed, in contrast, naïve HCC827 cells were sensitive to pemetrexed. Error bars indicated means ± standard deviation of three independent experiments, $p<0.05$ was considered statistically significant, * meant $p < 0.05$, calculated by Student unpaired t-test. One had previously published this figure in Liu et al., 2020 [1]

The mRNA expression levels of relevant Hh signaling pathway proteins were assessed by qPCR. Most of related Hh signaling pathway proteins were overexpressed in pemetrexed-resistant cells. Compared with naïve cells, Gli1, Gli2 and Gli3 were overexpressed by 3.7-, 4.3- and 3.5- fold respectively in pemetrexed-resistant cells, and the ligand SSH of Hh signaling pathway was overexpressed by 5.9-fold in pemetrexed-resistant cells. However, the inhibitor of the Hh signaling pathway HHIP and the receptor associated protein SMO were not obviously upregulated in pemetrexed-resistant cells (Figure 6).

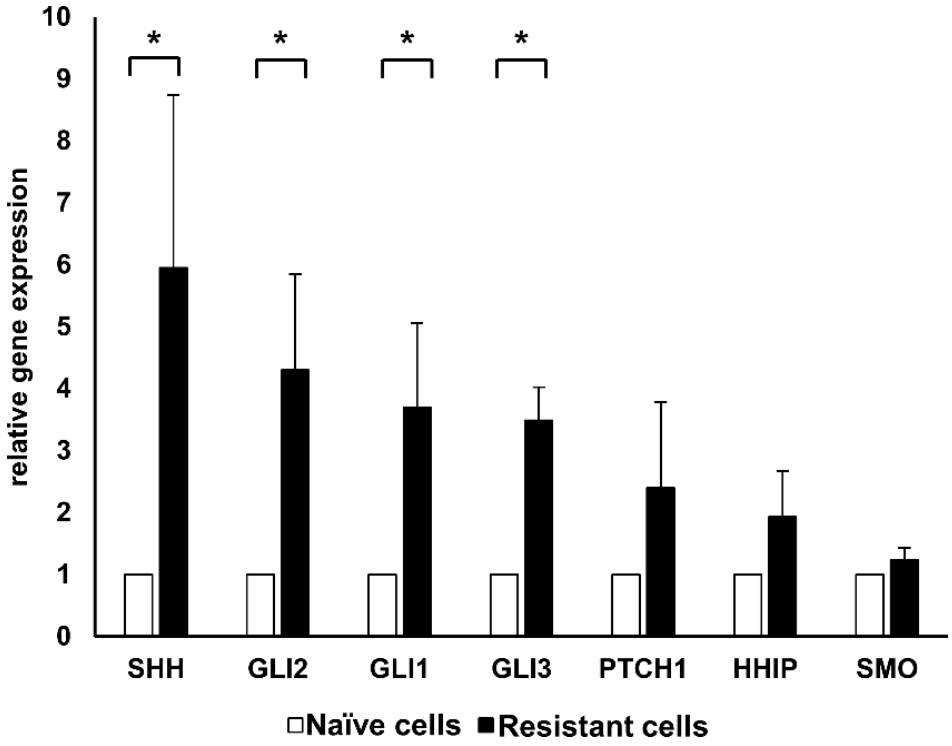


Figure 6. Relative mRNA gene expression levels of Hh signaling pathway proteins quantified by qPCR. Compared with naïve cells, SHH, Gli2, Gli1, Gli3 and PTCH1 were upregulated by 5.9-. 4.3-, 3.7-, 3.5-, 2.4-fold respectively in pemetrexed-resistant HCC827 cells. HHIP and SMO were indistinctly upregulated in pemetrexed-resistant cells. Error bars indicated means ± standard deviation of three independent experiments, $p<0.05$ was considered statistically significant, * meant $p < 0.05$, calculated by Student unpaired t-test. One had previously published this figure in Liu et al., 2020 [1].

4.3 Hh signaling pathway inhibitor Gant61 showed high inhibitory potency on pemetrexed-resistant cells and might suppress their pemetrexed resistance

The previous result showed SHH, Gli1, Gli2 and Gli3 were commonly detected in pemetrexed-resistant cells. As mentioned above, all of them had great effects on activating Hh signaling pathway and target genes. Therefore, there was also a second hypothesis that the pemetrexed resistance of HCC827 cells was reduced or the sensitivity to pemetrexed was improved by blocking Hh signaling pathway. To validate the hypothesis, the viability of resistant cells and non-resistant cells had been tested through CellTiter-Glo cell viability assay by three different treatments: solely pemetrexed, solely Gant61 and both pemetrexed and Gant61 together. Through preliminary experiments, the IC_{50} of naïve HCC827 cells to Gant61 was 27.7μM. There were four experimental groups: firstly

pemetrexed-resistant cells or naïve cells without treatment, secondly pemetrexed-resistant cells or naïve cells + pemetrexed (4μM), thirdly pemetrexed-resistant cells or naïve cells + Gant61 (27.7μM), and finally pemetrexed-resistant cells or naïve cells + pemetrexed (4μM) + Gant61 (27.7μM). As illustrated in figure 7A, the treatment with Gant61 significantly brought the cell viability down in pemetrexed-resistant cells, it would be more effective if Gant61 and pemetrexed were applied together. Whereas in figure 7B, not only the treatment with Gant61 plus pemetrexed but also Gant61 alone did not revealed distinct differences in reducing naïve cell viability compared with the treatment with pemetrexed alone. All the results indicated that chemoresistance of pemetrexed-resistant cells might be reduced, or the sensitivity might be improved or even restored by blocking Hh signaling pathway with Gnat61, which also further confirmed the first hypothesis that Hh signaling pathway might be involved in and crucial to chemoresistance of NSCLC.

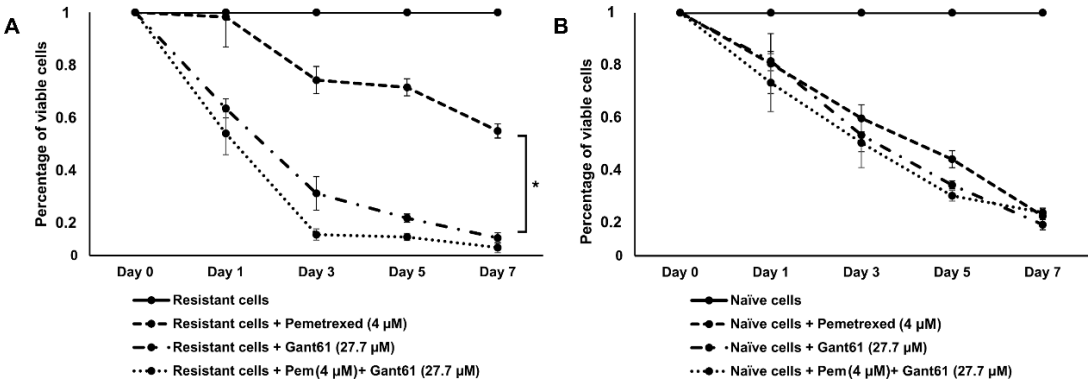


Figure 7. Inhibitory potencies of pemetrexed, Gant61 and their combination on pemetrexed-resistant and naïve cells detected by CellTiter-Glo cell viability assay. (A) The percentage of viable cells of resistant cells were tested under the influence of pemetrexed (4μM), Gant61 (27.7μM) and both of pemetrexed (4μM) and Gant61 (27.7μM) at different time. Pemetrexed-resistant cells were stably resistant to pemetrexed, Gant61 revealed inhibitory potency on pemetrexed-resistant cells, and was more effective in combination with pemetrexed. (B) The percentage of viable cells of naïve cells was tested under the influence of pemetrexed (4μM), Gant61 (27.7μM) and both of pemetrexed (4μM) and Gant61 (27.7μM) at different time. Naïve cells were sensitive to pemetrexed, Gant61 and the combination of Gant61 and pemetrexed did not appear obviously different inhibitory potency on pemetrexed-resistant cells. Error bars indicated means ± standard deviation of three independent experiments, $p<0.05$ was considered statistically significant, * meant $p < 0.05$, calculated by Student unpaired t-test. One had previously published this figure in Liu et al., 2020 [1].

To better exhibit the inhibitory potency of Gant61 on HCC827 cells, three experimental groups were set up as illustrated in figure 8: (1) naïve cells and pemetrexed-resistant cells (solid line); (2) naïve cells + Gant61 (27.7μM) (dashed line); (3) pemetrexed-resistant cells + Gant61 (27.7μM) (dotted line). The percentage of viable cells in all the group were tested through CellTiter-Glo cell viability assay. As the result showed, not only the viability of naïve cells but also the viability of pemetrexed-resistant cells was significantly reduced under the influence of Gant 61, which meaned both of them were sensitive to Gant61. Compared with naïve cells, however, pemetrexed-resistant cells revealed their higher susceptibility to Gant61, which implied the higher activation of the Hh

signaling pathway in these cells. All results showed that Hh signaling pathway might not only be connected with chemoresistance of NSCLC, but also be a decisive factor.

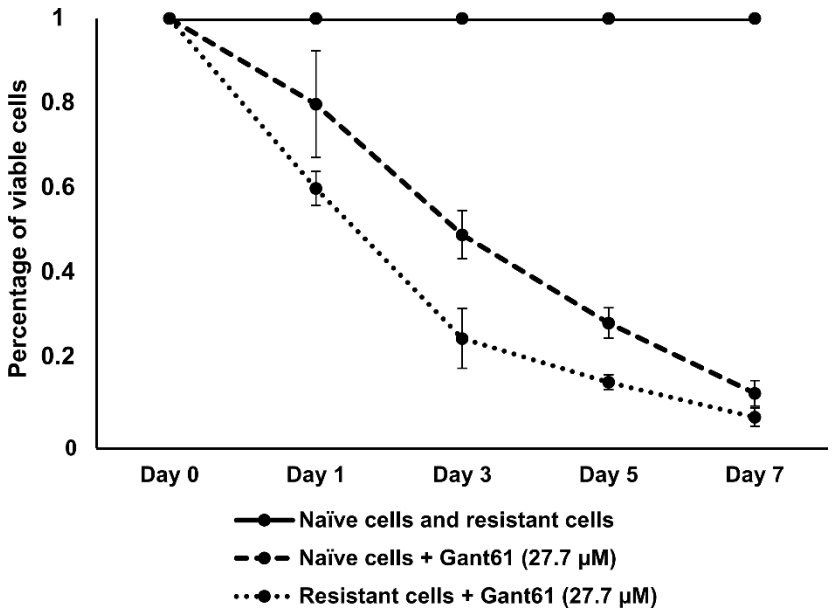


Figure 8. Inhibitory potency of Gant61 on pemetrexed-resistant and naïve cells detected by CellTiter-Glo cell viability assay. The result showed the percentages of viable cells under the influence of three different treatments: naïve cells and pemetrexed-resistant cells without treatment (solid line), naïve cells + Gant61 (dashed line), pemetrexed-resistant cells + Gant61 (dotted line) at different time. Gant61 revealed obviously inhibitory potency on pemetrexed-resistant cells and naïve cells. By contrast, Gant61 was more effective against the proliferation of pemetrexed-resistant cells. Error bars indicated means ± standard deviation of three independent experiments. One had previously published this figure in Liu et al., 2020 [1].

