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REVIEW ARTICLE

Non-small cell lung carcinoma (NSCLC): Implications on molecular pathology and advances in early diagnostics and therapeutics

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Abstract Continuous revision of the histologic and stage-wise classification of lung cancer by the World Health Organization (WHO) provides the foundation for therapeutic advances by promoting molecular targeted and immunotherapies and ensuring accurate diagnosis. Cancer epidemiologic data provide helpful information for cancer prevention, diagnosis, and management, supporting health-care interventions. Global cancer mortality projections from 2016 to 2060 show that cancer will overtake ischemic heart diseases (IHD) as the leading cause of death (18.9 million) immediately after 2030, surpassing non-small cell lung cancer (NSCLC), which accounts for 85 percent of lung cancers. The clinical stage at the diagnosis is the main prognostic factor in NSCLC therapies. Advanced early diagnostic methods are essential as the initial stages of cancer show reduced mortality compared to the advanced stages. Sophisticated approaches to proper histological classification and NSCLC management have improved clinical efficiency. Although immune checkpoint inhibitors (ICIs) and targeted molecular therapies have refined the therapeutic management of late-stage NSCLC, the specificity and sensitivity of cancer biomarkers should be improved by focusing on prospective studies, followed by their use as therapeutic tools. The liquid biopsy candidates such as circulating tumor cells (CTCs), circulating cell-free tumor DNA (cfDNA), tumor educated platelets (TEP), and extracellular vesicles (EVs) possess cancer-derived biomolecules and aid in tracing: driver mutations leading to cancer, acquired resistance caused by various generations of therapeutic agents, refractory disease, prognosis, and surveillance.

Introduction

Cancer will overtake ischemic heart disease (IHD) as the leading cause of death within the next four decades, with a 2.08-fold increase, according to World Health Organization (WHO) projections of mortality and causes of death from 2016 to 2060.[1] Among cancer mortality rates, lung cancer mortality is estimated to be the leading one.[2] Non-small cell lung cancer (NSCLC) is the most common epithelial lung cancer over small cell lung cancer (SCLC), accounting for about 85% of all lung cancer types.[3] Based on the Surveillance, Epidemiology, and End Result Program (SEER) database of the American Cancer Society (ACS), distant stage of lung cancer possesses a higher percentage of diagnosis (56%) with the most minor relative five-year survival (6.3%) than local and regional stages.[4] It signifies the necessity of advanced early diagnostic methods and new treatment strategies such as targeted and combination therapy.[5] The WHO 2021 classification of thoracic tumors is based on biomarker testing and immunohistochemistry rather than morphological features. In the recent update, numerous advanced pathologic diagnosis methods have resulted in more accurate pathologic and genetic classification of lung cancers, enabling improved therapeutic options.[6] As well as improving prognosis and aiding tumor care, biomarkers may also be helpful in better characterizing the risk of ambiguous nodules. The clinical implications of biomarkers are still under investigation, and their potential in future decision-making algorithms in screening and early lung cancer care is still up for debate.[7]

In this review, we discuss the current histologic and stage classification of NSCLC, focusing on its molecular pathology, diagnosis, and therapeutic values, and present lung cancer epidemiologic trends around the globe, focusing on the Asian continent using data retrieved from the official websites of Global Cancer Observatory (GLOBOCAN), WHO, ACS, National Cancer Registry Program (NCRP) and Cancer Samiksha, to substantiate trends in NSCLC therapeutic management. We also discuss the need for prospective and retrospective studies on the advances in early diagnostic methods, focusing on emerging candidates in liquid biopsies such as circulating tumor cells (CTCs), circulating cell-free DNA (cfDNA), tumor educated platelets (TEP), and extracellular vesicles (EVs) substantiating its necessity in NSCLC management, followed by recent progress in targeted therapy in the perspective of NSCLC therapeutics. We also discuss recent advances in biomarkers for NSCLC screening, prognosis, and prediction.

WHO classification of NSCLC histotypes, focusing on molecular pathology and diagnostic values

WHO 2021 update (fifth edition) on tumor classification follows molecular testing and immunohistochemistry approaches, ensuring precision in genetic and pathologic classification of lung tumors aiding in improved therapeutic strategies and patient management. The current classification majorly focuses on: genetic testing; small diagnostic samples; spread through air space (STAS) and its prognostic significance; reorganizing lymphoepithelial carcinoma over squamous cell carcinoma (SQCC); updating on neuroendocrine (NE) neoplasm (NEN); identification of SMARCA4-deficient undifferentiated thoracic tumor; recognition of ciliated muconodular papillary tumor (CMPT)/bronchiolar adenoma (BA); and formation of desirable and essential criteria for individual tumors. The classification also highlights the utilization of histologic patterns in invasive non-mucinous adenocarcinoma (ADC) for assisting formal grading system and redefining the recommendations by Tumor, Node, Metastasis (TNM)-VIII staging, specifically T-

factor size in part lepidic nonmucinous lung-ADC.[6,8] Even though molecular changes form part of the diagnostic criteria, many molecular alterations may not yet impact tumor subtype classification, which may affect clinical efficacy.[9] The approval of targeted therapy is significantly related to the relative decrease in NSCLC mortality rate comparing the incidence rate between 2013 and 2016. The use of inhibitors of anaplastic lymphoma kinase (ALK) and epidermal growth factor receptor (EGFR) that specifically target genomic abnormalities in NSCLC patients has improved clinical outcomes.[10]

The accuracy of histologic classification and staging of advanced lung cancers is unsatisfactory because 70% of lung cancers are unresectable at the time of diagnosis, limiting diagnostic samples to small numbers.[11] Managing small cytology and biopsy samples highlights the importance of obtaining an accurate diagnosis, including NSCLC-specific histologic type (10–13) and molecular testing (9). The international guidelines for NSCLC screening (ASCO-2020) suggest the driver mutations/fusions in the *ALK, EGFR,* ROS proto-oncogene 1 (*ROS1),* proto-oncogene B-RAF *(BRAF),* neurotrophic tyrosine receptor kinase *(NTRK1-3),* Kristen rat sarcoma *(KRAS),* tyrosine-protein kinase met (*MET),* and rearranged during transfection *(RET)* genes, as well as programmed death-ligand 1 (PD-L1) expression.[12–14] As measured by driver mutations and PD-L1 expression, tumor cellularity would vary and be influenced by prior treatments.[15]

The current update has classified ADC as invasive mucinous-ADC (IMA), invasive non-mucinous-ADC, fetal carcinoma, colloid carcinoma, and enteric type carcinoma. The most prevalent subtype of lung cancer is invasive non-mucinous-ADC, consisting of malignant epithelial tumors with immunohistochemical or morphological evidence of glandular differentiation. The staging and grading system has been updated with the measurement of invasion, specifically for nonmucinous-part lepidic-ADC. To determine the tumor grade and define the major histologic pattern (subtype), the percentage of each pattern is recorded in 5%–10% increments. The grade1, grade2, and grade3 possess histologic features of lepidic predominant with no or $<$20% high-grade pattern, acinar or papillary predominant with no or $<$20% high-grade pattern, and any tumor with $\geq$20% high-grade pattern, respectively. Any histologic subtype other than lepidic (papillary, micropapillary, acinar, solid, or less frequently colloid, fetal type, IMA, or enteric) or foci of tumor cells infiltrating myofibroblastic stroma are included in the assessment of tumor invasion.[6] The prognostic value of invasive nonmucinous-ADC can be relatively justified, but a formal grading system is lacking. Compared to acinar or other solid-predominant tumors, lepidic predominant tumors have a better prognosis.[16–20] The International Association for the Study of Lung Cancer (IASLC) pathology committee recommended a three-tiered grading system for invasive non-mucinous-ADCs to provide more significant prognostic information. This system is based on the combination of histologic and high-grade patterns such as solid, complex glandular, micropapillary, and cribriform, if they contribute $\leq$20% of the tumor.[21]

Tumor cells spread through air spaces (STAS) in ADC possess three morphologic patterns: discohesive single cells, micropapillary structures, and solid nests.[22] STAS in resected ADC has a poor clinical outcome and is worse in patients undergoing limited resection than those undergoing lobectomy.[23,24] IMA accounts for 3%–10% of invasive ADC, with nonmucinous types accounting for the remainder. IMA with its columnar or goblet cell morphology can be characterized by TTF1, CDX2 focal, CK7^{+}, CK20 focal, and HNF4α^{+} based on immunophenotypes. Substitution mutation in *KRAS*, loss of function mutation in *NKX2.1*, *ERBB2* alterations (insertion and amplification), *NRG1* fusion, *TP53*, and *EGFR* mutations can cause IMA. Furthermore, other rare alterations such as fusion of *ROS1, BRAF, NTRK1, ALK,* fibroblast growth factor receptor *(FGFR2/3), RET,* and nitrate regulatory gene2 (*NRG2)* and mutations of *BRAF* and *ERBB3 (HER3)* can be added to the above list.[25,26] IMA is distinguished by multilobar, multifocal, and bilateral presentation rather than intrapulmonary spread.[27] A colloid, enteric, and fetal-type variants are ADC's rare subtypes.[28] Colloidal and enteric types have similar origins and are usually distinguished by clinical terms, as both variants can be positive for intestinal markers (Villin, CDX2, and CK20) and negative for pneumocyte markers (Napsin A, and TTF1).[29,30] Low-grade or well-differentiated fetal-ADCs often show alterations in WNT-β-catenin pathways, and $>$50% high-grade morphology is needed for its diagnosis.[31,32]

Several therapeutic agents targeting the oncogenic driver mutations involved in lung ADCs are available now, while some are in clinical trials. Drivers such as echinoderm microtubule-associated protein-like4 (*EML4)-ALK* translocation, *EGFR* exon 19 deletions, exon 19-point mutations, in-frame deletions, point mutations, translocations, and splice variants in *ALK*,[33,34] *BRAF*,[35,36] *EGFR*,[37,38] *NTRK1-3*,[39] *RET*,[40,41] *MET*,[42,43] and *ROS1*,[40,44] possess several clinically approved therapeutic agents. At the same time, *KRAS*[45] and *ERBB2*[25] have emerging targetability. *EGFR* targeting has been expanded to patients with earlier stages, even though other molecular targets are primarily used for advanced stages.[46] Other mutations are seen alone or in combination with other alterations in lung ADCs, such as *TP53, STK11 (LKB1),* and kelch-like ECH-associated protein1 *(KEAP1)*, are not yet directly targetable but may be linked to tumor progression and resistance to immune checkpoint inhibitors (ICIs).[47] *KRAS* mutations are common in solid-predominant ADC and IMA, while the latter is characterized by specific translocations involving *NRG1*.[48,49] A mutation in catenin beta 1 (*CTNNB1)* is linked to well-differentiated fetal ADC. *EGFR* mutations are more common in ADC with a non-mucinous lepidic and papillary pattern, as well as in those that are TTF-1 positive. *ALK, ROS1*, and *RET*[50,51] translocations are found with signet ring patterns and cribriform/solid patterns.[52,53] Moreover, large cell neuroendocrine carcinoma (LCNEC) and SCLC may comprise NSCLC components primarily as ADC or SQCC, as SCLC adopts ADC characteristics as a resistance mechanism against EFGR-tyrosine kinase inhibitors (EGFR-TKIs).[54] It has been reported that NE differentiation occurs in 10%–20% of ADC and SQCC that lack NE morphology.[55] SQCC subtypes of NSCLC, such as basaloid carcinoma (reorganized to keratinizing and non-keratinizing subtypes), and lymphoepithelial carcinoma (positive staining for P63, P40, CK5/6, lymphoplasmacytic infiltrate, syncytial growth pattern, and association with Epstein-Barr virus (EBV)) are updated in WHO 2021 classification. The presence of EBER1 by in situ hybridization (ISH) is a desirable

criterion, as EBV positive and negative lymphoepithelioma occurs in European and Asian patients, respectively. Furthermore, *EGFR* mutations are more prevalent in East Asian populations, whereas KRAS mutations are more predominant in the American/European population,[56] emphasizing the epidemiologic and etiologic distribution of various cancers. Even though advances in SQCC molecular characteristics lag behind ADC, PD-L1 therapy alone or combined with chemotherapy is effective in SQCC.[12] NUT carcinoma is one of the SQCCs with P40 positivity and squamoid differentiation, but NUTM1 gene abnormalities in NUT progression should be considered in patients with malignant lung tumors.[57]

