Circulating tumor DNA (ctDNA) is DNA fragment shed from tumor cells and can be isolated from peripheral blood. ctDNA carries genomic information of cancer and is the most widely utilized indicator in liquid biopsy. The application of ctDNA is gaining traction due to its reproducible, non-invasive, and easy-to-obtain characteristics. With the development of detection approaches, ctDNA plays an important role in the management of patients with cancers. In this review, we summarize the basic ctDNA detection measurements and review its application in early screening, prognosis evaluation after the surgery, and efficacy prediction of different therapies in non-small cell lung cancer.

Keywords: Circulating tumor DNA; Non-small cell lung cancer; Early detection; Prognosis evaluation; Treatment selection

1. Introduction

1.1. The overview of lung cancer

Lung cancer is the leading cause of cancer-related deaths and has the second highest incidence rate of cancer worldwide[^4], among which non-small cell lung cancer (NSCLC) accounts for 85% of the total cases[^5]. According to the eighth edition of the TNM classification, 5-year survival for patients with Stage IA1 NSCLC is 92%, while for patients with Stage IV is <10%[^6]. To improve the prognosis, discerning and meddling with lung cancer during early stage could be a feasible strategy. Surgical resection is recommended to patients with NSCLC from Stage I to Stage IIIA, while 10 – 50% of patients after the operation experience metastatic relapse within 5 years[^7]. TNM staging system is the most widely utilized prognostic tool for recurrence prediction, but patients within the same staging group exhibit heterogeneity in clinical consequences and therapy responses. The variation of survival rate within the same TNM stage calls for additional prognostic indicators.

1.2. Circulating tumor DNA (ctDNA)

cDNA is a subset of cell-free DNA (cfDNA), a kind of fragmented DNA pieces in the blood stream shed by tumor cells undergoing apoptosis or necrosis, and sometimes released by active secretion[^8]. ctDNA carries the whole genome information of primary tumor tissue and holds the potential as a promising surrogate for tissue samples for
Figure 1. Origin of ctDNA and research progresses in lung cancer. ctDNA is a kind of fragmented DNA pieces in the bloodstream shed by tumor cells undergoing apoptosis or necrosis or through secretion. ctDNA has utility in the whole treatment process of lung cancer. In the early detection of cancer, ctDNA can be used as a sensitive tool to decide a suspicious nodule on CT. In postoperative MRD detection, ctDNA after surgery reflects the fluctuation of tumor burden and acts as an early warning of relapse before traditional detection. According to tumor-informed panel, ctDNA can be used to monitor intratumor subclonal evolution. ctDNA detection can also be used to monitor therapeutic effect and discern patients with high risk of relapse, which provides a clue as to whether escalate adjuvant therapy in order to prevent further development.
ctDNA is present relatively low in peripheral blood. This is because on average, at each cell's death, only 0.014% total DNA of a tumor cell is shed into the bloodstream. The detection of ctDNA is further confounded by constituent of cfDNA that originates from normal cells, especially fragmented DNA from germline of hematoepoietic lineage with strong metabolism, such as lymphoid and myeloid cell lines. This phenomenon is termed clonal hematopoiesis of indeterminate potential, which increases with age and is the most common interference factor in ctDNA detection. The concentration of cfDNA in peripheral blood is estimated to be 1 – 100 ng/ml but only 0.1 – 1% of which originates from tumor tissue. The density of ctDNA is even lesser in low tumor-burden individuals, such as patients with early stage cancers or patients after the resection surgery. The fragment length is another characteristic that differentiate ctDNA from cfDNA of non-cancerous origins. The size of cfDNA is around 166 bp, and it has been observed that ctDNA is 23 bp shorter than normal cfDNA in plasma. cfDNA shows strong relation to nucleosome and core histones and is conferred protection from endonuclease, while ctDNA presented a more fragmented pattern. However, the underlying reason for these differences remains unknown. In patients with NSCLC, ctDNA has a fast clearance, allowing for dynamic surveillance. Based on statistical analysis, the half-life of ctDNA after radical resection has been determined to be 35 min, so ctDNA can be detected in real-time and used to reflect tumor burden.