5. Discussion

Parts of the discussion and Methods were already published in Liu et al., 2020 [1]

With the rapid progress of social and economic changes in people's lifestyle, the morbidity of lung cancer is increasing year by year, and the treatments for it are getting more and more attention by patients and clinic researchers. According to the statistics, 80%-85% of patients with lung cancer was diagnosed as NSCLC. Most of them, however, were suffering from advanced NSCLC, even had lost the possibility for surgical treatment [170]. Therefore, chemotherapy is particularly crucial for the treatment of NSCLC. In order to precisely perform more individualized treatment and improve the efficacy of chemotherapy, discovering the mechanism of chemoresistance and looking for approaches to reduce or suppress the resistance of tumor cells to chemotherapeutic drugs have become hot topics in the field of oncology research.

5.1 Pemetrexed resistance in NSCLC

As is mentioned above, pemetrexed is an anti-cancer drug that can inhibit the metabolism of folate at multiple targets. Through a series of reaction processes, pemetrexed can be transformed into the form of polyglutamic acid after enters the cells, which can inhibit the activity of a variety of enzymes. Not only the synthesis of purine and pyrimidine is interfered, but also the mitosis and DNA replication process of cancer cells are greatly affected, so that the proliferation of tumor cells is inhibited [65, 66]. Due to its multi-target characteristics, pemetrexed is relatively less likely to cause chemoresistance in tumor cells, comparing with other single-target drugs for the treatment of tumors. The concentration of polyglutamic acid in tumor cells, as mentioned above, is much higher as its level in normal cells [65], which means that the activity of pemetrexed against folate metabolism is specifically targets tumor cells, and causes fewer side effects. Therefore, pemetrexed can be used as a first-line chemotherapeutic drug combined with platinum, or as a single agent for maintenance therapy in the treatment of NSCLC [171]. However, it must be admitted that resistance to pemetrexed always exists in the treatment of NSCLC due to complicated resistance mechanisms. Some report has suggested that NSCLC cells with higher expression of TS were more liable to resistance to pemetrexed [172], and ribonucleotide reductase subunit M1 (RRM1) has been proved to be closely connected with resistance to pemetrexed in NSCLC [173]. In view of the fact that many studies about chemoresistance of NSCLC cells to pemetrexed are based on the metabolites during chemical reaction, this research aims at analyzing the influence of Hh signaling pathway on resistance of NSCLC cells to pemetrexed.

5.2 P-gp is involved in chemoresistance in NSCLC

As the most significant treatment for lung cancer, the effectiveness of chemotherapy is severely weakened by MDR. MDR brings tumor cells into chemoresistance to chemotherapeutic products with different chemical constructions and therapeutic effects, which gives rise to the inefficacy of chemotherapy, and ultimately not only causes the unsuccessful outcomes of chemotherapy to patients with cancer but also the poor prognosis. As is mentioned above, P-gp, a transmembrane protein, is encoded by the MDR1 gene, which is located on human chromosome 7q21 [77]. Overexpressed P-gp is the classic mechanism for the formation of MDR of chemotherapeutic drugs. P-pg has the typical characteristic of active efflux pump, and can excrete chemotherapeutic drugs and other cytotoxic drugs to the outside of cells, which brings down the effective drug levels in the cells [77]. P-gp can be commonly detected in various human tissues, including NSCLC, breast cancer, ovarian cancer, esophageal cancer and so on [174, 175]. The expression level of P-gp is not only significantly increased in chemo-resistant cells of these types of cancers, but also in the recurrent breast cancer in accordance with some study [176]. Therefore, P-gp may be considered as the indicator that regulate the sensitivity of tumor cells to chemotherapeutic drugs.

P-gp can regulate apoptosis of chemo-resistant cells and decrease their apoptosis, which is especially essential for MDR of tumor resistant cells [177]. This may indicate that the occurrence of chemoresistance of tumor cells is closely connected with the reduction of tumor cell apoptosis. It is also believed that suppressing the function or expression level of P-gp and increasing the concentrations of intracellular chemotherapeutic drugs may reverse P-gp-mediated chemotherapeutic resistance of NSCLC cells and enhance the efficacy of chemotherapy [178]. A variety of medicines were detected to have the function of reversing MDR which was regulated by P-gp. These drugs mainly include the calcium channel blocker verapamil, the immunosuppressant cyclosporin A and so on [179]. Although these drugs have been confirmed that they have the function of decreasing the activity of P-gp, they have not been completely successful in clinical trials, mainly due to their complex pharmacokinetic effects and unavoidable side effects [179]. Therefore, it is necessary to find new mechanisms or discover new targets to reverse or inhibit the function of P-gp which regulate MDR of NSCLC cells.

5.3 Hh signaling pathway might mediate chemoresistance in NSCLC

In the process of signal transduction pathway, the related ligands outside of cells are effectively combined with corresponding receptors in the cell membrane, which causes the activation of effect proteins in the cytoplasm, and finally gives rise to a series of cascade reactions to activate the corresponding transcription factors. Hh signaling pathway and Wnt

signaling pathway that are involved in the occurrence and progress of tumors and negatively impact the prognosis of patients with cancers participate in MDR regulated by MDR1/P-gp. Queiroz et al. have reported that Hh signaling pathway may bring about chemoresistance by the mechanism of P-gp in myeloid leukemia cells [180]. Meanwhile, Katayama et al. have also reported that P-gp regulated chemoresistance of NSCLC cells to ceritinib [181]. In other word, Hh signaling pathway is closely connected with the expression of P-gp in this respect of chemoresistance. Some studies have suggested that Wnt signaling pathway mediate MDR of tumor cells by P-gp [128, 182]. All the results seem to be displayed on the basis of canonical Wnt/β-catenin signaling pathway which has been mentioned above. Non-canonical Wnt signaling pathway may take part in the chemoresistance of tumor cells by regulating P-gp. “JNK (c-Jun N-terminal kinase) is a member of the MAPK (mitogen-activated protein kinase) family that regulates a range of biological processes implicated in tumorigenesis and neurodegenerative disorders” [183]. In general, Wnt/JNK signaling pathway is considered as non-canonical Wnt/planar cell polarity signaling pathway which is mentioned above, when ligands attach to Fzd, Dvl would transmit the signal to Ras homologue GTPases (RhoGTPases) which includes Rho and Rac, they can stimulate the activation of JNK [184]. Activated JNK has been considered as the essential and positive factor on the expression of P-gp [185]. Oncoprotein c-Jun that could be activated by phosphorylated JNK is an essential part of activator protein-1 (AP-1) [186], which has been confirmed to be connected with high expression of P-gp in vinblastine resistant caco-2 Cells [187]. In brief, not only canonical Wnt signaling pathway may regulate MDR of tumor cells, but also non-canonical Wnt signaling pathway may be involved that. Interestingly, Gli3A, an activator in Hh signaling pathway, may improve the expression of Wnt5a [188], which can activate Wnt/JNK signaling pathway [189]. In other word, Hh signaling pathway may crosstalk with Wnt/JNK signaling pathway. There may be such a possibility, when Hh signaling pathway is activated by some reasons or sources, high expression of Gli3A promotes the expression of Wnt5a which induces the activation of Wnt/JNK signaling pathway. The expression of P-gp would be more effectively improved by synergistic effect of Hh and Wnt/JNK signaling pathway. MDR might be more likely to be detected in patients with tumors including NSCLC.

As is mentioned above, canonical Hh signaling pathway is that Hh ligands bind to its receptor PTCH1 to release the inhibition to Smo after the Hh signaling pathway is activated, then the signal can be transduced to the downstream transcription factors Gli1, Gli2 and Gli3, which are essential for Hh signaling pathway to regulate cell cycle, survival, migration and metabolism. Non-canonical Hh signaling pathway also regulates Hh/Gli signaling, and multiple pathways can activate Gli factors in tumor cells, such as RAS and P13K [150]. In view of the fact that vismodegib has less effect on inhibiting the growth of pemetrexed-resistant NSCLC cells compared with Gant61 in this study, non-canonical pathway may mainly induce the pemetrexed resistance of NSCLC cells. For the chemo-resistant cells suffering from canonical Hh signaling pathway, SMO antagonists may effectively inhibit their growth. But for the resistant cells that undergo non-canonical activation pathway, SMO antagonists may have less inhibitory effect on them due to activation of Gli. These two pathways also perhaps exist at the same time. However, whether it is a canonical Hh signaling pathway or a non-canonical Hh signaling pathway, it is ultimately necessary to

regulate the activity of Gli family, which is the terminal part of Hh signaling pathway and is also regulated by other signal pathways. Therefore, Gli may become an effective anti-tumor target. Gli is downstream protein of SMO, directly inhibiting Gli may fundamentally solve the current chemo-resistance problems caused by SMO mutations. Gant61, Hh signaling pathway inhibitor that targets Gli, binds to Gli1 and Gli2 to reduce the transcriptional activity of them and inhibits the activity of key components downstream of the Hh signaling pathway [165], regardless of the mode of Hh signal activation. Therefore, inhibiting Gli1 and Gli2 downstream of the Hh signaling pathway may have a greater effect, which is also consistent with our research result, so Gant61 was applied as a blocker of the Hh signaling pathway in the study. Selecting Gli as the target to develop targeted small molecule antagonists may be a promising treatment.