SMARCA4-deficient undifferentiated (SMARCA4-UT) is a new entity in the fifth edition, which possesses a rhabdoid phenotype and deficiency of SMARCA4. Because of its close resemblance to smoking-related NSCLC, it was added to the category of "other epithelial tumors of the lung," even though WHO classified it as a separate entity from conventional NSCLC. Up to 44% of SMARCA4-UT cases possess mutations in *STK11, KEAP1,* and *KRAS1*, which are common in smoking-associated NSCLC.[58] SMARCA4 deficiency affects about 5% of typical NSCLC patients. However, SMARCA4-UT is separate from SMARCA4-deficient NSCLC, with significant morphological, immunohistochemical, clinical, and prognostic distinctions.[58–60]

Stage classification of lung cancer

The stage-wise classification of lung cancer provides nomenclature for the anatomic extent of the disease and enables an understanding of disease severity, which aids in implementing disease strategies.[61] The Union for International Cancer Control (UICC) and the American Joint Committee on Cancer (AJCC) review and refine the stage system regularly, with assistance from the International Association for the Study of Lung Cancer (IASLC) and the Staging and Prognostic Factors Committee (SPFC).[62] The eighth edition compiled a database of 94,708 patients diagnosed between 1999 and 2010. It comprises three components: extent of the primary tumor (T), involvement of lymph nodes (N), and distant metastasis (M). Specific combinations of TNM and subtypes are grouped into stage groups (Table1). The T, N, and M components were analyzed respectively based on 10230 c-stage (clinical; before initiation of any treatment) and 22257 p-stage (pathologic; after resection)[63]; 38910 c-stage and 31426 p-stage; 1059 nonsurgically managed NSCLC M1 tumors.[64] Although stage classification and disease prognosis are related, there is a significant need for an accurate prognostic prediction model tailored to a specific patient. Figure 1 and Table 1 provide information on TNM staging in each lung cancer stage (I-IV).

Global epidemiologic trends of lung cancer

Cancer is one of the most prevalent diseases, with 14 million new cases diagnosed yearly and over 8.8 million deaths worldwide.[65] According to WHO updates, lung cancer is expected to overtake IHD as the leading cause of death by 2060.[66] Updating epidemiologic data is critical because it provides essential information on the disease's current status from geographical, statistical, and biological perspectives, allowing for the development of appropriate health care interventions.[65] We present a concise overview of lung cancer epidemiology for the entire world, the Asian continent, and South Asia, based on data obtained from the official websites of the WHO, GLOBOCAN, ACS, NCRP, and Cancer Samiksha. In addition, we compare GLOBOCAN-2018 and 2020 data to review the change in epidemiology trends from 2016 to 2020.

Global incidence, mortality, and prevalence rate of different cancers

Lung cancer is the first leading cancer in terms of mortality, second in terms of incidence, and fourth in terms of prevalence, according to the most recent GLOBOCAN update, which corresponds to the year 2020. In summary, 2.2 million new cases were reported in the age group 0–84, with 1.4 million male and 0.7 million female, while GLOBOCAN-2018 reported 2 million new cases, 1.3 of million male and 0.7 million female, with a 5.12 percent increase in total incidence. GLOBOCAN-2020 *vs*. 2018 data reveals the epidemiologic trends from 2016 to 2020 (Fig. 2). The most and least common cancer incidences in males and females and the percentage increase are discussed here. Lung (1.43 million, 4.6%), prostate (1.41 million, 9.7%), and colorectal cancer (1.06 million, 3.7%) are the most common cancers in males, while mesothelioma (21 k, −0.4%), Kaposi's sarcoma (23 k, −3.4%) and salivary glands (29 k, 1.4%) are the least common. Females have a higher incidence of breast cancer (2.2 million, 7.6%), colorectal (0.86 million, 4.8%), lung cancer (0.77 million, 5.8%), and the lowest incidence of mesothelioma (0.9 k, 5.6%), Kaposi's sarcoma (10 k, −24%), and hypopharynx (14 k, 6.3%).

Lung cancer, which accounts for 1.79 million deaths, has increased by 1.95 percent, while breast cancer, which has the highest incidence but accounts for 0.68 million deaths, has increased by 8.5 percent. Nonetheless, the difference in percent increase of each cancer corresponds to the "WHO global projection of mortality 2016–2060". The high mortality rate in males is primarily caused by lung cancer (1.18 million) and in females (0.60 million) after breast cancer (0.68 million). The five-year cancer prevalence (2016-20) shows 44 million new cases, with breast cancer (7.79 million), colorectal cancer (5.25 million), prostate (4.95 million), and lung cancer (2.6 million) dominating the list.[67]

WHO global projection, incidence, and mortality of lung cancer focusing Asia

According to the WHO estimated data on global projections of mortality and causes of death, 2016–2060, cancer will overtake IHD (16 million/year) as the leading cause of death immediately after 2030, with a 2.08-fold increase within four decades (Fig. 3A).[68] The cancer mortality rate will rise from 0.12% to 0.18% by 2060, while IHD mortality will increase from 0.13% to 0.16% during the same period, highlighting the importance of cancer therapeutics.

Table 1 TNM stage classification considering stage peculiarities and suggestive treatments.

STAGE OF TUMOR	STAGING TUMOR	NODE	METASTASIS	PECULIARITIES	SUGGESTED TREATMENTS
OCCULT CARCINOMA	TX	N0	M0	Primary tumor cannot be assessed or proven by the presence of malignant cells in sputum or bronchial washings but not visualized with imaging or bronchoscopy. No regional lymph node metastasis No distant metastasis	
STAGE 0	Tis	N0	M0	Carcinoma in situ	
STAGE IA1	T1a	N0	M0	Tumor $\leq$3 cm in greatest dimension, surrounded by lung or visceral pleura, without bronchoscopic evidence of invasion more proximal than the lobar bronchus Tumor $\leq$1 cm in greatest dimension	Surgical resection, Neoadjuvant PD1-PDL1 inhibition
STAGE IA2	T1b	N0	M0	Tumor $\leq$1 cm in greatest dimension	Surgical resection, Neoadjuvant PD1-PDL1 inhibition
STAGE IA3	T1c	N0	M0	Tumor $>$2 cm but $\leq$3 cm in greatest dimension	Surgical resection, Neoadjuvant PD1-PDL1 inhibition
STAGE IB	T2a	N0	M0	tumor with any of the following features: involvement of the main bronchus regardless of the distance from the carina; invasion of the visceral pleura; associated with partial or complete lung atelectasis or pneumonitis Tumor $>$3 cm but $\leq$4 cm in greatest dimension	Surgical resection, Neoadjuvant PD1-PDL1 inhibition
STAGE IIA	T2b	N0	M0	Tumor $>$4 cm but $\leq$5 cm in greatest dimension	Surgical resection, Neoadjuvant PD1-PDL1 inhibition
STAGE IIB	T1a-c	N1	M0	Metastasis in ipsilateral peribronchial and/	Surgical resection,
	T2a	N1	M0	or ipsilateral hilar lymph nodes and	Neoadjuvant PD1-
	T2b	N1	M0	intrapulmonary nodes, including	PDL1 inhibition
	T3	N0	M0	involvement by direct extension Tumor $>$5 cm but $\leq$7 cm in greatest dimension or one that directly invades any of the following structures: parietal pleura, chest wall (including superior sulcus tumors), phrenic nerve, parietal pericardium; or separate tumor nodule or nodules in the same lobe	
STAGE IIIA	T1a-c	N2	M0	Tumor measuring $>$7 cm in greatest	Chemotherapy
	T2a-b	N2	M0	dimension that invades any of the following	followed by
	T3	N1	M0	structures: mediastinum, diaphragm, heart,	radiation or
	T4	N0	M0	great vessels, trachea, recurrent laryngeal	surgery,
	T4	N1	M0	nerve, esophagus, vertebral body, carina; or separate tumor nodule or nodules in a different lobe of the same lung	Neoadjuvant PD1-PDL1 inhibition
STAGE IIIB	T1a-c	N3	M0	Metastasis in contralateral mediastinal,	Combination of
	T2a-b	N3	M0	contralateral hilar, ipsilateral or	chemotherapy and
	T3	N2	M0	contralateral scalene, or supraclavicular	radiation,
	T4	N2	M0	lymph nodes. Metastasis in ipsilateral mediastinal and/or subcarinal lymph nodes	Neoadjuvant PD1-PDL1 inhibition
STAGE IIIC	T3	N3	M0	Metastasis in contralateral mediastinal,	Combination of

Table 1 (*continued*)

STAGE OF TUMOR	STAGING TUMOR	NODE	METASTASIS	PECULIARITIES	SUGGESTED TREATMENTS
	T4	N3	M0	contralateral hilar, ipsilateral or contralateral scalene, or supraclavicular lymph nodes. Metastasis in ipsilateral mediastinal and/or subcarinal lymph nodes	chemotherapy and radiation, Neoadjuvant PD1-PDL1 inhibition
STAGE IVA	Any T Any T	Any N Any N	M1a M1b	Separate tumor nodule or nodules in the contralateral lung; malignant pleural effusion or pleural thickening or nodules or masses; malignant pericardial effusion or pericardial thickening or nodules or masses. Single distant (extrathoracic) metastasis in a single organ	Chemotherapy and palliative care with combination of either PD1-PDL1 or PD1-CTLA4
STAGE IVB	Any T	Any N	M1c	Multiple distant (extrathoracic) metastases in a single organ or multiple organs	Chemotherapy and palliative care with a combination of either PD1-PDL1 or PD1-CTLA4

T-the size of the tumor.
N- whether cancer spread to lymph node.
M-whether cancer spreads to distant organs.
[316–318].

Lung cancer will be the leading cause of death by 2060, with an estimated 2.4 million deaths yearly. Figure 3B depicts the epidemiology of the top five cancer causes from 2016 to 2060.[5] Validating the prediction, GLOBOCAN-2020 shows that liver cancer has surpassed stomach cancer in terms of mortality, with a 1.98-fold increase expected by 2060. Lung, colorectal, liver, stomach, and breast cancer are ranked in GLOBOCAN-2020 based on decreasing mortality rates, which corresponds to the WHO 2060 projection. These cancers have increased by 2.4, 1.79, 1.98, 1.99, and 2.12 times, respectively.

Lung cancer is one of the most commonly diagnosed cancers worldwide, with a poor prognosis.[69,70] Lung and breast cancer continue to be the most common cancers in men and women, respectively.[71] The incidence of lung cancer in males (age-standardized rate (ASR) per 100000) shows the highest values (ASR>60) in countries like Turkey (74.8), Serbia (68), and Hungary (66.6) while the lowest (ASR<2) in Burkina Faso (1.2), Niger (1.6). In females, the highest incidence is seen in Hungary (38.1) and the lowest in Niger (0.14).