Histopathological examination is the gold standard of NSCLC’s diagnosis, but ctDNA also shows unique advantages over other detection methods. First, ctDNA contains the whole genome information of the tumor tissue. Tumor cells consistently release ctDNA into the circulation and thus rendered as a surrogate of tumor tissue on the grounds of the same genomic information they carry. In late stage lung cancer, plasma ctDNA was found to possess at least one targeted driver mutations, which is similar to tumor tissue at a concordance of 81.6% and 50.4% in early stage NSCLC, probably due to low rate of ctDNA positivity in low tumor-burden individuals. Second, ctDNA is capable of capturing intratumor heterogeneity. Due to the fact that the cancer cells malignantly proliferate and release ctDNA into bloodstream, ctDNA is able to show the mutation landscape of tumor tissue, especially intratumor subclones, which generally is not captured by the traditional tissue histopathological examination. In a systematic comparative sequencing analysis among Tracking Non-Small-Cell Lung Cancer Evolution Through Therapy (Rx) (TRACERx) cohort conducted by Abbosh et al., intratumor single nucleotide variants (SNVs) were identified in ctDNA in 59% patients with early stage NSCLC. Thirdly, ctDNA enables dynamic monitoring of post-operative residual

Table 1. Research progress on the application of ctDNA in the treatment process in NSCLC patients

<table>
<thead>
<tr>
<th>Author/Ref.</th>
<th>Year</th>
<th>ctDNA assay</th>
<th>Number of patients involved</th>
<th>TNM stage of patients</th>
<th>Early detection</th>
<th>Prognostic evaluation</th>
<th>Prediction of therapeutic effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cohen et al.</td>
<td>2018</td>
<td>Cancer-SEEK</td>
<td>1850</td>
<td>Pan cancer</td>
<td>√</td>
<td></td>
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</tr>
<tr>
<td>Newman et al.</td>
<td>2014</td>
<td>CAPP-Seq (139 mutated genes)</td>
<td>407</td>
<td>I–IV</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chabon et al.</td>
<td>2020</td>
<td>Lung-CLip (255 mutated genes)</td>
<td>160</td>
<td>I–III</td>
<td>√</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>Abbosh et al.</td>
<td>2017</td>
<td>WES-PCR (personalized based on 16 SNVs)</td>
<td>100 First European cohort</td>
<td>I–III</td>
<td>√</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>Chaudhuri et al.</td>
<td>2017</td>
<td>CAPP-Seq (128 mutated genes)</td>
<td>40 First North America cohort</td>
<td>IB–III</td>
<td>√</td>
<td></td>
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</tr>
<tr>
<td>Chen et al.</td>
<td>2019</td>
<td>cSMART (9 genes)</td>
<td>36 First East Asian cohort</td>
<td>I–III</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Qiu et al.</td>
<td>2021</td>
<td>NGS-panel (139 genes)</td>
<td>91</td>
<td>I–IV</td>
<td>√</td>
<td></td>
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</tr>
<tr>
<td>Nabet et al.</td>
<td>2020</td>
<td>DIRect-On (ctDNA on treatment and immunotherapy response)</td>
<td>94</td>
<td>Unresectable IIIB–IV</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moding et al.</td>
<td>2020</td>
<td>CAPP-Seq (97 mutated genes)</td>
<td>65</td>
<td>Unresectable IIIB–III</td>
<td>√</td>
<td></td>
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</tr>
</tbody>
</table>
disease. Liquid biopsy is a noninvasive and convenient test, which can be used for multiple times in a short period of time. In the meantime, the short half-life of ctDNA further makes real-time surveillance a possibility. Fourth, ctDNA can act as a reference to tumor's volume. In ctDNA-positive patients, the mean plasma variant allele frequency (VAF) of clonal SNVs correlated with tumor volume tested by three-dimensional reconstruction technology of spiral computed tomography (CT). A linear relationship was found between log-transformed tumor size and log-transformed mean clonal VAF values in ctDNA-positive cohort. Based on the TRACERx data, a plasma VAF of 0.1% is corresponding to a tumor burden of 10 cm. Finally, ctDNA has the potential to be utilized throughout the whole clinical treatment course. The potential of ctDNA has been explored under different circumstances and the outcome lived up to our expectations. The use of ctDNA is advantageous to many fields, including lung cancer screening and early detection as well as monitoring of MRD for both prognostic evaluation and prediction of therapeutic effect (Table 1).

1.3. ctDNA mutation classification and detection technology

cDNA can barely be detected in peripheral blood. The precision of detection technology restricts the outcome of liquid biopsy analysis. Various highly sensitive methods that can be used to detect different mutational types have emerged.

cDNA has unique mutation patterns compared with normal tissue DNA and the following biomarkers have been reported in the previous research: (i) Genetic mutations and structural variations, such as SNVs and copy number variations (CNVs); (ii) fragmentomics characters, including motif, fragment length distribution, and nucleosome distribution patterns; and (iii) epigenetic changes, including DNA methylation, histone modification, and amount of special non-coding RNA (ncRNA).