In this study, the inhibitory effect of SMO inhibitor vismodegib on pemetrexed-resistant NSCLC cells was not as obviously potent as that of Gli inhibitor Gant61. As is mentioned above, some study reported that there was one common mutant point in SMO, which reduced the sensitivity of tumor cells to vismodegib, and improved their vismodegib-resistance [165]. These pemetrexed-resistant NSCLC cells in experimental group probably have the same mutant point or other unknown mutant points in SMO, which influence the efficacy of vismodegib. Or Hh signaling pathway may cooperate with other signaling pathways by transcription factors Gli family, such as Wnt/JNK signaling pathway that was mentioned above. Therefore, either efficacy of vismodegib was decreased or efficacy of Gant61 was increased in the light of these two possibilities. In order to show the results more clearly, Gant61 was applied in this study. According to the result which was obtained by qPCR, the gene of SMO in resistant NSCLS cells was not obviously overexpressed compared with that in naïve cells, while the expression of the other relative genes in resistant NSCLS cells which were crucial to Hh signaling pathway, such as SHH, Gli1, Gli2, Gli3 and so on, presented distinctly uptrend compared with that in naïve cells. The result further bears out that SMO inhibitor vismodegib is not as efficient as Gant61 on chemo-resistant NSCLC cells due to low expression of SMO. Previous research has suggested that the expression of SHH and Gli1 could be commonly detected in chemo-resistant NSCLC cells [190, 191], which was in accord with the result of this study. Hh signaling pathway probably take part in the pemetrexed resistance of NSCLC cells. After application of Gant61, pemetrexed-resistant NSCLC cells were more subject to pharmaceutical effect of pemetrexed compared with naïve NSCLC cells, in other words, the pemetrexed resistance of them was decreased. Some study reported that overexpression of Gli1 and Gli2 restrained the cell death induced by Gant61 through regulating NBS1 [192]. Taking in to account the results of this study, Gant61 perhaps is a sensitizer that is inclined to improve the sensitivity of resistant NSCLC cells to pemetrexed. Therefore, there may be a possibility that decreasing or reversing pemetrexed resistance by obstructing Hh signaling pathway.

5.4 Other factors related to Hh signaling pathway may regulate chemoresistance

However, the mechanism by which Hh signaling mediates chemotherapy resistance is not very clear. As is mentioned above, CSCs are a type of cells with stem cell-like characteristics, with unique self-renewal, proliferation and differentiation capabilities. CSCs are associated with malignant phenotypic characteristics such as tumor metastasis, resistance to chemotherapy and recurrence [193]. Some study reported that the formation of lung CSCs is correlated with Hh signaling pathway, and can be inhibited by obstructing Hh signaling pathway [194]. Hh signal pathway may mediate the self-renewal of CSCs and promote the expression of stemness-associated genes to sustain the characteristics of CSCs [193]. Therefore, Hh inhibitors may either directly target CSCs or target the correlated genes that provides support for CSCs.

Epithelial-mesenchymal transition (EMT) is a common physiological phenomenon, which is an important part of the morphogenesis of embryonic development [195]. In the process of EMT, epithelial cells acquire the characteristics of fibroblast-like cells: the intercellular adhesion is weakened, the tight junctions between cells and cell polarity are destroyed, and the motility of cells is enhanced [195]. EMT is closely related to tumor invasion, metastasis and chemoresistance [195]. And there is a close relationship between SHH and EMT, the main genes that regulate EMT are the target genes of Hh signal transcription factor Gli1 [196]. A study reported that Hh signaling pathway mediated the resistance of NSCLC cells to EGFR-TKIs induced by EMT, not only the CSCs markers were decreased, but also the chemoresistance was weakened [197]. Therefore, Hh signaling pathway may participate in the chemoresistance of NSCLC cells through a variety of known or unknown ways.

Although the process that Hh signaling pathway is involved in the occurrence, development and chemoresistance of tumors is very complicated, inhibitors that targets the Hh signaling pathway hold promise for inhibiting the occurrence and development of NSCLC and decreasing chemoresistance. In conclusion, activated Hh signaling pathway may be crucial to pemetrexed resistance of NSCLC cells, and the sensitivity of resistant NSLCL cells to pemetrexed may be improved or restored by blocking Hh signaling pathway. However, the results need to be confirmed in further experimental and clinical studies.

6. Outlook

It has been found that the Hh signaling pathway is not only involved in the occurrence and development of a variety of tumors, but also is connected with tumor chemoresistance. In NSCLC, the Hh signaling pathway is hyperactive. By applying Hh signaling pathway inhibitors, the chemoresistance of NSCLC cells is weakened compared with naïve NSCLC cells. This study further shows that the Hh signaling pathway has great effects on NSCLC cells, has also clarified parts of the chemoresistance mechanisms, provides a theoretical basis for future research on reducing tumor chemoresistance, and also provides ideas for clinical treatment of NSCLC patients with chemoresistance. However, the Hh signaling pathway is prone to cross-reactions with other signaling pathways to affect chemoresistance of tumors. How to quickly and effectively reduce the chemoresistance of tumors still needs further and more research.

References

1. Liu Y, Huber RM, Kiefl R, Tufman A, Kauffmann-Guerrero D. Hedgehog Pathway Activation Might Mediate Pemetrexed Resistance in NSCLC Cells. *Anticancer Research.* 2020;40(3):1451-1458.
2. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians.* 2018;68(6):394-424.
3. Taciak B, Pruszynska I, Kiraga L, Bialasek M, Krol M. Wnt signaling pathway in development and cancer. *Journal of Physiology and Pharmacology.* 2018;69(2).
4. Postmus PE, Kerr KM, Oudkerk M, Senan S, Waller DA, Vansteenkiste J, Escriu C, Peters S. Early and locally advanced non-small-cell lung cancer (NSCLC): ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Annals of Oncology.* 2017;28(suppl_4):iv1-iv21.
5. Planchard D, Popat S, Kerr K, Novello S, Smit E F, Faivre-Finn C, Mok T S, Reck M, Van Schil P E, Hellmann M D & Peters S. Metastatic non-small cell lung cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. 15 September 2020. European Society for Medical Oncology. https://www.esmo.org/content/download/347819/6934778/1/ESMO-CPG-mNSCLC-15SEPT2020.pdf. Accessed 16 June 2021. Accessed.
6. Zappa C, Mousa SA. Non-small cell lung cancer: current treatment and future advances. *Translational Lung Cancer Research.* 2016;5(3):288-300.
7. Paladugu RR, Benfield JR, Pak HY, Ross RK, Teplitz RL. Bronchopulmonary Kulchitzky cell carcinomas. A new classification scheme for typical and atypical carcinoids. *Cancer.* 1985;55(6):1303-1311.
8. Del Galdo F. Caveolin-1: a new therapeutic target in tissue fibrosis and scleroderma? *Cell Cycle.* 2011;10(21):3629.
9. Denisenko TV, Budkevich IN, Zhivotovsky B. Cell death-based treatment of lung adenocarcinoma. *Cell Death & Disease.* 2018;9(2):117.
10. McIntyre A, Ganti AK. Lung cancer-A global perspective. *Journal of Surgical Oncology.* 2017;115(5):550-554.
11. Xu Y, Zhang J, Han J, Pan X, Cao Y, Guo H, Pan Y, An Y, Li X. Curcumin inhibits tumor proliferation induced by neutrophil elastase through the upregulation of α1-antitrypsin in lung cancer. *Molecular Oncology.* 2012;6(4):405-417.
12. Nguyen DX, Chiang AC, Zhang XH, Kim JY, Kris MG, Ladanyi M, Gerald WL, Massagué J. WNT/TCF signaling through LEF1 and HOXB9 mediates lung adenocarcinoma metastasis. *Cell.* 2009;138(1):51-62.
13. Li X, Zhang Q, Cai L, Wang Y, Wang Q, Huang X, Fu S, Bai J, Liu J, Zhang G, Qi J. Inhibitor of growth 4 induces apoptosis in human lung adenocarcinoma cell line A549 via Bcl-2 family proteins and mitochondria apoptosis pathway. *Journal of Cancer Research and Clinical Oncology.* 2009;135(6):829-835.
14. Youlden DR, Cramb SM, Baade PD. The International Epidemiology of Lung Cancer: geographical distribution and secular trends. *Journal of Thoracic Oncology.*

2008;3(8):819-831.