The mortality rate of males is highest in countries like Turkey (67.5), Serbia (59.6), and Hungary (58.6), while the lowest in Burkina Faso (1.1) and Niger (1.6), but in females, the highest is in Hungary (30.6), Denmark (25.2) and the lowest in Niger (0.14). The ASR of lung cancer incidence and mortality, with a comparison of males and females from all countries, using data from GLOBOCAN-2018 and 2020, is provided in Table S1.[67]

Asia has 4.7 billion people, accounting for roughly 60% of the global population.[72] The incidence and mortality of lung cancer in both sexes in Asia (data from GLOBOCAN-2018 and 2020) are depicted in Figure S1. The estimated incidence (ASR) of lung cancer among males is highest in Turkey (74.8), Armenia (56.8), Korea (48.2), and lowest in Saudi Arabia (6.2), while among females, it is highest in Korea (28.7), Brunei Darussalam (28), and least in Pakistan (2.7) and Oman (2.9). Lung cancer mortality among males is highest in Turkey (67.5), Armenia (52.4), Korea (41.9), and lowest in Saudi Arabia (5.5) and Yemen (6.5), while among females, it is highest in Korea (22.6) and least in Pakistan (2.4)[67]. India is the world's second-most populous country after China. The most common types of cancer in India are breast cancer and cervix uteri in females, lip and oral cavity cancers in males, followed by lung cancer[73](See the subsequent reading for the epidemiologic trends in India and South India).

Influence of diagnostic stage over survival rate of lung cancer

The survival rate denotes the percentage of people with the same type and stage of cancer with a 5-year life expectancy after being diagnosed. In collaboration with the National Institutes of Health (NIH) and the National Cancer Institute (NCI), the SEER database program provides cancer statistics for the US population.[74] The ACS relies on information from the NCI's SEER database to provide survival statistics for various types of cancer. Lung cancer is classified into three types based on its pathology: localized (no evidence of cancer spreading outside the lung), regional (cancer has spread outside the lung to nearby structures or lymph nodes), and distant (spread to distant parts of the

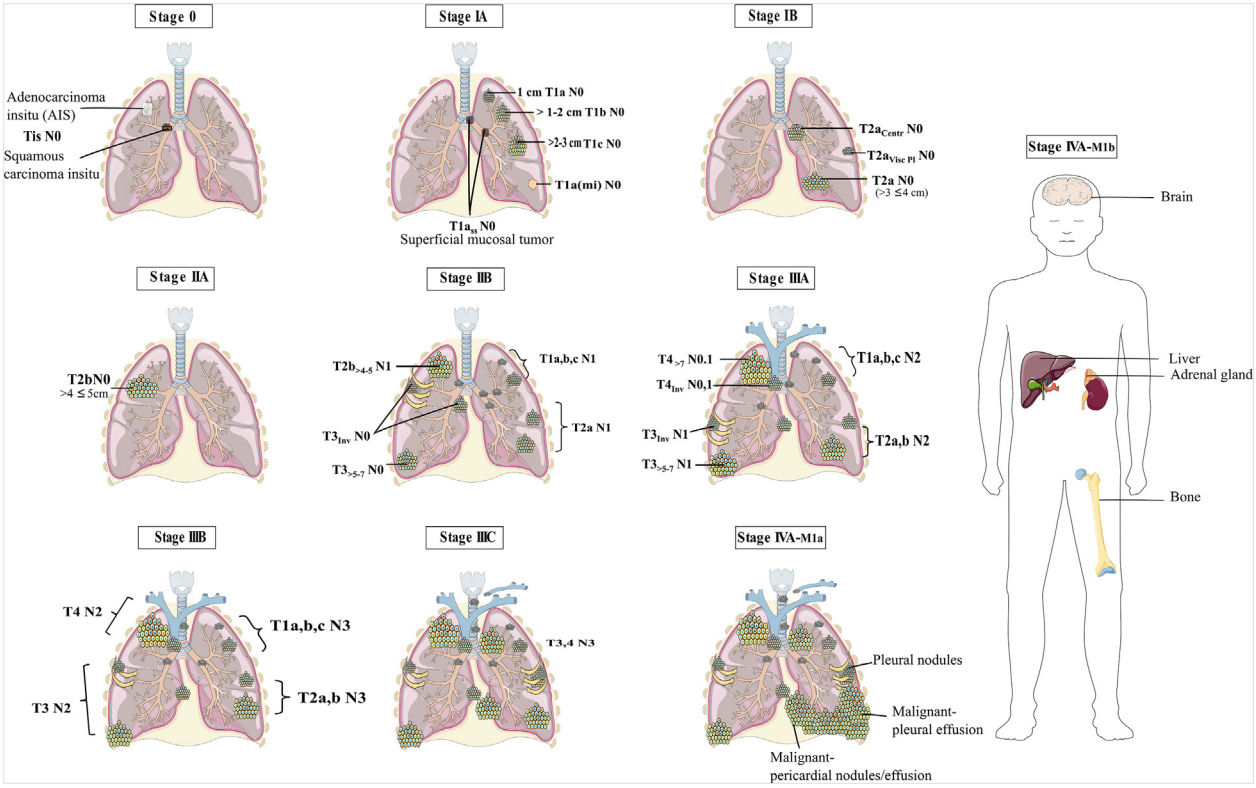

Figure 1 Lung cancer stage classification. Stage 0: carcinoma in situ. Stage IA: tumor ≤3 cm and no spread to lymph nodes. Tia (mi)- minimally invasive carcinoma and Tia (ss)- superficial spreading tumor in central airways is seen. Stage IB: tumor size between 3 and 4 cm, no spread to lymph nodes. Stage IIA: tumor size between 4 and 5 cm. Stage IIB: (Note: stage IIB is unilobar, the illustration shows bilobar as to reduce picture clustering) tumor size range between 5 and 7 cm, invade parietal pleura, chest wall, phrenic nerve, parietal pericardium, separate tumor nodule, or nodules in the same lobe, metastasis in ipsilateral pulmonary or hilar lobes. Stage IIIA: tumor measuring greater than 7 cm dimension. Invades mediastinum, heart, great vessels, trachea, recurrent laryngeal nerve, esophagus, vertebral body, carina; or separate tumor nodules or nodules in a different lobe of the same lung. Stage IIIB: tumor size greater than 7 cm and spread to contralateral mediastinal, contralateral hilar, ipsilateral, or supraclavicular lymph nodes. Stage IIIC: invade chest wall, pericardium, phrenic nerve, or separate tumor nodule in same lobe, diaphragm, heart, great vessels, recurrent laryngeal nerve, carina, trachea, esophagus, and spine. Stage IV: malignant pleural or pericardial effusion, separate tumor nodules in contralateral lobe, single or multiple extra thoracic metastases. The details of T, N, and M grades in different stages of cancers are given in Table 1. Abbreviation: Tia (mi), minimally invasive carcinoma; Tia (ss), superficial spreading tumor.

body, such as the brain, bones, liver, or the other lung).[70] According to the SEER database, the distant stage has a higher percentage of diagnosis but the lowest relative five-year survival rate, while the localized stage has the lowest percentage of diagnosis but the highest relative five-year survival rate.

The SEER database was used to obtain NSCLC survival statistics. It shows 2.3 lakh new lung and bronchus cancer cases in 2021, accounting for 12.4% of all new cancer cases, and 1.3 lakh deaths, accounting for 21.7% of all cancer deaths. The median age at diagnosis is 71, while the median age at death is 72, indicating a high prevalence in post-reproductive age. Furthermore, according to SEER 2011—2017 data, the five-year relative survival rate is 21.7%. The percentage of localized, regional, and distant lung cancer during diagnosis is 18%, 22%, and 56%, respectively, while the relative five-year survival is 59.8% for localized, 32.9% for regional, and 6.3% for distant lung cancers. The bar diagram representing relative five-year survival and percent of lung cancer during diagnosis is shown in Figure 4. It implies that most lung cancers are diagnosed in advanced stages, resulting in a meager five-year survival rate, highlighting the need for advancements in biomarker-associated early detection methods.

Risk factors of lung cancer

Smoking, history of TB, asthma, chronic obstructive pulmonary disease (COPD), and occupational exposure to asbestos and radon are the significant risk factors for lung cancer we know yet. Sex, age, smoking status, and geographic locations have been used to investigate the frequency of these changes in lung ADC. Light or never smokers are more likely to have *EGFR* mutations and *ROS1*, *ALK*, and *RET* translocations, whereas heavy smokers are more likely to have *KRAS* mutations, particularly transversion-type mutations.[75] Other mutations, such as *MET* and *BRAF*, are found in both smokers and non-smokers; *NRAS*,[76] *MAP2K1*,[77] and *TP53*[78] are more common in

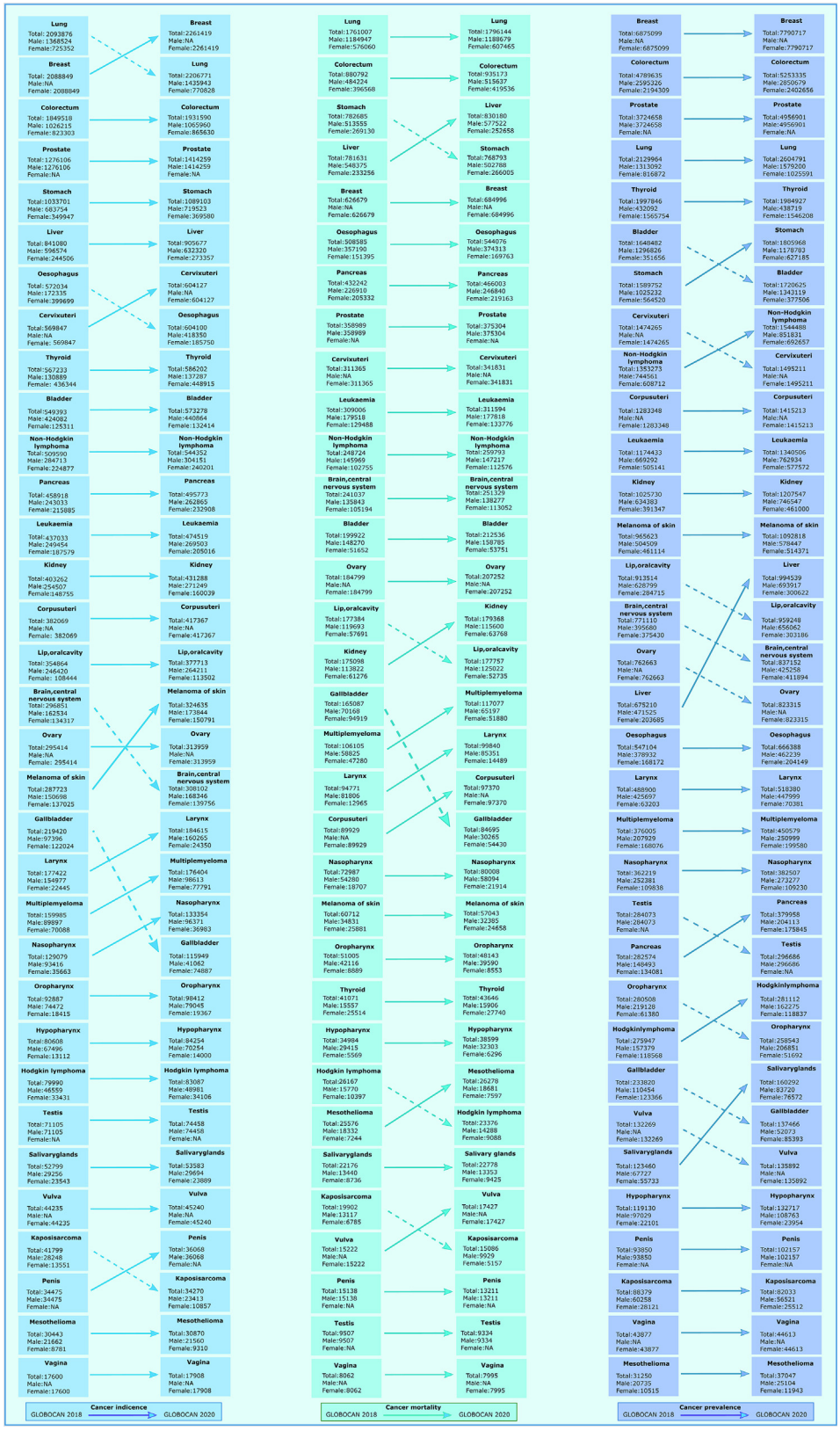

Figure 2 Comparison of epidemiologic trends of cancer incidence, mortality, and prevalence using GLOBOCAN-2018 and 2020 data.