1.3.1. Genetic mutations and structural variations

To detect DNA mutations, polymerase chain reaction (PCR) and next generation sequencing (NGS) are recommended. Real-time PCR, droplet digital PCR, amplification refractory mutation system, and BEAMing (that stands for beads, emulsion, amplification, magnetics) can be classified as PCR-based methods. These techniques have high sensitivity and specificity with temperate cost for limited known mutation spot, but with a disability of discerning structural variations, such as copy variations and gene fusions. NGS-based method can overcome this problem by determining the order of nucleotides in entire genomes or targeted regions of ctDNA and spotting every DNA mutation a sample may have. However, with the width of NGS-based detection, it inevitably costs more time and money to run a test and also requires professional researchers equipped with abundant relevant knowledge and sufficient training to manipulate the detection. Cancer personalized profiling by deep sequencing (CAPP-Seq) and tagged-amplicon deep sequencing are classical and widely recognized NGS-based methods. PCR-based method is a high-throughput technique that involves the selection of targeted main mutation driver genes or the detection of specific mutation spots, which can be meaningful to the outcome of adjuvant therapy, while NGS-based method has high sensitivity, especially in early diagnosis, for its ability in detecting unknown mutations. As for structural variations, NGS-based technology is the only means for detection. The analytical tools which extract different features from NGS outputs allow for the detection of CNVs by low-coverage sequencing of the genome accompanied by normalization algorithms. Since CNVs are also originally present in healthy individuals and susceptible to tumor conditions, CNVs in ctDNA are quite difficult to discern. Recent technical development on CNV's detection, within-sample aneupLoidy detection (WALDO), has set the detection threshold to 1% of cfDNA fragment concentration in sample plasma, but there is still insufficient proof for its clinical applications. NGS is also applicable to the detection of genomic rearrangements.

1.3.2. Fragmentomics characters

Gel electrophoresis and electron microscopy were the first detection methods used to detect the length of cfDNA, and NGS-based methods are mainly used to work out the fragmentomics characters at present. Except for fragment coverage and size, various innovative fragmentation patterns can be delineated by corresponding analytical techniques. DNA evaluation of fragments for early interception (DELFI) used ~1× low-coverage whole-genome sequencing (WGS) to detect genomic fragment abnormalities, including fragment coverage, size distribution chromosome-arm copy number, and mitochondrial aligned reads from cfDNA. Ulz et al. developed another detection method to estimate the overall binding affinity of the transcription factor by cfDNA WGS. Transcription factors at specific genetic spots are protected from endonuclease and transcription factor's affinity consequently influenced ctDNA's fragment distribution. The score of binding affinity exhibited its ability in early detection and primary classification of cancer.

1.3.3. Epigenetic changes

Epigenetics refer to heritable chromatin modifications that impact gene expression without a direct effect on the coding sequence of the DNA. DNA methylation is the most studied epigenetic regulatory mechanism and the methylation
focuses on silencing cancer suppressor genes by inactivating specific chromatin structures. DNA methylation often happens on CpG islands (CGIs) around the 5’ untranslated regions (5’UTRs) of genes, which are always associated with promoters and are rich in GC base-pairs. The methyl groups on CGIs are offered by S-adenosyl-methionine to the position 5 of cytosine and catalyzed by DNA methyltransferases, including DNA methyltransferase 1 (DNMT1), DNMT3A, and DNMT3B, among which DNMT1 plays the main part of maintaining the methylated status\(^2^7\). Moreover, tumor’s genes are highly methylated and consequently occupies the majority of DNMT1. As a result, the methylation landscape in a patient with tumor burden turns to be highly methylated in CGIs with generally global demethylation\(^2^8\).

For DNA methylation that has no nucleotide sequence changes, the way to detect methylation is quite different from the above. Three mainstream detection methods are applied to differentiate methylated DNA spots. Bisulfite sequencing first converts unmethylated cytosine into uracil under the effect of bisulfite, then transferred into thymine by PCR, with methylated nucleotide theoretically intact. This bisulfite method is cheap and accurate, and honored as a gold standard of methylation detection. However, its default is also apparent, and the bisulfite transformation weakens the DNA double-chains’ stability, which may cause bias to the result\(^2^9\). The second detection tool is using methylation-sensitive restriction enzymes before amplification, aiming to digest unmethylated CGIs before gel electrophoresis or PCR sequencing across the restriction site. Besides, affinity-enrichment-based methods are considered in the methylation detection. Researchers also designed methyl-binding protein or antibodies specific for 5mC, intending to elevate the concentration of methylated spots. By running sequencing techniques in a high density of targeted DNA, this affinity-enrichment-based method achieved high-sensitivity detection with less efforts\(^3^0,3^1\).