15. Lianidou E, Alix-Panabières C. Early detection of lung cancer based on DNA methylation analysis in sputum and plasma. *Translational Cancer Research.* 2017:S51-S53.
16. Blandin Knight S, Crosbie PA, Balata H, Chudziak J, Hussell T, Dive C. Progress and prospects of early detection in lung cancer. *Open Biology.* 2017;7(9).
17. Fedor D, Johnson WR, Singhal S. Local recurrence following lung cancer surgery: incidence, risk factors, and outcomes. *Surgical Oncology.* 2013;22(3):156-161.
18. Uramoto H, Tanaka F. Recurrence after surgery in patients with NSCLC. *Translational Lung Cancer Research.* 2014;3(4):242-249.
19. Park JH, Shim YM, Baek HJ, Kim MS, Choe DH, Cho KJ, Lee CT, Zo JI. Postoperative adjuvant therapy for stage II non-small-cell lung cancer. *Annals of Thoracic Surgery.* 1999;68(5):1821-1826.
20. Douillard JY, Rosell R, De Lena M, Carpagnano F, Ramlau R, Gonzáles-Larriba JL, Grodzki T, Pereira JR, Le Groumellec A, Lorusso V, Clary C, Torres AJ, Dahabreh J, Souquet PJ, Astudillo J, Fournel P, Artal-Cortes A, Jassem J, Koubkova L, His P, Riggi M, Hurteloup P. Adjuvant vinorelbine plus cisplatin versus observation in patients with completely resected stage IB-IIIA non-small-cell lung cancer (Adjuvant Navelbine International Trialist Association [ANITA]): a randomised controlled trial. *Lancet Oncology.* 2006;7(9):719-727.
21. Tanaka F, Yoneda K. Adjuvant therapy following surgery in non-small cell lung cancer (NSCLC). *Surgery Today.* 2016;46(1):25-37.
22. Tyldesley S, Boyd C, Schulze K, Walker H, Mackillop WJ. Estimating the need for radiotherapy for lung cancer: an evidence-based, epidemiologic approach. *International Journal of Radiation Oncology, Biology, Physics.* 2001;49(4):973-985.
23. Hav M, Libbrecht L, Geboes K, Ferdinande L, Boterberg T, Ceelen W, Pattyn P, Cuvelier C. Prognostic value of tumor shrinkage versus fragmentation following radiochemotherapy and surgery for rectal cancer. *Virchows Archiv.* 2015;466(5):517-523.
24. Wei W, Zhou J, Zhang Q, Liao DH, Liu QD, Zhong BL, Liang ZB, Zhang YC, Jiang R, Liu GY, Xu CY, Li Zhou H, Zhu SY, Yang N, Jiang W, Liu ZG. Postoperative intensity-modulated radiation therapy reduces local recurrence and improves overall survival in III-N2 non-small-cell lung cancer: A single-center, retrospective study. *Cancer Medicine.* 2020;9(8):2820-2832.
25. Armstrong JG, Burman C, Leibel S, Fontenla D, Kutcher G, Zelefsky M, Fuks Z. Three-dimensional conformal radiation therapy may improve the therapeutic ratio of high dose radiation therapy for lung cancer. *International Journal of Radiation Oncology, Biology, Physics.* 1993;26(4):685-689.
26. Harada K, Katoh N, Suzuki R, Ito YM, Shimizu S, Onimaru R, Inoue T, Miyamoto N, Shirato H. Evaluation of the motion of lung tumors during stereotactic body radiation therapy (SBRT) with four-dimensional computed tomography (4DCT) using real-time tumor-tracking radiotherapy system (RTRT). *Physica Medica.* 2016;32(2):305-311.
27. Wee P, Wang Z. Epidermal Growth Factor Receptor Cell Proliferation Signaling Pathways. *Cancers.* 2017;9(5).

28. Sebastian S, Settleman J, Reshkin SJ, Azzariti A, Bellizzi A, Paradiso A. The complexity of targeting EGFR signalling in cancer: from expression to turnover. *Biochimica et Biophysica Acta.* 2006;1766(1):120-139.
29. Duyster J, Bai RY, Morris SW. Translocations involving anaplastic lymphoma kinase (ALK). *Oncogene.* 2001;20(40):5623-5637.
30. Hallberg B, Palmer RH. Mechanistic insight into ALK receptor tyrosine kinase in human cancer biology. *Nature Reviews: Cancer.* 2013;13(10):685-700.
31. Soda M, Takada S, Takeuchi K, Choi YL, Enomoto M, Ueno T, Haruta H, Hamada T, Yamashita Y, Ishikawa Y, Sugiyama Y, Mano H. A mouse model for EML4-ALK-positive lung cancer. *Proceedings of the National Academy of Sciences of the United States of America.* 2008;105(50):19893-19897.
32. Camidge DR, Bang YJ, Kwak EL, Iafrate AJ, Varella-Garcia M, Fox SB, Riely GJ, Solomon B, Ou SH, Kim DW, Salgia R, Fidias P, Engelman JA, Gandhi L, Jänne PA, Costa DB, Shapiro GI, Lorusso P, Ruffner K, Stephenson P, Tang Y, Wilner K, Clark JW, Shaw AT. Activity and safety of crizotinib in patients with ALK-positive non-small-cell lung cancer: updated results from a phase 1 study. *Lancet Oncology.* 2012;13(10):1011-1019.
33. Zhang Q, Qin N, Wang J, Lv J, Yang X, Li X, Nong J, Zhang H, Zhang X, Wu Y, Zhang S. Crizotinib versus platinum-based double-agent chemotherapy as the first line treatment in advanced anaplastic lymphoma kinase-positive lung adenocarcinoma. *Thorac Cancer.* 2016;7(1):3-8.
34. Davies KD, Doebele RC. Molecular pathways: ROS1 fusion proteins in cancer. *Clinical Cancer Research.* 2013;19(15):4040-4045.
35. Shaw AT, Ou SH, Bang YJ, Camidge DR, Solomon BJ, Salgia R, Riely GJ, Varella-Garcia M, Shapiro GI, Costa DB, Doebele RC, Le LP, Zheng Z, Tan W, Stephenson P, Shreeve SM, Tye LM, Christensen JG, Wilner KD, Clark JW, Iafrate AJ. Crizotinib in ROS1-rearranged non-small-cell lung cancer. *New England Journal of Medicine.* 2014;371(21):1963-1971.
36. Ou SH, Tan J, Yen Y, Soo RA. ROS1 as a 'druggable' receptor tyrosine kinase: lessons learned from inhibiting the ALK pathway. *Expert Review of Anticancer Therapy.* 2012;12(4):447-456.
37. Xalkori: European Public Assessment Report - Summary for the public. European Medicines Agency. 06 February 2018. https://www.ema.europa.eu/en/documents/overview/xalkori-epar-summary-public_en.pdf. Accessed 16 June 2016.
38. Oikonomou E, Pintzas A. Cancer genetics of sporadic colorectal cancer: BRAF and PI3KCA mutations, their impact on signaling and novel targeted therapies. *Anticancer Research.* 2006;26(2a):1077-1084.
39. Schaeffer HJ, Weber MJ. Mitogen-activated protein kinases: specific messages from ubiquitous messengers. *Molecular and Cellular Biology.* 1999;19(4):2435-2444.
40. Śmiech M, Leszczyński P, Kono H, Wardell C, Taniguchi H. Emerging BRAF Mutations in Cancer Progression and Their Possible Effects on Transcriptional Networks. *Genes.* 2020;11(11).
41. Greaves WO, Verma S, Patel KP, Davies MA, Barkoh BA, Galbincea JM, Yao H, Lazar

AJ, Aldape KD, Medeiros LJ, Luthra R. Frequency and spectrum of BRAF mutations in a retrospective, single-institution study of 1112 cases of melanoma. *Journal of Molecular Diagnostics.* 2013;15(2):220-226.

42. Alvarez JGB, Otterson GA. Agents to treat BRAF-mutant lung cancer. *Drugs Context.* 2019;8:212566.
43. Wu PK, Park JI. MEK1/2 Inhibitors: Molecular Activity and Resistance Mechanisms. *Seminars in Oncology.* 2015;42(6):849-862.
44. Tafinlar : European Public Assessment Report - Medicine overview. European Medicines Agency. 11 September 2018. https://www.ema.europa.eu/en/documents/overview/tafinlar-epar-medicine-overview_en.pdf. Access 16 June 2021.
45. Ascierto PA, Marincola FM. 2015: The Year of Anti-PD-1/PD-L1s Against Melanoma and Beyond. *EBioMedicine.* 2015;2(2):92-93.
46. Chikuma S. Basics of PD-1 in self-tolerance, infection, and cancer immunity. *International Journal of Clinical Oncology.* 2016;21(3):448-455.
47. Gettinger S, Rizvi NA, Chow LQ, Borghaei H, Brahmer J, Ready N, Gerber DE, Shepherd FA, Antonia S, Goldman JW, Juergens RA, Laurie SA, Nathan FE, Shen Y, Harbison CT, Hellmann MD. Nivolumab Monotherapy for First-Line Treatment of Advanced Non-Small-Cell Lung Cancer. *Journal of Clinical Oncology.* 2016;34(25):2980-2987.
48. Garon EB, Rizvi NA, Hui R, Leighl N, Balmanoukian AS, Eder JP, Patnaik A, Aggarwal C, Gubens M, Horn L, Carcereny E, Ahn MJ, Felip E, Lee JS, Hellmann MD, Hamid O, Goldman JW, Soria JC, Dolled-Filhart M, Rutledge RZ, Zhang J, Lunceford JK, Rangwala R, Lubiniecki GM, Roach C, Emancipator K, Gandhi L. Pembrolizumab for the treatment of non-small-cell lung cancer. *New England Journal of Medicine.* 2015;372(21):2018-2028.
49. Mu CY, Huang JA, Chen Y, Chen C, Zhang XG. High expression of PD-L1 in lung cancer may contribute to poor prognosis and tumor cells immune escape through suppressing tumor infiltrating dendritic cells maturation. *Medical Oncology.* 2011;28(3):682-688.
50. Herbst RS, Giaccone G, de Marinis F, Reinmuth N, Vergnenegre A, Barrios CH, Morise M, Felip E, Andric Z, Geater S, Özgüroğlu M, Zou W, Sandler A, Enquist I, Komatsubara K, Deng Y, Kuriki H, Wen X, McCleland M, Mocci S, Jassem J, Spigel DR. Atezolizumab for First-Line Treatment of PD-L1-Selected Patients with NSCLC. *New England Journal of Medicine.* 2020;383(14):1328-1339.
51. Antonia SJ, Villegas A, Daniel D, Vicente D, Murakami S, Hui R, Kurata T, Chiappori A, Lee KH, de Wit M, Cho BC, Bourhaba M, Quantin X, Tokito T, Mekhail T, Planchard D, Kim YC, Karapetis CS, Hiret S, Ostoros G, Kubota K, Gray JE, Paz-Ares L, de Castro Carpeño J, Faivre-Finn C, Reck M, Vansteenkiste J, Spigel DR, Wadsworth C, Melillo G, Taboada M, Dennis PA, Özgüroğlu M. Overall Survival with Durvalumab after Chemoradiotherapy in Stage III NSCLC. *New England Journal of Medicine.* 2018;379(24):2342-2350.
52. Imfinzi : European Public Assessment Report - Medicine overview. European Medicines Agency. 30 October 2018.

https://www.ema.europa.eu/en/documents/overview/imfinzi-epar-medicine-overview_en.pdf. Accessed 16 June 2021.