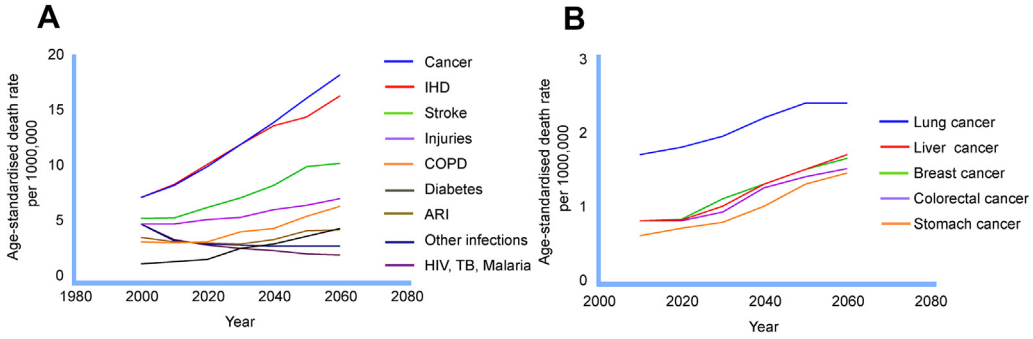

Figure 3 Epidemiologic trend of "WHO projection on cancer mortality. **(A)** The estimated epidemiologic trend of "WHO projection on cancer mortality" shows the frequency of major nine causes of deaths from the years 2016—2060. **(B)** The estimated epidemiologic trend of "WHO projection on cancer mortality" shows the frequency of deaths caused by leading five different types of cancers during 2016—2060. (x-axis; years: y-axis; numbers in million).

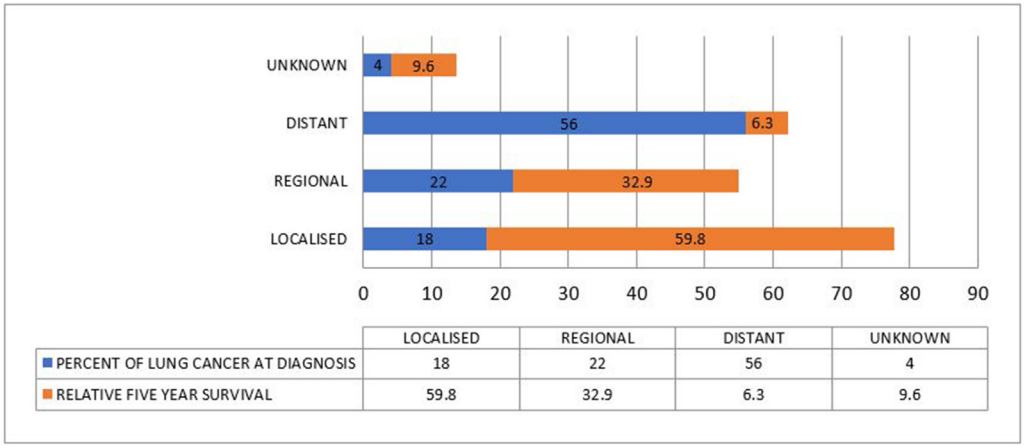

Figure 4 Percent of lung cancer cases at diagnosis and five-year relative survival by stage. The numbers are based on people diagnosed with NSCLC between 2011 and 2017.

smokers; *EGFR* mutations are more common in women and young patients, but *ROS1*, *RET*, and *ALK* mutations are more common in young patients irrespective of gender.[56]

The significant risk factors of lung cancer include tobacco smoking (association magnitude of 20-fold increased risk),[79—81] use of cigars, pipes, and water pipes (1.9—4.6 fold),[82,83] history of chlamydia pneumonia (1.2—2.4 fold),[84] history of tuberculosis (48—76 fold),[85—87] chronic bronchitis (2—3 fold),[86,87] and HIV infection (2 fold).[88] Second-hand smoke (25%—28% increased risk),[89,90] radon (14%—29%),[91—93] asbestos (12%—24%),[94,95] history of asthma (28%—44%),[96] and history of pneumonia (30%—57%)[97—100] add to the list of risk factors. Exposure to other cancer-causing agents in the workplace like radioactive ores such as uranium, inhaled chemicals such as arsenic, beryllium, cadmium, silica, vinyl chloride, nickel compounds, chromium compounds, coal products, mustard gas, and chloromethyl ethers, and taking certain dietary supplements like beta carotene supplements, can also cause lung cancer.[101,102]

Cancer susceptibility is also proportional to the individual's genetic history.[103] Point mutations (missense, nonsense, silent, loss of function, gain of function, and dominant-negative mutations), chromosomal alterations (deletion or insertion, inversion, translocation, aneuploidy, and gene amplification), epigenetic variations (histone acetylation and DNA methylation),[104] and chromothripsis[105] may lead to cancer. Genetic instability due to nucleotide alteration, gross chromosome rearrangements, whole chromosome instability due to spindle checkpoint dysfunction, centromere over-duplication, chromatid cohesion defect, and merotelic attachment can induce tumor heterogeneity.[104,106] Somatic mutation and alterations in NSCLC are seen in: *EGFR*, 10%—35%; *KRAS*, 15%—25%; *FGFR1*, 20%; phosphatase and tensin homolog (*PTEN4*),8%; discoidin domain-containing receptor tyrosine kinase (*2DDR2*), 4%; *ALK*, 3%—7%; *HER2*, 2%—4%); *MET*, 2%—4%; *BRAF*, 1%—3%; phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*), 1%—3%; protein kinase b (*AKT*), 1%; *MEK*1, 1%; neuroblastoma-ras (*NRAS*), 1%; *RET*, 1%; and *ROS*, 1%.[70] These genes' circulating transcriptional and translational products can be used in the early diagnosis of NSCLC, promising a hike in the overall survival rate (OS).[107,108]

Emerging candidates in liquid biopsy as NSCLC early diagnostic tools

The clinical-stage at diagnosis is the most important prognostic factor in NSCLC therapeutics, highlighting the

importance of early detection because late stages are associated with higher mortality and shorter survival. Although immune checkpoint inhibitors and targeted molecular therapies have improved the therapeutic management of late-stage NSCLC, the specificity and sensitivity of cancer biomarkers should be improved by focusing on prospective studies.[109] Different tumor-derived components, such as circulating cell-free tumor DNA (ctDNA), cell-free tumor RNA (ctRNA), EVs, tumor educated platelets (TEP), circulating tumor cells (CTC), and microRNA (miRNA), are isolated from the body fluids such as blood, saliva, cerebrospinal fluid (CSF), urine, bronchoalveolar fluid, and pleural effusion, aid in capturing the molecular heterogeneity of tumor microenvironment.[109–112] Liquid biopsy candidates such as CTCs, cfDNA, circulating miRNA (ci-miRNA), EV, and TEP possesses a competitive advantage.[113] The fact that cfDNA is the only FDA-approved tool for detecting NSCLC highlights the importance of improving reliable cut-offs, standardizing procedures, and focusing on prospective studies.[114,115] They reflect parental molecular profiles, which aid in tracing driver mutations that lead to cancer-acquired resistance caused by different generations of therapeutic agents, refractory disease, prognosis, and surveillance.

Circulating cell-free tumor DNA (cfDNA)

Cancer patients have higher levels of cfDNA and ctDNAs in their blood than healthy individuals.[116] Their release is thought to be primarily mediated by apoptotic and necrotic cells, with macrophages involved in the phagocytosis of apoptotic bodies and necrotic tumor cells playing an essential role in cfDNA release.[109,110,117–123] The presence of circulating cfDNA is higher than ctDNA (125), which can be detected in healthy individuals at low concentrations of 5–10 ng/mL (180 bp) and is thought to be associated with nucleosomes.[124] ctDNA is likely to represent the entire genomic landscape of a tumor. However, the cfDNA-based screening is not very specific, as TP53 mutations, a biomarker, was detected in 11% of 225 non-cancerous controls, posing significant challenges for developing screening tools.[125] Other epigenetic modifications like gene methylation can also be used as a marker of cfDNA. They can be analyzed using methylation-specific PCRs like methylation-specific tumor suppressor genes, including O^6-methyl guanine DNA methyltransferase (*MGMT*), *P16*, Ras association domain family 1 isoform A (*RASSF1A*), death-associated protein kinase (*DAPK*) and retinoic acid receptor beta (*RARB*).[126] The presence of a member of the homeobox family gene, short stature homeobox 2 (*SHOX2*), that encodes DNA binding transcription factors has emerged as a specific and sensitive biomarker for lung cancer.[127,128]

Meta-analysis and systematic reviews done by Cargin et al on 1723 patients enrolled in 16 studies demonstrate a correlation between progression-free survival (PFS), OS, and baseline cfDNA levels ($P < 0.001$), indicating an inverse relationship.[129–132] The ability to detect genomic alterations in ctDNA assists in molecular profiling of NSCLC, especially in *EGFR* gene mutations, and tracing acquired resistance caused by EGFR-TKIs, T790M led to U.S. FDA approval of ctDNA in 2016 as the first liquid biopsy test.[133] The phase-II biomarker study using digital PCR (dPCR) in evaluable 57 EGFR mutated NSCLC patients pretreated with afatinib showed that 62.5% of patients were positive for plasma EGFR. Significantly longer PFS and OS were noted in those patients with negative plasma EGFR, respectively, as 13.6 months *vs*. 5.1 months ($P < 0.0001$).[134] Acquired resistance caused by TKIs can effectively be traced by cfDNA, such as *ALK* rearrangements and point mutations.[135–138] Bordi et al conducted a mutational analysis in 20 ALK-positive crizotinib pretreated NSCLC patients and noted novel *ALK* point mutations in five patients, suggesting cfDNA as a tool for monitoring acquired *ALK* mutations.[135] The use of cfDNA may be exploited to trace specific mutations and molecular targets for personalized therapies, albeit the concordance between the mutational status of tumor DNA and cfDNA is not satisfactory. However, cfDNA from sites close to metastases such as pleural effusion, ascites, and cerebrospinal fluid results in more effective methods for detecting relevant mutations.[139]

Circulating tumor cells (CTC)