53. Burdett S, Pignon JP, Tierney J, Tribodet H, Stewart L, Le Pechoux C, Aupérin A, Le Chevalier T, Stephens RJ, Arriagada R, Higgins JP, Johnson DH, Van Meerbeeck J, Parmar MK, Souhami RL, Bergman B, Douillard JY, Dunant A, Endo C, Girling D, Kato H, Keller SM, Kimura H, Knuuttila A, Kodama K, Komaki R, Kris MG, Lad T, Mineo T, Piantadosi S, Rosell R, Scagliotti G, Seymour LK, Shepherd FA, Sylvester R, Tada H, Tanaka F, Torri V, Waller D, Liang Y. Adjuvant chemotherapy for resected early-stage non-small cell lung cancer. *Cochrane Database of Systematic Reviews.* 2015(3):Cd011430.
54. Ramnath N, Dilling TJ, Harris LJ, Kim AW, Michaud GC, Balekian AA, Diekemper R, Detterbeck FC, Arenberg DA. Treatment of stage III non-small cell lung cancer: Diagnosis and management of lung cancer, 3rd ed: American College of Chest Physicians evidence-based clinical practice guidelines. *Chest.* 2013;143(5 Suppl):e314S-e340S.
55. Zarogoulidis K, Zarogoulidis P, Darwiche K, Boutsikou E, Machairiotis N, Tsakiridis K, Katsikogiannis N, Kougioumtzi I, Karapantzos I, Huang H, Spyratos D. Treatment of non-small cell lung cancer (NSCLC). *Journal of Thoracic Disease.* 2013;5 Suppl 4(Suppl 4):S389-396.
56. Sculier JP, Moro-Sibilot D. First- and second-line therapy for advanced nonsmall cell lung cancer. *European Respiratory Journal.* 2009;33(4):915-930.
57. Pirker R. Adjuvant chemotherapy of non-small cell lung cancer. *Tanaffos.* 2012;11(1):12-17.
58. Helm CW, States JC. Enhancing the efficacy of cisplatin in ovarian cancer treatment - could arsenic have a role. *Journal of Ovarian Research.* 2009;2:2.
59. Arriagada R, Bergman B, Dunant A, Le Chevalier T, Pignon JP, Vansteenkiste J. Cisplatin-based adjuvant chemotherapy in patients with completely resected non-small-cell lung cancer. *New England Journal of Medicine.* 2004;350(4):351-360.
60. Mastrangelo M. Cisplatin-Based Therapy and CINV: Optimal Antiemetics During Germ Cell Testicular Cancer Treatment. *Clinical Journal of Oncology Nursing.* 2018;22(2):E31-e36.
61. Martinho N, Santos TCB, Florindo HF, Silva LC. Cisplatin-Membrane Interactions and Their Influence on Platinum Complexes Activity and Toxicity. *Frontiers in Physiology.* 2018;9:1898.
62. Sharp SY, Rogers PM, Kelland LR. Transport of cisplatin and bis-acetato-ammine-dichlorocyclohexylamine Platinum(IV) (JM216) in human ovarian carcinoma cell lines: identification of a plasma membrane protein associated with cisplatin resistance. *Clinical Cancer Research.* 1995;1(9):981-989.
63. Rabik CA, Dolan ME. Molecular mechanisms of resistance and toxicity associated with platinating agents. *Cancer Treatment Reviews.* 2007;33(1):9-23.
64. Rajeswaran A, Trojan A, Burnand B, Giannelli M. Efficacy and side effects of cisplatin- and carboplatin-based doublet chemotherapeutic regimens versus non-platinum-based doublet chemotherapeutic regimens as first line treatment of metastatic non-small cell lung carcinoma: a systematic review of randomized controlled trials. *Lung*

Cancer. 2008;59(1):1-11.

65. Adjei AA. Pemetrexed (Alimta): a novel multitargeted antifolate agent. *Expert Review of Anticancer Therapy.* 2003;3(2):145-156.
66. Miller DS, Blessing JA, Krasner CN, Mannel RS, Hanjani P, Pearl ML, Waggoner SE, Boardman CH. Phase II evaluation of pemetrexed in the treatment of recurrent or persistent platinum-resistant ovarian or primary peritoneal carcinoma: a study of the Gynecologic Oncology Group. *Journal of Clinical Oncology.* 2009;27(16):2686-2691.
67. Hanna N, Shepherd FA, Fossella FV, Pereira JR, De Marinis F, von Pawel J, Gatzemeier U, Tsao TC, Pless M, Muller T, Lim HL, Desch C, Szondy K, Gervais R, Shaharyar, Manegold C, Paul S, Paoletti P, Einhorn L, Bunn PA, Jr. Randomized phase III trial of pemetrexed versus docetaxel in patients with non-small-cell lung cancer previously treated with chemotherapy. *Journal of Clinical Oncology.* 2004;22(9):1589-1597.
68. Li X, Wei S, Chen J. Critical appraisal of pemetrexed in the treatment of NSCLC and metastatic pulmonary nodules. *OncoTargets and Therapy.* 2014;7:937-945.
69. Noble RL, Beer CT, Cutts JH. Role of chance observations in chemotherapy: Vinca rosea. *Annals of the New York Academy of Sciences.* 1958;76(3):882-894.
70. Moudi M, Go R, Yien CYS, Nazre M. Vinca alkaloids. *International Journal of Preventive Medicine.* 2013;4(11):1231-1235.
71. Capasso A. Vinorelbine in cancer therapy. *Current Drug Targets.* 2012;13(8):1065-1071.
72. Gregory RK, Smith IE. Vinorelbine--a clinical review. *British Journal of Cancer.* 2000;82(12):1907-1913.
73. Le Chevalier T, Brisgand D, Douillard JY, Pujol JL, Alberola V, Monnier A, Riviere A, Lianes P, Chomy P, Cigolari S, et al. Randomized study of vinorelbine and cisplatin versus vindesine and cisplatin versus vinorelbine alone in advanced non-small-cell lung cancer: results of a European multicenter trial including 612 patients. *Journal of Clinical Oncology.* 1994;12(2):360-367.
74. Chang A. Chemotherapy, chemoresistance and the changing treatment landscape for NSCLC. *Lung Cancer.* 2011;71(1):3-10.
75. Baguley BC. Multiple drug resistance mechanisms in cancer. *Molecular Biotechnology.* 2010;46(3):308-316.
76. Juliano RL, Ling V. A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochimica et Biophysica Acta.* 1976;455(1):152-162.
77. Jose M, Thomas SV. Role of multidrug transporters in neurotherapeutics. *Annals of the Indian Academy of Neurology.* 2009;12(2):89-98.
78. Kantharidis P, El-Osta S, Silva M, Lee G, Hu XF, Zalcberg J. Regulation of MDR1 gene expression: emerging concepts. *Drug Resist Updat.* 2000;3(2):99-108.
79. Ziemann C, Bürkle A, Kahl GF, Hirsch-Ernst KI. Reactive oxygen species participate in mdr1b mRNA and P-glycoprotein overexpression in primary rat hepatocyte cultures. *Carcinogenesis.* 1999;20(3):407-414.
80. Gao F, Liu J, Dong WW, Wang W, Wang Y, Cai D, Zheng Z, Sun K. Investigation of the mechanism involved in the As2O3-regulated decrease in MDR1 expression in leukemia cells. *Oncology Reports.* 2014;31(2):926-932.

81. Bentires-Alj M, Barbu V, Fillet M, Chariot A, Relic B, Jacobs N, Gielen J, Merville MP, Bours V. NF-kappaB transcription factor induces drug resistance through MDR1 expression in cancer cells. *Oncogene.* 2003;22(1):90-97.
82. Becker JP, Depret G, Van Bambeke F, Tulkens PM, Prévost M. Molecular models of human P-glycoprotein in two different catalytic states. *BMC Structural Biology.* 2009;9:3.
83. Safa AR. Identification and characterization of the binding sites of P-glycoprotein for multidrug resistance-related drugs and modulators. *Current Medicinal Chemistry: Anti-Cancer Agents.* 2004;4(1):1-17.
84. Abolhoda A, Wilson AE, Ross H, Danenberg PV, Burt M, Scotto KW. Rapid activation of MDR1 gene expression in human metastatic sarcoma after in vivo exposure to doxorubicin. *Clinical Cancer Research.* 1999;5(11):3352-3356.
85. Nooter K, Sonneveld P. Clinical relevance of P-glycoprotein expression in haematological malignancies. *Leukemia Research.* 1994;18(4):233-243.
86. Abe Y, Ohnishi Y, Yoshimura M, Ota E, Ozeki Y, Oshika Y, Tokunaga T, Yamazaki H, Ueyema Y, Ogata T, Tamaoki N, Nakamura M. P-glycoprotein-mediated acquired multidrug resistance of human lung cancer cells in vivo. *British Journal of Cancer.* 1996;74(12):1929-1934.
87. Fang L, Sheng H, Wan D, Zhu C, Jiang R, Sun X, Feng J. Prognostic role of multidrug resistance-associated protein 1 expression and platelet count in operable non-small cell lung cancer. *Oncology Letters.* 2018;16(1):1123-1132.
88. Lili X, Xiaoyu T. Expression of PKCα, PKCε, and P-gp in epithelial ovarian carcinoma and the clinical significance. *European Journal of Gynaecological Oncology.* 2015;36(2):181-185.
89. Chen YL, Yang TY, Chen KC, Wu CL, Hsu SL, Hsueh CM. Hypoxia can impair doxorubicin resistance of non-small cell lung cancer cells by inhibiting MRP1 and P-gp expression and boosting the chemosensitizing effects of MRP1 and P-gp blockers. *Cellular Oncology (Dordrecht).* 2016;39(5):411-433.
90. Chan KT, Lung ML. Mutant p53 expression enhances drug resistance in a hepatocellular carcinoma cell line. *Cancer Chemotherapy and Pharmacology.* 2004;53(6):519-526.
91. Wang J, Zhang J, Zhang L, Zhao L, Fan S, Yang Z, Gao F, Kong Y, Xiao GG, Wang Q. Expression of P-gp, MRP, LRP, GST-π and TopoIIα and intrinsic resistance in human lung cancer cell lines. *Oncology Reports.* 2011;26(5):1081-1089.
92. Ferguson GD, Bridge WJ. The glutathione system and the related thiol network in Caenorhabditis elegans. *Redox Biology.* 2019;24:101171.
93. Lushchak VI. Glutathione homeostasis and functions: potential targets for medical interventions. *Journal of Amino Acids.* 2012;2012:736837.
94. Jamali B, Nakhjavani M, Hosseinzadeh L, Amidi S, Nikounezhad N, H Shirazi F. Intracellular GSH Alterations and Its Relationship to Level of Resistance following Exposure to Cisplatin in Cancer Cells. *Iranian journal of pharmaceutical research : IJPR.* 2015;14(2):513-519.
95. Yu DS, Ma CP, Chang SY. Establishment and characterization of renal cell carcinoma cell lines with multidrug resistance. *Urological Research.* 2000;28(2):86-92.
96. Zhu Z, Du S, Du Y, Ren J, Ying G, Yan Z. Glutathione reductase mediates drug

resistance in glioblastoma cells by regulating redox homeostasis. *Journal of Neurochemistry.*