The detachment of cells from the tumor mass into circulation leads to CTC formation, promoting cancer progression and metastasis. Due to the harsh environment in endothelial cells, one out of thousands of CTCs can only metastasize, making them rare in peripheral blood, accounting for one to ten CTCs per 1 mL of the whole blood.[140] Platelets protect CTC and carry them to sites of inflammation.[141] The potential role of CTCs as a biomarker is demonstrated in various studies.[110,142–145] Laboratory procedures for CTC isolation are complex due to their fragility, but it allows comprehensive analysis of DNA, RNA, protein, lipid, and miRNA.[146] Indeed, isolation of EpCAM (epithelial cell adhesion molecule) positive CTCs in NSCLC is lower than in other epithelial tumors, and the change in the molecular composition of CTCs during epithelial-mesenchymal transition is a drawback of using CTC markers.[147–150] The detection and quantification of CTCs can be done using the CellSearch assay, which is the only FDA-approved system for monitoring CTCs in NSCLC progression.[151] The PDL1+CTCs flare in 127 samples from NSCLC patients using label-independent microfluidic Parsortix TM system, based on the size and rigidity of CTCs, demonstrated acquired resistance towards immunotherapy.[152] EGFR and HER3 are upregulated in CTCs and metastatic tissue compared to primary tumors, demonstrating the clinical significance of CTCs in NSCLC.[153] The prognostic significance of CTCs in 101 baselines and pemetrexed NSCLC patients showed significantly better PFS (2.4 months) and OS (4.3 months) in those having less than five CTCs compared to those with greater or equal to five CTCs.[154] The TRACERx trial on the prognostic value of pulmonary venous CTCs in 100 early-stage NSCLC patients demonstrates that patients with detectable CTCs are an indicator of disease recurrence, even though further studies are needed to confirm the sensitivity and specificity of CTCs as a diagnostic tool.[155] The acquired resistance caused by TKIs can also be traced using CTCs, albeit the current focus on *EGFR*-associated therapies is in the hike. The comparison in the expression of EGFR T790M mutation associated with CTCs and cfDNA in 27 metastatic NSCLC

patients showed 11 and 4 T790M^{+}, respectively, indicating CTCs as a valid option to monitor emerging mutations.[156]

Tumor educated platelets (TEP)

TEP is formed when platelets associated with circulating cancer cells sequester tumor-associated biomolecules or by directly ingesting circulating mRNA, miRNA, or proteins.[157] Specific splice variants of pre-mRNAs in circulating platelets induced by external signals produce distinct profiles used in cancer diagnostics.[158–162] Methods for isolation and analysis of spliced TEP mRNA have been developed for cancer detection.[157] TEP mRNA cannot distinguish between non-metastasized and metastasized tumors, indicating their inability to show different stages of cancer, even though oncogenic drivers such as HER2/KRAS/EGFR and PIK3CA mutations can.[163] Thus, more robust data derived from extensive case-control studies and homogenous populations are required to ensure the diagnostic value of TEP.[111] RNA sequencing data of TEPs from 402 NSCLC patients and 231 healthy controls revealed 48 genes that are deregulated in NSCLC, implying that TEP can be considered an early detection tool for NSCLC.[164] Large retrospective and prospective studies and clinical trials are required before TEPs can be used as a diagnostic tool for NSCLC diagnosis and prognosis.

MicroRNAs

Small EVs, large EVs, miRNA complexes, and TEPs are known to carry short non-coding miRNAs commonly deregulated in cancers.[110] A single miRNA targets hundreds of mRNAs based on its homology to the target sequence's 3' UTR, thereby regulating various signaling pathways that lead to tumor growth, dissemination, metastasis, and even acquired resistance.[165] miRNA can be used as a non-invasive biomarker due to its stability, tissue specificity, and uniqueness in various cancer profiles, including lung cancer.[166,167] Ci-miRNAs (Table 2) are released from the tumor cells either actively or passively. The active release occurs following the necrosis or apoptosis of tumor clones, whereas the passive release is intended for intercellular communication, in which miRNAs are found associated with exosomes, microvesicles, Argonaute2 (Ago2) proteins, or high-density lipoprotein (HDL).[168–170]

Various studies have demonstrated the significant difference in expression profiles of miRNA in healthy and NSCLC patients,[171–174] as Solexa technology identified 63 new miRNAs in NSCLC patients.[167] Another study evaluated miRNA in NSCLC serum samples using fluorescence quantum dots liquid bead array revealed that five miRNAs were significantly downregulated (miR-16-5p, miR-17b-5p, miR-20a-5b, miR-19-3p, and miR-92-3p) while miR-15b-5p was upregulated. Moreover, miR-15b-5p, miR-16-5p, and miR-20a-5b have been chosen clinically as the independent diagnostic marker for NSCLC.[175] The RNA sequencing data by Hu et al demonstrated 30 long survivors with OS 49.5 months and 30 short survivors with OS 9.5 months based on the expression difference of 11 miRNAs. The qPCR data further validate the direct relation of four out of 11 miRNAs with OS of early-stage NSCLC patients.[176] The wide range of parameters used in different studies and differences in sample size and methodology necessitates additional validations. An in-depth functional analysis of miRNAs and their target genes should be performed to use miRNAs as potential biomarkers. Table 2 shows the regulation, target sites, and roles of significant ci-miRNAs linked to NSCLC.

Extracellular vesicles

EVs are heterogeneous groups of membrane-bounded particles of varying sizes that are actively released by various cell types, including cancer cells.[177] EVs such as microvesicles, microparticles, ectosomes, endosomes, apoptotic bodies, and exosomes have been subtyped based on their size, cellular origin, and biogenesis mechanism. A definite nomenclature and classification of EVs have not yet been established. EVs are partially characterized based on their cellular components and size ranging from 30 nm to over 2000 nm. Although it lacks the asymmetric distribution of lipid bilayers, the lipid particles of EVs are comparable to those of cell membranes, indicating similarity to parent cells.[178] EVs are typically isolated from bodily fluids such as blood, saliva, urine, broncho-alveolar lavage (BAL) fluid, and ascites, but no standardized protocol for increased purity and yield has been developed[179] (Fig. 5). They can transport proteins, lipids, nucleic acids such as DNA, RNA, long non-coding RNAs (lncRNAs), and miRNAs to recipient cells, promoting intercellular communication, disease pathogenesis including inflammation, and immune regulation.[180] Because they resemble their parent cells, NSCLC-derived EVs can be used to detect the complexity of cancer. They can also be used as a biomarker because they contain conserved molecules such as tetraspanins, glycoproteins, unique cytoplasmic constituents such as miRNA and RNA, and specific lipid bilayer compositions (Table 3).

Microvesicles are intermediate-sized EVs known as microparticles, ectosomes, or oncosomes if tumor-derived, and are formed by direct budding of plasma membrane without participating in the endolysosomal pathway for multivesicular body (MVB) formation.[181] Apoptotic bodies, which are made up of organelles, cytosolic components, and nuclear fragments, form due to necrosis and apoptosis in a growing tumor mass. They can transfer biological components to their recipient cells horizontally, thereby promoting metastasis.[182] As defined by Johnstone et al, exosomes are pathologically and physiologically significant nanosized particles formed by all cell types via the endosomal pathway, with a density of 1.13—1.19 g/ml and a diameter of 40—100 nm.[183] Exosome vesicles contain cargos like proteins, lipids, nucleic acids, mRNA, miRNA, lncRNA, transfer RNA (tRNA), viral RNA,[180,184–186] small fragments of single-stranded DNA, and large fragments of double-stranded DNA[187] protected by a lipid bilayer derived from parent cells. CD63, CD81, CD9, TSG101, ALG2-interacting protein-X (ALIX), and heat shock protein (HSP70) are the conserved proteins in exosomes of all cell types, thus regarded as the exosomal biomarkers.[188] 1010 kinds of lipids, over 9690 types of proteins, more than 3300 types of mRNA, 1400 types of miRNAs, 18 types of ribosomal RNA (rRNA), 60 types of tRNA, 110 types of small nucleolar RNA (snoRNA), 27 types of small nuclear RNA (snRNA), 6 types of

Table 2 ci-miRNAs' regulation, target, significant actions, and biomarker potential in NSCLC.

miRNA	Expression	Target	Function in NSCLC	Use as a biomarker	References
miR-422a	Up-regulated	*E2F2, TGFBR2, CISH, PFKFB2, PTCD1*	Lymphatic metastasis, control apoptosis	Yes	319
miR-22	Up-regulated	*MTHFR, Rb, E2F, WNT1, CDX2*	Regulate cell cycle, proliferation and differentiation	Yes	320,321
miR-24	Up-regulated	*ZNF367, NAIF1*	Regulate invasion, migration and proliferation	Yes	320,322
miR-1246	Up-regulated	*MT1G*	Tumor initiation and metastasis	Yes	323
miR-1290	Up-regulated	*MT1G*	Tumor initiation and metastasis	Yes	323
miR-574-5p	Up-regulated	*PTPRU*	Migration and invasion, enhance tyrosine phosphorylation of β-catenin	Yes	324
miR-125b	Up-regulated	*TP53, TP53INP1*	Reduce excessive apoptosis and regulate homeostasis	Yes	325,326
miR-200b	Up-regulated	*ZEB1, ZEB2*	Regulate EMT by targeting E-cadherin	Yes	325
miR-34b	Up-regulated	*TP53*	Reduce excessive apoptosis	Yes	325
miR-203	Up-regulated	*SMAD3, RGS17*	Repress TGFβ induced EMT; inhibit cell proliferation, invasion, and migration	Yes	327
miR-205	Up-regulated	*ZEB1, ZEB2*	Regulate EMT by targeting E-cadherin	Yes	325
miR-429	Up-regulated	*DLC1*	Promote proliferation	Yes	325,328
miR-448	Up-regulated	*CXCL12, KLF5, SIRT1*	Progression, migration of NSCLC	Yes	329,330
miR-4478	Up-regulated	*E2F1*	miR-4478 and E2F1 feedback loop promote NSCLC proliferation and migration	Yes	329,331
miR-182	Up-regulated	*EGR1/ZNF268*	Tumor cell growth and migration	Yes	332
miR-183	Up-regulated	*EGR1/ZNF268*	Tumor cell growth and migration	Yes	332
miR-210	Up-regulated	*HIF*	Regulate hypoxia	Yes	332
miR-20a-5p	Up-regulated	*KLF9*	Accelerate proliferation and invasion of NSCLC	Yes	175,333
miR-324-3p	Up-regulated	*ID4, CREBBP*	Regulate TGF-β signaling	Yes	334
miR-106a-5p	Up-regulated	*PTEN, ABCA1*	Regulate MAPK and mTOR signaling, angiogenesis, cell proliferation, and metastasis, enhance chemoresistance to cisplatin	Yes	335
miR-20a-5p	Up-regulated	*TβRII*	Enhance cell proliferation and differentiation by downregulating MARK1, regulating MAPK and mTOR signaling, promoting growth, and inhibiting apoptosis	Yes	335
miR-93-5p	Up-regulated	*FUS1, DAB2, ZNRF3, LATS2*	Regulate MAPK and mTOR signaling, (LATS2); enhanced angiogenesis, metastasis, (ZNRF3); activation of Wnt signaling	Yes	335
miR-31	Up-regulated	*ABCB9*	Inhibit cisplatin induced apoptosis	Yes	336,337
miR-944	Up-regulated	*EPHA7, SOCS*	Promote tumor growth, proliferation, and squamous differentiation	Yes	338,339
miR-3662	Up-regulated	*CLDN, TIMP3*	Regulate NSCLC progression	Yes	339
miR-210-3p	Up-regulated	*SIN3A*	Regulate proliferation and apoptosis of NSCLC cells	Yes	340,341
miR-146-b	Up-regulated	*IRAK1*	Regulate EGFR, MAPK, AKT, ERBB, mTOR, Hippo, and T cell receptor signaling pathways and promote EGFR-TKI resistance.	Yes	342,343
miR-205	Up-regulated	*PTEN*	Tumor growth, metastasis, and chemoresistance	Yes	201
miR-30b	Up-regulated	*RAB18*	Cell proliferation	Yes	201
miR-141	Up-regulated	*KLF6*	Regulate expression of PHLPP1 and PHLPP2; enhance secretion of VEGFA by downregulating KLF6	Yes	344

(*continued on next page*)

Table 2 (*continued*)

miRNA	Expression	Target	Function in NSCLC	Use as a biomarker	References
miR-21	Up-regulated	*PTEN, SESN1, CAB39L*	Repress PTEN and promote growth, invasion, mTOR reduction, and AMPK activation.	Yes	345—347
miR-21-5p	Up-regulated	*SMAD7*	Enhance NSCLC proliferation, invasion and migration	Yes	348,349
miR-223-3p	Up-regulated	*E2F8*	Regulate NSCLC growth and metastasis	Yes	348,350
miR-9-5p	Up-regulated	*TGFBR2*	Promote cell growth and metastasis in NSCLC	Yes	348,351
miR-486	Up-regulated	*PTEN*	Assist ADC and SQCC progression	Yes	352,353
miR-19b-3p	Up-regulated	*MYPT1*	Trigger EMT, invasion, migration, and repress apoptosis, a biomarker for monitoring EGFR-TKI treatment	Yes	354,355
miR-221-3p	Up-regulated	*P27*	Promote growth of NSCLC through controlling cell cycle	Yes	354,356
miR-409-3p	Up-regulated	*SOD1, SETDB1*	SOD1 mediated cell proliferation, invasion, and migration	Yes	354,357
miR-425-5p	Up-regulated	*PTEN, PI3K, Akt*	Enhance cell proliferation and survival through up-regulated PTEN, PI3K/AKT pathways	Yes	354,358
miR-584-5p	Up-regulated	*MMP14, WWP1, ROCK1, KLRG1*	Decrease MMP14 promoter activity and regulate migration and invasion	Yes	354,359
let-7c	Down-regulated	*MYB, ABCC2, BCL-XL*	Regulate proliferation, modulate PI3/AKT, MEK/ERK pathways	Yes	360
miR-152	Down-regulated	*DNMT1*	Cell proliferation, colony formation, invasion, migration	Yes	360
miR-126	Down-regulated	*VEGFA*	Regulate tumor angiogenesis, mediate Crk expression	Yes	332,361
let-7a	Down-regulated	*RAS, MYB, CYCLIND1, HMGA2, EGFR*	Enhance cytotoxicity of target genes	Yes	361,362
miR-506	Down-regulated	*GATA6, YAP1*	Growth and metastasis	Yes	329
miR-15b-5p	Down-regulated	*MYCN*	Cell invasion and migration	Yes	175,363
miR-1285	Down-regulated	*CREBBP, DVL2, MSH6, HSP90AA1, SLC2A1, STAT5B, APPL1*	Regulate proliferation and metastasis of NSCLC via down-regulating CDH1 and SMAD4	Yes	334,364
miR-145	Down-regulated	*C-MYC*	Inhibit cancer cell growth in EGFR mutant lung ADC Inhibit angiogenesis, invasion, and cell growth	Yes	365,366
miR-223	Down-regulated	*EPB41L3, IGF1R*	Affect cell cycle by regulating E2F1, regulate migration and invasion, proliferation and tumor growth, regulate AKT/mTOR pathway/P70S6K signaling pathway	Yes	365,367
miR-339-5p	Down-regulated	*BCL6*	Regulate EMT	Yes	346
miR-28	Down-regulated	*PTEN*	Cell proliferation and metastasis	Yes	368,369
miR-362	Down-regulated	*PIK3C2B*	Regulate PI3K pathway, migration, and proliferation	Yes	368,370
miR-660-5p	Down-regulated	*MDM2*	Regulate apoptosis, cell proliferation	Yes	368,371

Table 2 (*continued*)

miRNA	Expression	Target	Function in NSCLC	Use as a biomarker	References
miR-15a-5p	Down-regulated	*ACSS2*	Regulate metastasis and lipid metabolism by suppressing histone H4 acetylation	Yes	372,373
miR-320a	Down-regulated	*VDAC1*, *ELF4*, *IGF1R*, *STAT3*, *SND1*	Induce cell proliferation and invasion by activating VDAC1, STAT3, and PI3K/Akt signaling	Yes	360,374–377
miR-25-3p	Down-regulated	*LATS2/YAP*	Promote proliferation, invasion, and migration by targeting LATS2/YAP signaling pathway	Yes	372,378
miR-192-5p	Down-regulated	*RB1*, *XIAP*, *TRIM44*	Regulate progression of NSCLC	Yes	372,379
miR-148a-3p	Down-regulated	*SOS2*, *ROCK1*, *STAT3*, *WNT1*	Lymph node metastasis, pleural effusion, reduced cell proliferation, and EMT	Yes	372
miR-92a-3p	Down-regulated	Neurofibromin2	Regulate migration, proliferation, and resistance to apoptosis	Yes	372
let-7d	Down-regulated	*KRAS*, *MYC*, *IGF1R*, *TGFBR1*, *IGF2BP1*, and *HMGA2*	Regulate cell cycle, DNA synthesis and replication	Yes	372,380
let-7e-5p	Down-regulated	*KRAS*, *MYC*, *IGF1R*, *TGFBR1*, *IGF2BP1*, *HMGA2*, chemokine receptor7	Inhibit proliferation and metastasis	Yes	372,380,381
miR-34a	Down-regulated	*MTHFR*, *TNF*α, *IL6*	Exert anti-inflammatory effects	Yes	320,382

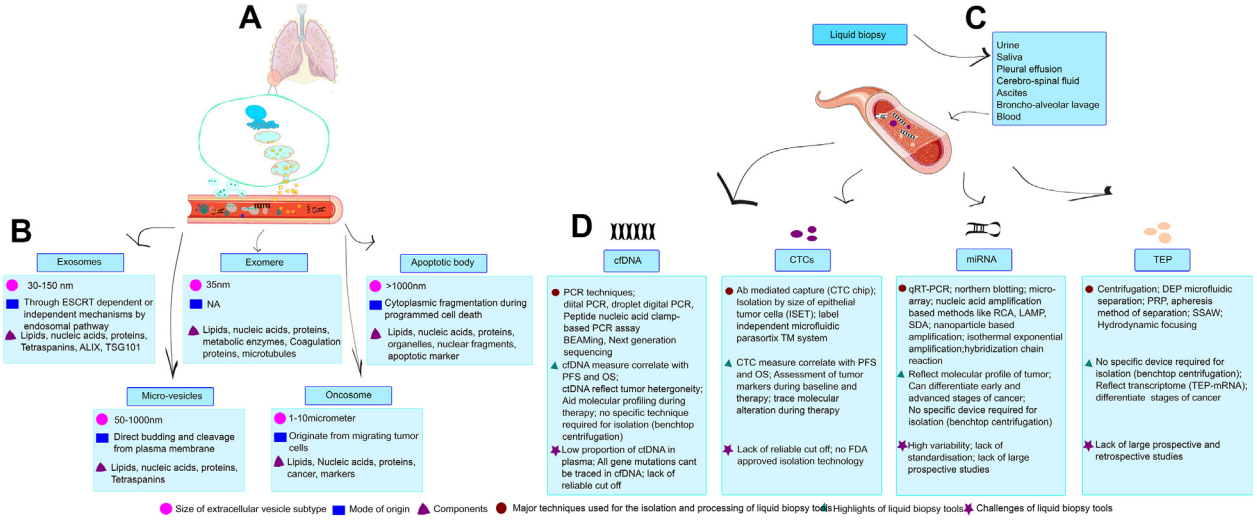

Figure 5 Representation of Extracellular vesicle subtypes and liquid biopsy candidates. **(A)** EV biogenesis through MVB formation and direct plasma budding. **(B)** Figure showing extracellular vesicle types, mode of origin, and biological constituents. Exosome, exomere, multi vesicle, oncosomes, and apoptotic bodies belong to extracellular vesicle types. They act by both paracrine and endocrine mode to exert biological roles in target places. **(C)** Various sources for the isolation and processing of liquid biopsy candidates. **(D)** Major techniques used for isolation and processing of liquid biopsy tools, highlights, and challenges. Abbreviation: ALIX, ALG-2 interacting protein x; ESCRT, endosomal sorting complexes required for transport; EV, extracellular vesicle; HSP, heat shock protein; MHC, major histocompatibility complex; TSG101, tumor susceptibility gene.

Table 3 EV subtypes with their size, biogenesis, and contents.

Types of EV	Size(nm)	Biogenesis	Content	Reference
Exosomes	30–150	Through ESCRT dependent or independent mechanisms by endosomal pathway.	Lipids Nucleic acids Proteins Tetraspanins ALIX TSG101	383–387
Microvesicles/Microparticles/ Ectosomes	50–1000	Direct budding and cleavage from the plasma membrane	Lipids Nucleic acids Proteins Tetraspanins	181,383,388
Exomeres	~35	NA	Lipids Nucleic acids Proteins Metabolic enzymes Coagulation proteins microtubules	389
Oncosomes	1–10 μm	Originate from migrating tumor cells	Lipids Nucleic acids Proteins Cancer marker	390
Apoptotic bodies	>1000	Cytoplasmic fragmentation during programmed cell death	Lipids Nucleic acids Proteins Organelle Nuclear fragment Apoptotic marker	182,391

lncRNA, 3 types of long intergenic noncoding RNA (lincRNA) and 5 types of non-coding RNA (ncRNA) are present in exosomes in different cell lines of different species under different conditions which helps for intercellular communications.[180,189] Currently, exocarta (exosome content database) contains about 41860 proteins, 3408 mRNA, 2838 miRNA, and 1116 lipids in human exosomes.[190] Various exosome proteins include membrane transport and fusion proteins, GTPases, heat shock proteins, signal transduction proteins, cytoskeleton proteins, MVB biogenesis proteins, and metabolic enzymes.[191,192] The lipid constituents of exosomes like sphingolipids, cholesterol, phospholipids, glycerophospholipids, phosphatidylserine, and diglycerides help for signaling and exosome internalization.[193]

NSCLC exosomes contain several tumor-associated proteins like tyrosine kinase receptor-b (TRKB), EGFR, KRAS, extracellular matrix-metalloproteinase inducer (EMMPRIN), HSP, claudins, and RAB family proteins. Several proteins, including EGFR, TRKB, and HSP, are either over- or under-expressed in exosomes in various cancers, including NSCLC, promoting malignancy in the target site via paracrine or endocrine mechanisms. This lends credence to the use of exosome proteins as cancer biomarkers. Exosomes containing HSP72 activate the signal transducer and activator of the transcription3 (STAT3) signaling pathway, suppressing T cell activation by mimicking the immunosuppressive effect of myeloid-derived suppressor cells (MDSC).[194,195] As a result of EGFR expression on exosomes, CD8 cells are suppressed by the generation of tolerogenic dendritic cells (DC) and Treg cells. Urinary exosomes containing leucine-rich alpha 2 glycoproteins (LRG1) positively correlate with the primary tumor, implying that they could be used as a biomarker for NSCLC.[196]

Tumor-derived EVs can promote immune-suppressive stroma by promoting apoptosis of NK cells and T cells through PD1/PD-L1 and FAS/FASL pathways. Nonetheless, they alter immune surveillance cells through functional polarization, activation, and inhibition of immune cells.[197] The polarization of immune cells mainly involves resident macrophages. TAM with M2 phenotype is polarized from M1 phenotypes.[198–200] Tumor-derived exosomes activate the MAPK pathway in monocyte by delivering functional TKIs, leading to inhibition of apoptosis-related caspases.[201] PD-L1 and CD47 and the FAS/FASL alteration promote T cell apoptosis and protect CTCs.[202] Functional inhibition of immune cells involves normal anti-tumor responses mediated by NK cells, DCs, and T-lymphocytes.[203] Activating checkpoint pathways such as PD-L1/PD1 inhibits $CD8^+$ T cells.[204] EVs harbor tumor cells and transfer them to many cancer cells, DCs, and macrophages, inducing an immune-suppressive environment.[197] The level of exosomal PD-L1 in NSCLC patients correlates with tumor size, stage, number of positive lymph nodes, and metastasis.[205] EV PD-L1 dynamics predict durable response to ICIs and survival in NSCLC patients.[206] Tumor-derived EVs harbor immunoinhibitory factors like FASL and TNF, inducing immune cell apoptosis.[207,208]

Advances in the targeted therapy of NSCLC

In recent years, new drugs that block the activities of cancer cell signaling pathways have opened up the possibility of new treatments for precision medicine.[209] EGFR, rat

Table 4 FDA-approved drugs used in targetable therapies against major oncogenes in lung ADC and their notable resistance patterns.