97. Bhattacharjee P, Paul S, Banerjee M, Patra D, Banerjee P, Ghoshal N, Bandyopadhyay A, Giri AK. Functional compensation of glutathione S-transferase M1 (GSTM1) null by another GST superfamily member, GSTM2. *Scientific Reports.* 2013;3:2704.
98. Gu JD, Hua F, Mei CR, Zheng DJ, Wang GF, Zhou QH. HapMap-based study on the association between MPO and GSTP1 gene polymorphisms and lung cancer susceptibility in Chinese Han population. *Acta Pharmacologica Sinica.* 2014;35(5):636-644.
99. Raijmakers MT, Steegers EA, Peters WH. Glutathione S-transferases and thiol concentrations in embryonic and early fetal tissues. *Human Reproduction.* 2001;16(11):2445-2450.
100. Cheng X, Kigawa J, Minagawa Y, Kanamori Y, Itamochi H, Okada M, Terakawa N. Glutathione S-transferase-pi expression and glutathione concentration in ovarian carcinoma before and after chemotherapy. *Cancer.* 1997;79(3):521-527.
101. Rybárová S, Hodorová I, Mihalik J, Mirossay L. MRP1 and GSTp1 expression in non-small cell lung cancer does not correlate with clinicopathological parameters: A Slovakian population study. *Acta Histochemica.* 2014;116(8):1390-1398.
102. Bax BD, Murshudov G, Maxwell A, Germe T. DNA Topoisomerase Inhibitors: Trapping a DNA-Cleaving Machine in Motion. *Journal of Molecular Biology.* 2019;431(18):3427-3449.
103. Goffart S, Hangas A, Pohjoismäki JLO. Twist and Turn-Topoisomerase Functions in Mitochondrial DNA Maintenance. *International Journal of Molecular Sciences.* 2019;20(8):2041.
104. Deweese JE, Osheroff N. The use of divalent metal ions by type II topoisomerases. *Metallomics : integrated biometal science.* 2010;2(7):450-459.
105. Naito S, Hasegawa S, Yokomizo A, Koga H, Kotoh S, Kuwano M, Kumazawa J. Non-P-glycoprotein-mediated atypical multidrug resistance in a human bladder cancer cell line. *Japanese journal of cancer research : Gann.* 1995;86(11):1112-1118.
106. Nielsen D, Maare C, Skovsgaard T. Cellular resistance to anthracyclines. *General Pharmacology.* 1996;27(2):251-255.
107. Yusenko M, Jakobs A, Klempnauer KH. A novel cell-based screening assay for small-molecule MYB inhibitors identifies podophyllotoxins teniposide and etoposide as inhibitors of MYB activity. *Scientific Reports.* 2018;8(1):13159.
108. Yang F, Teves SS, Kemp CJ, Henikoff S. Doxorubicin, DNA torsion, and chromatin dynamics. *Biochimica et Biophysica Acta.* 2014;1845(1):84-89.
109. de Jong S, Zijlstra JG, de Vries EG, Mulder NH. Reduced DNA topoisomerase II activity and drug-induced DNA cleavage activity in an adriamycin-resistant human small cell lung carcinoma cell line. *Cancer Research.* 1990;50(2):304-309.
110. Matsumoto Y, Takano H, Kunishio K, Nagao S, Fojo T. Incidence of mutation and deletion in topoisomerase II alpha mRNA of etoposide and mAMSA-resistant cell lines. *Japanese Journal of Cancer Research.* 2001;92(10):1133-1137.
111. Takano H, Kohno K, Ono M, Uchida Y, Kuwano M. Increased phosphorylation of DNA topoisomerase II in etoposide-resistant mutants of human cancer KB cells. *Cancer*

Research. 1991;51(15):3951-3957.

112. Wahid M, Bano Q. Camptothecin and its analogs antitumor activity by poisoning topoisomerase I, their structure activity relationship and clinical development perspective of analogs. *Journal of Applied Pharmacy.* 2014;6:386-395.
113. Rasheed ZA, Rubin EH. Mechanisms of resistance to topoisomerase I-targeting drugs. *Oncogene.* 2003;22(47):7296-7304.
114. Lee YC, Lee CH, Tsai HP, An HW, Lee CM, Wu JC, Chen CS, Huang SH, Hwang J, Cheng KT, Leiw PL, Chen CL, Lin CM. Targeting of Topoisomerase I for Prognoses and Therapeutics of Camptothecin-Resistant Ovarian Cancer. *PloS One.* 2015;10(7):e0132579.
115. Jensen NF, Agama K, Roy A, Smith DH, Pfister TD, Rømer MU, Zhang HL, Doroshow JH, Knudsen BR, Stenvang J, Brünner N, Pommier Y. Characterization of DNA topoisomerase I in three SN-38 resistant human colon cancer cell lines reveals a new pair of resistance-associated mutations. *Journal of Experimental and Clinical Cancer Research.* 2016;35:56.
116. Tsujimoto Y, Cossman J, Jaffe E, Croce CM. Involvement of the bcl-2 gene in human follicular lymphoma. *Science.* 1985;228(4706):1440-1443.
117. Wang X, Welsh N. Bcl-2 maintains the mitochondrial membrane potential, but fails to affect production of reactive oxygen species and endoplasmic reticulum stress, in sodium palmitate-induced β-cell death. *Upsala Journal of Medical Sciences.* 2014;119(4):306-315.
118. Delbridge AR, Grabow S, Strasser A, Vaux DL. Thirty years of BCL-2: translating cell death discoveries into novel cancer therapies. *Nature Reviews: Cancer.* 2016;16(2):99-109.
119. Murphy KM, Ranganathan V, Farnsworth ML, Kavallaris M, Lock RB. Bcl-2 inhibits Bax translocation from cytosol to mitochondria during drug-induced apoptosis of human tumor cells. *Cell Death and Differentiation.* 2000;7(1):102-111.
120. Voehringer DW, McConkey DJ, McDonnell TJ, Brisbay S, Meyn RE. Bcl-2 expression causes redistribution of glutathione to the nucleus. *Proceedings of the National Academy of Sciences of the United States of America.* 1998;95(6):2956-2960.
121. Sartorius UA, Krammer PH. Upregulation of Bcl-2 is involved in the mediation of chemotherapy resistance in human small cell lung cancer cell lines. *International Journal of Cancer.* 2002;97(5):584-592.
122. Kowaltowski AJ, Fiskum G. Redox mechanisms of cytoprotection by Bcl-2. *Antioxidants & redox signaling.* 2005;7(3-4):508-514.
123. Olie RA, Hafner C, Küttel R, Sigrist B, Willers J, Dummer R, Hall J, Stahel RA, Zangemeister-Wittke U. Bcl-2 and bcl-xL antisense oligonucleotides induce apoptosis in melanoma cells of different clinical stages. *Journal of Investigative Dermatology.* 2002;118(3):505-512.
124. Nusse R, Varmus HE. Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. *Cell.* 1982;31(1):99-109.
125. Nakashima T, Liu D, Nakano J, Ishikawa S, Yokomise H, Ueno M, Kadota K, Huang CL. Wnt1 overexpression associated with tumor proliferation and a poor prognosis in

non-small cell lung cancer patients. *Oncology Reports.* 2008;19(1):203-209.

126. Huang CL, Liu D, Ishikawa S, Nakashima T, Nakashima N, Yokomise H, Kadota K, Ueno M. Wnt1 overexpression promotes tumour progression in non-small cell lung cancer. *European Journal of Cancer.* 2008;44(17):2680-2688.
127. Gao Y, Liu Z, Zhang X, He J, Pan Y, Hao F, Xie L, Li Q, Qiu X, Wang E. Inhibition of cytoplasmic GSK-3β increases cisplatin resistance through activation of Wnt/β-catenin signaling in A549/DDP cells. *Cancer Letters.* 2013;336(1):231-239.
128. Zhang H, Zhang X, Wu X, Li W, Su P, Cheng H, Xiang L, Gao P, Zhou G. Interference of Frizzled 1 (FZD1) reverses multidrug resistance in breast cancer cells through the Wnt/β-catenin pathway. *Cancer Letters.* 2012;323(1):106-113.
129. Biehl JK, Russell B. Introduction to stem cell therapy. *Journal of Cardiovascular Nursing.* 2009;24(2):98-103; quiz 104-105.
130. Ayob AZ, Ramasamy TS. Cancer stem cells as key drivers of tumour progression. *Journal of Biomedical Science.* 2018;25(1):20-20.
131. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nature Medicine.* 1997;3(7):730-737.
132. Yang L, Shi P, Zhao G, Xu J, Peng W, Zhang J, Zhang G, Wang X, Dong Z, Chen F, Cui H. Targeting cancer stem cell pathways for cancer therapy. *Signal Transduct Target Ther.* 2020;5(1):8.
133. Qi C, Wang X, Shen Z, Chen S, Yu H, Williams N, Wang G. Anti-mitotic chemotherapeutics promote apoptosis through TL1A-activated death receptor 3 in cancer cells. *Cell Research.* 2018;28(5):544-555.
134. Gomis RR, Gawrzak S. Tumor cell dormancy. *Molecular Oncology.* 2017;11(1):62-78.
135. Nair A, Chauhan P, Saha B, Kubatzky KF. Conceptual Evolution of Cell Signaling. *International Journal of Molecular Sciences.* 2019;20(13).
136. Nüsslein-Volhard C, Wieschaus E. Mutations affecting segment number and polarity in Drosophila. *Nature.* 1980;287(5785):795-801.
137. Gupta S, Takebe N, Lorusso P. Targeting the Hedgehog pathway in cancer. *Therapeutic Advances in Medical Oncology.* 2010;2(4):237-250.
138. Tukachinsky H, Kuzmickas RP, Jao CY, Liu J, Salic A. Dispatched and scube mediate the efficient secretion of the cholesterol-modified hedgehog ligand. *Cell Reports.* 2012;2(2):308-320.
139. Veenstra VL, Dingjan I, Waasdorp C, Damhofer H, van der Wal AC, van Laarhoven HW, Medema JP, Bijlsma MF. Patched-2 functions to limit Patched-1 deficient skin cancer growth. *Cellular Oncology (Dordrecht).* 2018;41(4):427-437.
140. Yu FY, Hong YY, Qu JF, Chen F, Li TJ. The large intracellular loop of ptch1 mediates the non-canonical Hedgehog pathway through cyclin B1 in nevoid basal cell carcinoma syndrome. *International Journal of Molecular Medicine.* 2014;34(2):507-512.
141. McCabe JM, Leahy DJ. Smoothened goes molecular: new pieces in the hedgehog signaling puzzle. *Journal of Biological Chemistry.* 2015;290(6):3500-3507.
142. Rohatgi R, Milenkovic L, Scott MP. Patched1 regulates hedgehog signaling at the primary cilium. *Science.* 2007;317(5836):372-376.
143. Lin EH, Kao YR, Lin CA, Kuo TY, Yang SP, Hsu CF, Chou TY, Ho CC, Wu CW. Hedgehog pathway maintains cell survival under stress conditions, and drives drug