Oncogene	Drugs used in targeted therapies	Estimated frequency in Lung ADC (%)	Study	Notable resistance patterns	Reference
EGFR	Erlotinib, Afatinib, Gefitinib, Osimertinib, Dacomitinib, Osimertinib	15	FLAURA	T790M mutation, rare transformation to small cell lung cancer	392—395
ALK	Crizotinib, Ceritinib, Alectinib, Brigatinib, Lorlatinib, Dabrafenib, Trametinib	5	ALEX, ALTA-1L, ASCEND-4	Mutations conferring to crizotinib (eg; L1196M and G1269A)	392,393,396—399
ROS1	Lorlatinib, Crizotinib, Ceritinib, Brigatinib	2	ALKA, STARTRK-1/2, PROFILE1001	Secondary ROS1 mutations, conferring resistance to crizotinib (e.g., G2032R, D2033N, and S1986F) [47]	44,393,400—402
BRAF	Dabrafenib, Trametinib, Vemurafenib	2	NCT01336634	Resistance is common with monotherapy, but the mechanism is poorly understood.	392,393,403
RET	Vandetanib, Sorafenib, Sunitinib, Cabozantinib	2	LIBRETTO-001, ARROW	Not well understood	392,393,404,405
KRAS	Trametinib, Selumetinib, Sotorasib In combination with other therapeutic agents	25—33	NCT03704688	Resistance commonly develops causing a decreased response to TKIs	392,393,403
HER 2	Herceptin, trastuzumab	2	NCT02289833	Cells are less sensitive to the trastuzumab in the second-line therapy (eg;MCF7)	393,403,406
MET	Capmatinib	3	GEOMETRY	Acquired resistance to capmatinib	43,393,407
PD1-PDL1	Pembrolizumab, nivolumab	33	KEYNOTE-024, IMpower, KEYNOTE-042, CheckMate 227	Ineffective in a significant percentage of patients	393,408—413
CTLA4	Ipilimumab		NCT02221739	Causes advanced resistant SQCC	403,414

Abbreviation: ALK anaplastic lymphoma kinase; BRAF b-raf proto-oncogene; CTLA4 cytotoxic T lymphocyte-associated antigen-4; EGFR epidermal growth factor receptor; HER2 human epidermal growth factor receptor 2; KRAS k-ras proto-oncogene; MET/HGFR hepatocyte growth factor receptor; PDL/R programmed death-ligand/receptor; RET rearranged during transfection; ROS c-ros oncogene.

sarcoma-mitogen activated protein kinase (RAS-MAPK), just another kinase-signal transducer and activator of transcription (JAK-STAT), NTRK/ROS1, and phosphoinositide 3 kinase/protein kinase B/mechanistic target of rapamycin (P13K/AKT/mTOR) have all been identified as targetable pathways in lung ADC.[210—213] Some of them have now been replaced as first-line treatment with chemotherapy, such as EGFR inhibitors (erlotinib and gefitinib), PI3K/AKT/mTOR inhibitors (everolimus), and NTRK/ROS1 inhibitors (entrectinib).[214—217] Major mutations yet studied in NSCLC include EGFR, KRAS, BRAF, HER2/MEK, RET, ROS1, hepatocyte growth factor receptor (HGFR/MET), and ALK.[218,219] Table 4 shows the frequency of mutations, FDA-approved drugs, and clinical trials. EGFR-TKIs are the evidence-based first-line treatment for advanced NSCLC, acting against L858R substitutions in EGFR exon 21 and exon 19 deletions. The most common cause of acquired resistance to EGFR TKIs is a second-site EGFR T790M mutation that negates their inhibitory action. Osimertinib is a third-generation oral EGFR TKI that targets the EGFR T790M mutation while leaving the wild-type EGFR intact. It also reduces the activity of ERBB2-4, BLK, and ACK1 at clinically relevant concentrations.[220] AURA3 (NCT02151981), ADAURA (NCT02511106), and FLAURA (NCT02296125) studies show phase III clinical trials of osimertinib. The AURA3 trial is an open-label, randomized trial with a primary endpoint of PFS. The study compares osimertinib to platinum-based doublet chemotherapy as second-line therapy for patients with locally advanced or metastatic NSCLC who have the EGFR T790M mutation with patients in the chemotherapy arm developing progressive disease and being able to switch to osimertinib treatment. In the ADAURA study, osimertinib was compared to placebo in patients with EGFR mutation-positive stage Ib—IIIa NSCLC after total tumor resection in a double-blind, randomized, global Phase III trial with the primary endpoint of disease-free survival. The FLAURA trial, a double-blind, randomized, international Phase III trial with a primary endpoint of PFS, compares osimertinib to gefitinib or erlotinib first-line therapy in treatment-naive patients with locally advanced or metastatic NSCLC.[220,221]

Oncogenic drivers are usually mutually exclusive within a tumor in the untreated state. These abnormalities are

evident in early lesions such as atypical adenomatous hyperplasia[222,223] and AIS,[47] supporting the theory that they represent early events in tumor formation when mutually exclusive. Furthermore, the mutual exclusivity of molecular modifications may support the staging of independent primaries.[25,224,225] Testing is significant for the first-line use of ICIs, which is precluded by EGFR mutations.[226] EGFR mutations and ALK translocations prevent ICIs from being used as a first-line treatment. Other oncogenic changes may be linked to a poor response to ICIs, but they have no bearing on first-line ICI therapy decisions.[227] Changes in STK11/LKB1, for instance, may result in PD-1 inhibitor resistance, but this does not rule out the use of PD-1 inhibitors as first-line therapy.[228]

Immunotherapy

Modified immunologic effector cell-mediated killing of tumor cells has been referred to as adoptive cellular immunotherapy (ACI). Specific ACI stands for those activated by tumor antigen stimulation or factors like T-cell receptor-T (TCR-T) and chimeric antigen receptor T-cell immunotherapy (CAR-T). Non-specific API is activated by cytokines or lymphocytes specifically present in peripheral blood like natural killer cells (NKC), DCs, tumor-infiltrating lymphocytes (TIL), cytokine-induced killer cells (CIK), etc.[229] With the emergence of ICIs, many NSCLC patients are responsive to anti-PD1 antibodies (nivolumab and pembrolizumab). CHECKMATE-227 study showed a median OS of 17.1 months for patients positive for PD-L1 with $\geq$1% receiving nivolumab and ipilimumab compared with the group treated with platinum-doublet chemotherapy.[230] Cancer cells may reduce antigens and express checkpoint proteins to hide from immune cells. Antibodies against inhibitory factors produced by cancer cells (anti-IL10, anti-TGFβ, IDO inhibitor) and checkpoint proteins (anti-PD1-PDL1, anti CTLA4), i*n vitro*, activated leukocytes, use of T cell-stimulating agents (IL-2, IL-7, IL-12, and IL-15), vaccines prepared from cancer cell-specific membranes, proteins and DNA are used to enhance the survival rate. Pembrolizumab, atezolizumab, nivolumab, and ipilimumab are used for checkpoint blockade.[231]

The development of novel immunotherapy agents like PD-1 checkpoint inhibitors (anti-PD-1 and anti-PDL-1 antibodies) that improve the immune system's capacity to recognize and delete tumors, including lung cancer, has been prompted by a better understanding of immunology and antitumor immune responses. In advanced or metastatic non-small cell lung cancer, two anti-PD-1 (nivolumab and pembrolizumab) and one anti-PD-L1 (MPDL-3280A) medicines are currently in clinical trials (NSCLC). Among them, nivolumab showed a 41% reduction in the risk of death compared to docetaxel in resistant SQCC (median OS: 9.2 against 6.0 months; objective response rate (ORR): 20% versus 9%). However, improving the immune response to cancer by targeting specific immune regulatory checkpoints has a different hazard profile than traditional chemotherapeutic drugs and molecularly targeted therapies. Immunotherapy's success is dependent on the continuing examination, detection, and treatment of immune-related adverse effects.[232]

Anti-angiogenic therapy

Anti-angiogenic drugs, such as bevacizumab, target VEGF, VEGFR, and fibroblast growth factor (FGF), are widely used to induce apoptosis or secrete enzymes that may cause endothelial cell disintegration in tumor cells. It also aids in the prevention of matrix remodeling and integrin reformation, restoring the typical structure of the vasculature. Thalidomide, a matrix metalloprotease (MMP) inhibitor, blocks angiogenesis by inhibiting the degradation of extracellular matrix (ECM), cell adhesion molecules (CAM), and the release of cytokines.[233] Four types of anti-angiogenic agents approved for NSCLC malignant stages include anti-VEGF mAb, bevacizumab; anti-VEGFR mAb, ramucirumab; VEGF-trap receptor, aflibercept; TKIs, nintedanib, axitinib, sorafenib, sunitinib, vatalanib, and lenvatinib. The HELPER-II study with unresectable stage III NSCLC using end star combined with chemoradiotherapy has shown a median OS of 34.7 months.[234] Even though anti-angiogenic therapy improves therapy efficacy mechanically, clinical outcomes are poor. The ECOG4599 trial used combination therapy with paclitaxel, carboplatin, and bevacizumab in patients with recurrent NSCLC, and the PFS and median OS were 6.2 months and 12.3 months, respectively.[235]

Neoadjuvant and adjuvant therapy

Neoadjuvant studies possess better efficacy over adjuvant studies as tumor immune activation might be boosted by neoantigens and intra-tumoral immune cells.[236–238] Nonetheless, clinical trials are required for validation; neoadjuvant chemotherapy trials show survival benefits in cases with less than or equal to 10% viable residual tumor.[239–242] ICI therapy with the combination of atezolizumab, durvalumab, and nivolumab has shown improved pathologic responses in 14%–45% of patients.[243–245] The PACIFIC study has the most mature data for stage III NSCLC, with patients receiving adjuvant durvalumab having PFS and OS of 12 months after chemoradiotherapy, respectively, and OS of 66.3 percent after two years.[246–248] Early-stage ALK and EGFR positive NSCLC are now trends in clinical trials for examining adjuvant TKIs.[249–251] The ADAURA trial with resected stage IB-III EGFR positive NSCLC to osimertinib or placebo up to three years after standard adjuvant chemotherapy showed OS of 89 percent and 53 percent, respectively, in the osimertinib and placebo groups.[252,253] Nonetheless, there is no evidence to suggest that in patients with resected EGFR-positive NSCLC, adjuvant chemotherapy could be replaced with adjuvant osimertinib.

Advances in the biomarkers for the early-stage management of NSCLC

A clinically significant biomarker should be measured using cost-effective and reproducible procedures and should offer valid information for the clinical management of lung cancer.[254] Blood-borne biomarkers are ideal as they recapitulate tumor heterogeneity and represent both metastatic lesions and primary tumors. Potential sources of biomarkers rather than blood include BAL, sputum, bronchial aspirates, urine,

and saliva.[255–257] Nodify XL2, early CDT-Lung, and Percepta are the commercially available biomarker tests with relatively better specificity.[258] This section discusses significant biomarkers in lung cancer management with screening, prognosis, and predictive values other than CTC, TEP, EVs, miRNA, and cfDNA, as discussed previously.

Autoantibodies (AAbs) are groups of potential biomarkers for cancer screening, which are secreted in response to tumor antigens and may be found in the plasma of NSCLC patients. AAbs panel in various screening cohorts shows their potential in classifying benign and malignant tumors.[259–262] The high specificity of AAbs shows their potential to improve the diagnostic performance of composite biomarker panels and complement the findings of high sensitivity imaging studies.[263] CDT-Lung test, the most advanced test for AAbs, shows specificity of 90% and sensitivity of 40%.[264] The score from the early CDT-Lung test to randomize 12,000 high-risk individuals into either standard of care follow-up or CT screening is published in a clinical trial in Scotland. The intervention arm received an early CDT-Lung test. The group with a positive result was treated with low-dose CT scanning for two years. Those with negative results and the control arm received a standard clinical trial. Patients in the intervention arm and control arm were diagnosed with stage III/IV as 58.9% and 73.2% (95%CI; sensitivity, 32.1%; specificity, 90.3%).[265] Protein biomarkers for lung cancer other than AAbs in the blood include LG3BP and C16A, which can be optimized for evaluating low-risk nodules by integrating them with other clinical risk factors such as nodule size, location, and spiculation, patient's age, and smoking history.[266]

Cancer cells activate the complement system through classical pathways. C4D, a component of the classical complement pathway, is high in the biological fluids of lung cancer patients.[267–269] C4D is valued for its possibility in lung cancer early diagnosis and management of pulmonary intermediate nodules.[270]

Prognostic factors vary for individual histotypes of NSCLC.[271] Lymphovascular invasion (LVI) is associated with worse recurrence-free survival (RFS) (CI = 95%; $n = 1147$).[272] Moreover, meta-analysis data indicate that Ki-67 expression is inversely proportional to OS in NSCLC patients.[273] High TILs are proportional to improved disease-free survival (DFS). Nonetheless, CD56/57$^+$ NK cells, CD20$^+$ B cells, and CD8$^+$ Tc cells positively correlate OS and DFS, while an inverse correlation between FOXP3$^+$ Treg cells and OS.[274] Other biomarkers identified by immunohistochemistry (Cyclins, P53, EGFR, and HER2), genotyping, and gene expression signatures are also potent NSCLC prognostic factors.[275] STAS is another important prognostic factor (30%–40% prevalence) in sub lobar resections of ADC.[24,276] Microarray technology has emerged as a potent tool for evaluating the expression of hundreds of genes in many samples, but the lack of validation and reproducibility constrains its application in routine clinical practice. Using genome-wide expression profiling in formalin-fixed paraffin-embedded (FFPE) samples, Xie and colleagues created a 59-gene predictive pattern for NSCLC.[277] Kratz and colleagues developed a 14-gene prognostic profile for non-squamous NSCLC using qPCR analysis on the same kind of tissues.[278,279] Wistuba and colleagues proposed a proliferation-based expression profile of 31 genes for the ADC histological subtype.[280] Moreover, Bianchi and colleagues created and validated a 10-gene prognostic signature in a sizable cohort of lung ADC patients using qPCR and FFPE samples.[281] They are all promising, but validation is still needed. Interestingly, efforts in artificial intelligence (AI) based on comprehensive computational pathological image analysis are trending toward predicting cancer prognosis.[282,283]

The prognostic biomarkers help decide suitable therapy for NSCLC patients. Ribonucleotide reductase regulatory subunit M1 (RRM1), excision repair cross-complement group 1 (ERCC1), breast cancer-specific tumor suppressor protein 1 (BRCA1), and receptor-associated protein 80 (RAP80) are just a few of the molecular markers that have been evaluated as a predictive biomarker for adjuvant therapy but dropped due to ineffectiveness. Thymidylate synthase (TS) is in the ITACA trial but with pending results.[284–288]

Assays or technologies corresponding to NSCLC biomarkers

Mass spectrometry (MS) allows non-hypothesis-driven total protein analysis of NSCLC early stage, which uses purpose-oriented sample preparation together with liquid chromatography prior to tandem MS scan and peptide ionization.[289] It involves sample digestion, peptide titration, ionization, and biomarker characterization.[290,291] These include streamlining the preparation workflow (MStern blotting, immune-depletion/filter-aided sample preparation [FASP], suspension trapping [S-trap]), altering the MS scanning modes (data-dependent acquisition (DDA), data-independent acquisition (DIA)), developing quantification techniques (isobaric labeling/label-free), and improving instrumentation (trapped ion mobility spectrometry (TIMS), high-field asymmetric ion mobility spectrometry (FAIMS)).[289,292] Hundreds to thousands of proteins can be characterized in a single MS run using blood or sera.[289,290] MS-based liquid biopsies have been done in multiple cancers, including NSCLC.[291] Proximity extension assays (PEA) work based on the principle of conventional sandwich ELISA and DNA-readout technologies.[293] The serological profiling using PEA seek minimal sample requirement with a full range. Multiple antibody pairs for the protein of interest are labeled using complementary DNA oligo sequences to allow high-fidelity discriminative hybridization.[294] The resulting DNA sequences are amplified, followed by NGS.[295] The advanced PEA assay possesses 3072 targets avoiding cross-reactivity issues common in conventional multiplexed immunoassayas.[294] However, high plex (>96 plex) PEA possesses issues with a trade-off in library preparation, NGS, and analytical factors.[296]

Reverse-phase protein arrays (RPPA) are a high content targeted, high-throughput proteomic technology superior to tissue-based profiling for tracing proteins, including their post-translational modifications within signaling networks.[297] Fully-denatured proteins are immobilized into substrates with dilution series. Highly specific, RPPA-validated antibodies are used to probe sample-containing slides, and fluorescence detection or colorimetric amplification is used to identify quantitative signals.[297–299] Exosomes with oncoproteins are becoming a significant hotspot for RPPA, which can be extended to liquid biopsy.[300] Though it needs sophisticated workflow, RPPA analysis of 276

cellular proteins, finding seven protein biomarkers of breast cancers is traced.[301]

Aptamers are short single-stranded DNA, RNA, or peptides that can bind to protein targets in native states with high affinity and specificity.[302–304] Slow-off rate modified aptamers (SOMA) scan assay use binding molecules (SOMAmers) attached to fluorescent labels and photocleavable linkers, followed by biotin-mediated purification and UV-based cleavage and tagging of bound proteins with biotin. SOMAmers with proteins are eluted and quantified via DNA hybridization techniques.[305] It enables high throughput ultra-plex screening approach with >7000 protein profiling in parellel,[306] though the difficulty of designing high-quality aptamers for novel targets remains.[307]

Sandwich ELISA-based and bead-based arrays are limited to medium/low-plex proteomic profiling.[308] Antibody/Antigen arrays use planar and bead antibody arrays together with proteome arrays due to their similarity in analytical and biochemical properties. It involves immobilizing specific antibodies onto substrates through affinity binding, covalent binding, and physical entrapment.[309,310] It is highly robust and practically characterizes thousands of proteins with minimal immunogenic cross-reactivity induced from antibody reaction mixtures. Antibody arrays have the ultrasensitive performance to overcome cross-sensitivity issues caused by untargeted proteins. Antigen arrays can theoretically investigate protein interaction with proteins, cells, small molecules, lipids, antibodies, and nucleic acids. To date, the most comprehensive human proteome array reaches 21,000 protein forms (over 81% proteome coverage), making it a robust tool.[311,312] Moreover, high-plex protein arrays are used to design a panel of lung cancer early diagnostic AAbs against H-RAS, P53, and ETHE1.[313]

Conclusion

Although much progress has been made in early cancer diagnosis, prevention, and treatment, lung cancer remains a severe health hazard worldwide, and it is expected to be the leading cause of death within four decades, even overtaking the current leading cause of death, IHD. The epidemiologic trends of various cancers based on the GLOBOCAN-2020 update agree with WHO mortality projections. Lung cancer has a high incidence, prevalence, and mortality rate, emphasizing the need for effective intervention and therapeutic strategies. Cancer detection in localized stages has advantages over regional and distant stages in terms of improved relative five-year survival, implying the importance of early cancer detection.

The poor prognosis of NSCLC patients continues to be caused by a lack of reliable, non-invasive diagnostic tools in the early stages of the disease. As a result, even though targeted therapy, combination therapy, and precision medicine have been shown to improve the median PFS and OS of NSCLC patients, surgical treatment remains the most commonly used treatment option. As molecular pathology advances and more potential therapies become available, the need for comprehensive molecular testing data to determine the best treatment will grow. Individual differences in treatment sensitivity and drug resistance highlight the importance of considering precision therapy.

Biomarkers may be vital in improving risk assessment and patient care, thereby enhancing DFS in early-stage NSCLC. Using liquid biopsy to detect circulating biomarkers such as RNA, microRNA, cfDNA, onco-proteins, AAbs, complement proteins, TEP, and CTCs can significantly alter cancer management in screening, prognosis, and prediction. It can improve disease stratification using intrinsic molecular characteristics, monitoring residual molecular disease, and identifying therapeutic targets. Liquid biopsy, a revolutionizing cancer early detection method, possesses emerging tools that have emerging roles as biomarkers in early cancer diagnosis. Tools such as cfDNA and CTCs can be used to track disease progression and driver mutations and study acquired resistance caused by tyrosine kinase inhibitors such as gefitinib and erlotinib in a more reliable and less invasive manner. Identifying cytomorphological and histological profiles of tumors using LBT can be justified as miRNA, and specific proteins differentiate ADC and SQCC histologically. However, liquid biopsy tools are still in their infancy and need to advance further before they can be used in clinical practice. Liquid biopsy tests (LBT) face significant challenges due to a lack of reliable cut-offs, standardized procedures, and prospective and retrospective studies. Nevertheless, the technological advancement and the use of LBT, either alone or in combination, raises the prospect of an era of early detection and lower NSCLC mortality rates.

Although there are concepts of early detection using liquid biopsy followed by targeted therapy, evasion and *de novo* mutations are the significant barriers to cures.[314] The fluctuating levels of various resistance genes and their ability to reprogram the treated tumor cells into a pool of drug-resistant cells complicate cancer therapy.[315] The drugs or radiations cause genetic changes in the surrounding normal cancer cells, which may cause them to become cancerous; they also increase resistance to pre-existing cancer through various signaling pathways. Clinical efficacy remains unsatisfactory as the drug design is challenging to combat specific mutations caused by drug resistance. A combinatorial approach that targets hotspot candidates in the signaling pathways could improve the efficacy. Combination therapy includes personalized medicine, immune therapy, and targeted therapy, all of which can be used to treat cancer, though much progress in the respective fields is required. Candidates in combination therapy and other newly developing approaches like ribozymes, telomerase inhibitors, antisense treatment, mechanistic target of rapamycin (mTOR) inhibitor, heat shock protein (HSP) inhibitor, apoptosis stimulators, proteasome inhibitor offer hope for improved clinical efficacy. Moreover, epigenetic approaches (histone acyl transferase (HAT)/histone deacetylase (HDAC) inhibitors, DNA methylation inhibitors), bacteriolytic therapy, clustered regularly interspersed short palindromic repeats (CRISPR/Cas9), and siRNA/RNA interference-based approaches can also use in combination therapy for better therapeutic outcome.

Author contributions

Conceptualization, A.G., A.V.G., A.D. and resources and data curation, H.P., J.V., M.C.J., G.K.R., C.M.W. and

M.A.Y.; writing—original draft preparation, H.P., J.V., M.C.J., G.K.R., C.M.W., M.A.Y. and K.R., S.D., R.S., A.G.M., U.R.W; writing—review and editing, K.R., A.G.M., U.R.W, A.V.G., A.G.; visualization, R.S., H.P, A.G., A.V.G. and A.D.; supervision, A.G.; project administration, A.G. All authors have read and agreed to the published version of the manuscript.

Conflict of interests

The authors declare that there are no conflict of interests.

Appendix A. Supplementary data

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