resistance in lung adenocarcinoma. *Oncotarget.* 2016;7(17):24179-24193.

144. Niewiadomski P, Niedziółka SM, Markiewicz Ł, Uśpieński T, Baran B, Chojnowska K. Gli Proteins: Regulation in Development and Cancer. *Cells.* 2019;8(2).
145. Kugler MC, Joyner AL, Loomis CA, Munger JS. Sonic hedgehog signaling in the lung. From development to disease. *American Journal of Respiratory Cell and Molecular Biology.* 2015;52(1):1-13.
146. Chen Y, Sasai N, Ma G, Yue T, Jia J, Briscoe J, Jiang J. Sonic Hedgehog dependent phosphorylation by CK1α and GRK2 is required for ciliary accumulation and activation of smoothened. *PLoS Biology.* 2011;9(6):e1001083.
147. Doheny D, Manore SG, Wong GL, Lo HW. Hedgehog Signaling and Truncated GLI1 in Cancer. *Cells.* 2020;9(9).
148. Armas-López L, Zúñiga J, Arrieta O, Ávila-Moreno F. The Hedgehog-GLI pathway in embryonic development and cancer: implications for pulmonary oncology therapy. *Oncotarget.* 2017;8(36):60684-60703.
149. Skoda AM, Simovic D, Karin V, Kardum V, Vranic S, Serman L. The role of the Hedgehog signaling pathway in cancer: A comprehensive review. *Bosnian Journal of Basic Medical Sciences.* 2018;18(1):8-20.
150. Hanna A, Shevde LA. Hedgehog signaling: modulation of cancer properies and tumor mircroenvironment. *Molecular Cancer.* 2016;15:24.
151. Yockey LJ, Iwasaki A. Interferons and Proinflammatory Cytokines in Pregnancy and Fetal Development. *Immunity.* 2018;49(3):397-412.
152. Watkins DN, Berman DM, Burkholder SG, Wang B, Beachy PA, Baylin SB. Hedgehog signalling within airway epithelial progenitors and in small-cell lung cancer. *Nature.* 2003;422(6929):313-317.
153. Chi S, Huang S, Li C, Zhang X, He N, Bhutani MS, Jones D, Castro CY, Logrono R, Haque A, Zwischenberger J, Tyring SK, Zhang H, Xie J. Activation of the hedgehog pathway in a subset of lung cancers. *Cancer Letters.* 2006;244(1):53-60.
154. Abe Y, Tanaka N. The Hedgehog Signaling Networks in Lung Cancer: The Mechanisms and Roles in Tumor Progression and Implications for Cancer Therapy. *BioMed Research International.* 2016;2016:7969286.
155. Singh S, Wang Z, Liang Fei D, Black KE, Goetz JA, Tokhunts R, Giambelli C, Rodriguez-Blanco J, Long J, Lee E, Briegel KJ, Bejarano PA, Dmitrovsky E, Capobianco AJ, Robbins DJ. Hedgehog-producing cancer cells respond to and require autocrine Hedgehog activity. *Cancer Research.* 2011;71(13):4454-4463.
156. Kobune M, Takimoto R, Murase K, Iyama S, Sato T, Kikuchi S, Kawano Y, Miyanishi K, Sato Y, Niitsu Y, Kato J. Drug resistance is dramatically restored by hedgehog inhibitors in CD34+ leukemic cells. *Cancer Science.* 2009;100(5):948-955.
157. Chen YJ, Kuo CD, Chen SH, Chen WJ, Huang WC, Chao KS, Liao HF. Small-molecule synthetic compound norcantharidin reverses multi-drug resistance by regulating Sonic hedgehog signaling in human breast cancer cells. *PloS One.* 2012;7(5):e37006.
158. Cui D, Xu Q, Wang K, Che X. Gli1 is a potential target for alleviating multidrug resistance of gliomas. *Journal of the Neurological Sciences.* 2010;288(1-2):156-166.
159. Das S, Harris LG, Metge BJ, Liu S, Riker AI, Samant RS, Shevde LA. The hedgehog pathway transcription factor GLI1 promotes malignant behavior of cancer cells by up-

regulating osteopontin. *Journal of Biological Chemistry.* 2009;284(34):22888-22897.

160. Kasper M, Schnidar H, Neill GW, Hanneder M, Klingler S, Blaas L, Schmid C, Hauser-Kronberger C, Regl G, Philpott MP, Aberger F. Selective modulation of Hedgehog/GLI target gene expression by epidermal growth factor signaling in human keratinocytes. *Molecular and Cellular Biology.* 2006;26(16):6283-6298.
161. Chen JK, Taipale J, Cooper MK, Beachy PA. Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened. *Genes and Development.* 2002;16(21):2743-2748.
162. Tremblay MR, Nevalainen M, Nair SJ, Porter JR, Castro AC, Behnke ML, Yu LC, Hagel M, White K, Faia K, Grenier L, Campbell MJ, Cushing J, Woodward CN, Hoyt J, Foley MA, Read MA, Sydor JR, Tong JK, Palombella VJ, McGovern K, Adams J. Semisynthetic cyclopamine analogues as potent and orally bioavailable hedgehog pathway antagonists. *Journal of Medicinal Chemistry.* 2008;51(21):6646-6649.
163. Erivdege: European Public Assessment Report - Summary for the public European Medicines Agency. 17 November 2016. https://www.ema.europa.eu/en/documents/overview/erivedge-epar-summary-public_en.pdf. Accessed 16 June 2021.
164. LoRusso PM, Rudin CM, Reddy JC, Tibes R, Weiss GJ, Borad MJ, Hann CL, Brahmer JR, Chang I, Darbonne WC, Graham RA, Zerivitz KL, Low JA, Von Hoff DD. Phase I Trial of Hedgehog Pathway Inhibitor Vismodegib (GDC-0449) in Patients with Refractory, Locally Advanced or Metastatic Solid Tumors. *Clinical Cancer Research.* 2011;17(8):2502-2511.
165. Sinx KAE, Roemen GMJM, van Zutven V, Janssen R, Speel E-JM, Steijlen PM, van Geel M, Mosterd K. Vismodegib-resistant basal cell carcinomas in basal cell nevus syndrome: Clinical approach and genetic analysis. *JAAD case reports.* 2018;4(5):408-411.
166. Agyeman A, Jha BK, Mazumdar T, Houghton JA. Mode and specificity of binding of the small molecule GANT61 to GLI determines inhibition of GLI-DNA binding. *Oncotarget.* 2014;5(12):4492-4503.
167. Riaz SK, Ke Y, Wang F, Kayani MA, Malik MFA. Influence of SHH/GLI1 axis on EMT mediated migration and invasion of breast cancer cells. *Scientific Reports.* 2019;9(1):6620.
168. Ghanbari A, Cheraghzadeh Z, Mahmoudi R, Zibara K, Hosseini E. GLI inhibitors overcome Erlotinib resistance in human pancreatic cancer cells by modulating E-cadherin. *Journal of Chemotherapy.* 2019;31(3):141-149.
169. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research.* 2001;29(9):e45.
170. Bareschino MA, Schettino C, Rossi A, Maione P, Sacco PC, Zeppa R, Gridelli C. Treatment of advanced non small cell lung cancer. *Journal of Thoracic Disease.* 2011;3(2):122-133.
171. Cohen MH, Cortazar P, Justice R, Pazdur R. Approval summary: pemetrexed maintenance therapy of advanced/metastatic nonsquamous, non-small cell lung cancer (NSCLC). *The oncologist.* 2010;15(12):1352-1358.
172. Zhang D, Ochi N, Takigawa N, Tanimoto Y, Chen Y, Ichihara E, Hotta K, Tabata M,

Tanimoto M, Kiura K. Establishment of pemetrexed-resistant non-small cell lung cancer cell lines. *Cancer Letters.* 2011;309(2):228-235.

173. Zhou J, Oliveira P, Li X, Chen Z, Bepler G. Modulation of the ribonucleotide reductase-antimetabolite drug interaction in cancer cell lines. *Journal of Nucleic Acids.* 2010;2010:597098.
174. Lu Y, Yu LQ, Zhu L, Zhao N, Zhou XJ, Lu X. Expression of HIF-1α and P-gp in non-small cell lung cancer and the relationship with HPV infection. *Oncology Letters.* 2016;12(2):1455-1459.
175. Robinson K, Tiriveedhi V. Perplexing Role of P-Glycoprotein in Tumor Microenvironment. *Frontiers in Oncology.* 2020;10:265.
176. Gregorcyk S, Kang Y, Brandt D, Kolm P, Singer G, Perry RR. p-Glycoprotein expression as a predictor of breast cancer recurrence. *Annals of Surgical Oncology.* 1996;3(1):8-14.
177. Zu Y, Yang Z, Tang S, Han Y, Ma J. Effects of P-glycoprotein and its inhibitors on apoptosis in K562 cells. *Molecules (Basel, Switzerland).* 2014;19(9):13061-13075.
178. Liao XZ, Gao Y, Sun LL, Liu JH, Chen HR, Yu L, Chen ZZ, Chen WH, Lin LZ. Rosmarinic acid reverses non-small cell lung cancer cisplatin resistance by activating the MAPK signaling pathway. *Phytotherapy Research.* 2020;34(5):1142-1153.
179. Choi CH. ABC transporters as multidrug resistance mechanisms and the development of chemosensitizers for their reversal. *Cancer Cell International.* 2005;5:30.
180. Queiroz KC, Ruela-de-Sousa RR, Fuhler GM, Aberson HL, Ferreira CV, Peppelenbosch MP, Spek CA. Hedgehog signaling maintains chemoresistance in myeloid leukemic cells. *Oncogene.* 2010;29(48):6314-6322.
181. Katayama R, Sakashita T, Yanagitani N, Ninomiya H, Horiike A, Friboulet L, Gainor JF, Motoi N, Dobashi A, Sakata S, Tambo Y, Kitazono S, Sato S, Koike S, John Iafrate A, Mino-Kenudson M, Ishikawa Y, Shaw AT, Engelman JA, Takeuchi K, Nishio M, Fujita N. P-glycoprotein Mediates Ceritinib Resistance in Anaplastic Lymphoma Kinase-rearranged Non-small Cell Lung Cancer. *EBioMedicine.* 2016;3:54-66.
182. Shen DY, Zhang W, Zeng X, Liu CQ. Inhibition of Wnt/β-catenin signaling downregulates P-glycoprotein and reverses multi-drug resistance of cholangiocarcinoma. *Cancer Science.* 2013;104(10):1303-1308.
183. Davies C, Tournier C. Exploring the function of the JNK (c-Jun N-terminal kinase) signalling pathway in physiological and pathological processes to design novel therapeutic strategies. *Biochemical Society Transactions.* 2012;40(1):85-89.
184. Mezzacappa C, Komiya Y, Habas R. Activation and function of small GTPases Rho, Rac, and Cdc42 during gastrulation. *Methods in Molecular Biology.* 2012;839:119-131.
185. Liu XY, Liu SP, Jiang J, Zhang X, Zhang T. Inhibition of the JNK signaling pathway increases sensitivity of hepatocellular carcinoma cells to cisplatin by down-regulating expression of P-glycoprotein. *European Review for Medical and Pharmacological Sciences.* 2016;20(6):1098-1108.
186. Papachristou DJ, Batistatou A, Sykiotis GP, Varakis I, Papavassiliou AG. Activation of the JNK-AP-1 signal transduction pathway is associated with pathogenesis and progression of human osteosarcomas. *Bone.* 2003;32(4):364-371.
187. Chen Q, Bian Y, Zeng S. Involvement of AP-1 and NF-κB in the up-regulation of P-gp

in vinblastine resistant Caco-2 cells. *Drug Metabolism and Pharmacokinetics.* 2014;29(2):223-226.

188. Wuelling M, Schneider S, Schröther VA, Waterkamp C, Hoffmann D, Vortkamp A. Wnt5a is a transcriptional target of Gli3 and Trps1 at the onset of chondrocyte hypertrophy. *Developmental Biology.* 2020;457(1):104-118.
189. Liu A, Chen S, Cai S, Dong L, Liu L, Yang Y, Guo F, Lu X, He H, Chen Q, Hu S, Qiu H. Wnt5a through noncanonical Wnt/JNK or Wnt/PKC signaling contributes to the differentiation of mesenchymal stem cells into type II alveolar epithelial cells in vitro. *PloS One.* 2014;9(3):e90229.
190. Giroux Leprieur E, Vieira T, Antoine M, Rozensztajn N, Rabbe N, Ruppert AM, Lavole A, Cadranel J, Wislez M. Sonic Hedgehog Pathway Activation Is Associated With Resistance to Platinum-Based Chemotherapy in Advanced Non-Small-Cell Lung Carcinoma. *Clinical Lung Cancer.* 2016;17(4):301-308.
191. Dong Z, Wang Y, Ding V, Yan X, Lv Y, Zhong M, Zhu F, Zhao P, He C, Ding F, Shi H. GLI1 activation is a key mechanism of erlotinib resistance in human non-small cell lung cancer. *Oncology Letters.* 2020;20(4):76.
192. Agyeman A, Mazumdar T, Houghton JA. Regulation of DNA damage following termination of Hedgehog (HH) survival signaling at the level of the GLI genes in human colon cancer. *Oncotarget.* 2012;3(8):854-868.
193. Cochrane CR, Szczepny A, Watkins DN, Cain JE. Hedgehog Signaling in the Maintenance of Cancer Stem Cells. *Cancers.* 2015;7(3):1554-1585.
194. Choi HS, Kim SL, Kim JH, Lee DS. The FDA-Approved Anti-Asthma Medicine Ciclesonide Inhibits Lung Cancer Stem Cells through Hedgehog Signaling-Mediated SOX2 Regulation. *International Journal of Molecular Sciences.* 2020;21(3).
195. Yang J, Antin P, Berx G, Blanpain C, Brabletz T, Bronner M, Campbell K, Cano A, Casanova J, Christofori G, Dedhar S, Derynck R, Ford HL, Fuxe J, García de Herreros A, Goodall GJ, Hadjantonakis AK, Huang RJY, Kalcheim C, Kalluri R, Kang Y, Khew-Goodall Y, Levine H, Liu J, Longmore GD, Mani SA, Massagué J, Mayor R, McClay D, Mostov KE, Newgreen DF, Nieto MA, Puisieux A, Runyan R, Savagner P, Stanger B, Stemmler MP, Takahashi Y, Takeichi M, Theveneau E, Thiery JP, Thompson EW, Weinberg RA, Williams ED, Xing J, Zhou BP, Sheng G. Guidelines and definitions for research on epithelial-mesenchymal transition. *Nature Reviews: Molecular Cell Biology.* 2020;21(6):341-352.
196. Ohta H, Aoyagi K, Fukaya M, Danjoh I, Ohta A, Isohata N, Saeki N, Taniguchi H, Sakamoto H, Shimoda T, Tani T, Yoshida T, Sasaki H. Cross talk between hedgehog and epithelial-mesenchymal transition pathways in gastric pit cells and in diffuse-type gastric cancers. *British Journal of Cancer.* 2009;100(2):389-398.
197. Ahmad A, Maitah MY, Ginnebaugh KR, Li Y, Bao B, Gadgeel SM, Sarkar FH. Inhibition of Hedgehog signaling sensitizes NSCLC cells to standard therapies through modulation of EMT-regulating miRNAs. *Journal of Hematology & Oncology.* 2013;6(1):77.

Acknowledgements

From the topic selection and data collection to the completion of the thesis, I have received enthusiastic help from Prof. Dr. med. Rudolf Maria, Huber, Dr. med. Amanda Tufman, Dr. med. Diego Kauffmann-Guerrero, Dr. rer. biol. hum. Tian Fei, Rosemarie Kiefl and other colleagues.

To Prof. Dr. med. Rudolf Maria Huber. First and foremost, thank you very much to offered me an opportunity to continue to learn and do the research about non-small cell lung cancer as a doctoral candidate. You always encouraged me when I encountered difficulties in the search. You also helped me solve problems in my research with your rich experimental experience and scientific research knowledge. Therefore, I did not only complete doctoral thesis, but also learned much more than before in the field of oncology, which would be beneficial for my future career.

To Dr. med. Amanda Tufman. Thanks very much for your advice to my research. Especially you were always very patient to point out my problems and put forward valuable suggestions in our seminars. I would not be able to make progress in my scientific research without your advice.

To Dr. med. Diego Kauffmann-Guerrero. Thank you for your assistance in data analysis and many helpful discussions for the research. You would take time to discuss experiments with me and make suggestions even after your busy clinical work. When my experiment encountered obstacles, you were always patient and put forward reasonable suggestions to help me solve these problems.

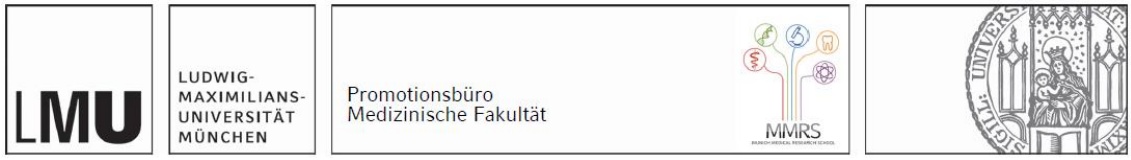
To Dr. rer. biol. hum. Tian Fei. Thank you for your valuable suggestions on the designs of my experiments and technical help for my experiments. I had been able to solve the problems in my experiments well with your assistance.

To Rosemarie Kiefl. Thank you for teaching me how to perform related experiments and for your assistance in ordering laboratory reagents, and also thank you for your advice to research. You always ordered agents for my experiments in time, which ensured the smooth progress of my experiment. I was also happy to work with you in the office.

To other colleagues who were working in the department of internal medicine group V, thoracic oncology center of LMU. Thanks for your help and collaboration.

Finally, I would like to t express my deepest gratitude towards my family and friends, thank you all for your support and understanding.

Affidavit



Affidavit

Liu Yichao
Surname, first name

Ziemssenstrasse 1
Street

80336, Munich, Germany.
Zip code, town, country

I hereby declare, that the submitted thesis entitled “Hedgehog pathway activation might mediate pemetrexed resistance in NSCLC cells” is my own work. I have only used the sources indicated and have not made unauthorised use of services of a third party. Where the work of others has been quoted or reproduced, the source is always given.

I further declare that the submitted thesis or parts thereof have not been presented as part of an examination degree to any other university.

Munich, 30.05.2022
place, date

Yichao Liu
Signature doctoral candidate

List of publications

Liu Y, Huber RM, Kiefl R, Tufman A, Kauffmann-Guerrero D. Hedgehog Pathway Activation Might Mediate Pemetrexed Resistance in NSCLC Cells. *Anticancer Research.* 2020;40(3):1451-1458.