In comparison, methylation-sensitive restriction enzyme method is limited to recognizable unmethylated CpG loci out of the enzyme’s specificity range, but the bisulfite-based sequencing and affinity-enrichment-based method are capable for the whole genome. However, bisulfite-based technique has a large working load, and the accuracy of affinity-enrichment-based method is closely related to the protein-target binding. As for target preference, bisulfite sequencing is repeatable and sensitive especially for single CpG resolution. Methylation-sensitive restriction enzymes have high efficiency on low-CpG-density regions and affinity-enrichment-based method performs better on CpG-rich ones\(^3^2\).

Histone modification and the amount of special non-coding RNA (ncRNA) are additional epigenetic changes that can be used in cancer detection. Histone proteins can be modified in a variety of ways, including acetylation, methylation, and phosphorylation. Core histones’ N-terminal tails protrude from nucleosomes and are embellished with post-translational modifications. Based on the functions, the enzymes are categorized into four groups: Acetylation by HATs, deacetylation by HDACs, methylation by HMTs, and demethylation by HDMs. Modifications on histones inactivate cancer suppressor genes, and posttranslational modifications complement by promoting or inhibiting cell metabolism\(^3^3\). However, posttranslational modifications on histones are neither exclusive nor independent in tumor tissues, and there are numerous combinations that form modification crosstalk. Figuring the tumor-specific modifications or combinations are one of its greatest challenges\(^3^4\).

MicroRNA (miRNA), long ncRNA (lncRNA), circular RNA (circRNA), and PIWI interacting RNA (piRNA) comprised the majority of ncRNA in liquid biopsy. Single miRNA can target more than 400 targeted mRNA and it is believed that more than half of human’s genes are directly modulated by miRNA\(^3^5\). Researchers realized the great diagnostic and therapeutic potential of miRNA soon after the discovery and identification, and miRNA is widely used in various studies compared with other ncRNA biomarkers. miRNA specifically binds to targeted mRNA by complementary sequence and forms RNA-induced silencing complex to silence or degrade mRNA. Many investigations had confirmed that miR-126 and miR-155 were oncogene in many kinds of cancers, while let-7 miRNA and miR-34a were regarded as tumor suppressors\(^3^6\). miRNA can be best extracted from plasma and the extraction methods can be grossly divided into three groups, namely, organic extraction methods, filter-based methods, and magnetic particles-based methods. When combined with other extraction methods, the accuracy of these methods can be further improved\(^3^7\).

2. Early detection and screening

2.1. Status quo of lung cancer screening

As discussed above, lung cancer is a vital disease, but the prognosis is tightly associated with the stage when it was first discovered. Low-dose CT (LDCT) among high-risk population is one of the current lung cancer screening strategies, which is recommended as a screening guidance of lung cancer in many countries\(^3^8\). National Lung Screening Trial (NLST) was the first to verify LDCT’s effect in reducing mortality in large-scale trial. A multi-centered cohort comprising 53,454 high-risk patients who were randomly subjected to regular LDCT or single-view posteroanterior chest radiography examination for 3 years was analyzed for the following lung cancer-related death. LDCT group had a relative reduction

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of mortality from lung cancer by 20% in a 2-year follow-up visit\[35\]. Similar small-scale investigations emerged in succession, but none of them yielded findings of comparable significance or reliability of NLST trial outcomes until the Dutch-Belgian lung-cancer screening trial (NELSON). This population-based, randomized, and controlled trial recruited 30,959 persons with high-risk lung cancer in four selected regions in the Netherlands and Belgium. The experimental group reduced 0.80 deaths per 1000 person-years after four rounds of LDCT screening compared with the control group at 10 years of follow-up, further revealing LDCT’s benefits in high-risk people with lung cancer\[36\]. These two influential clinical trials consolidated LDCT as a reliable tool in early screening of lung cancer.

However, health-care personnel face numerous clinical problems using LDCT. First, LDCT has high false positive rate. The false positive rate of LDCT in NLST reached 96.4%, while the positive predictive value in NELSON test was only 43.5%\[37,38\]. Second, over-diagnosis causes unnecessary invasive treatments. A meta-analysis revealed that 1.7% negative positive patients received invasive diagnostic measures and serious complications happened in 0.4% of them\[39\]. Third, radiation exposure in the regular screening routine may act as the induction of other cancers. The effective dose from 256-slice LDCT is 0.71 mSv each time, which is much lower than that from regular CT examination. However, this extent of exposure still increases nominal lifetime intrinsic risks related to radiogenic cancer by 0.13% and 0.30% in male and female populations, respectively\[40\]. Fourth, the targeted screening population of the previous LDCT screening is restrained to high-risk group, mostly smoking and aging population, but the incidence of NSCLC in people free of these risks, are rising\[41\]. For these low-risk population, whether LDCT brings more benefits or harms remains unknown.

To improve the early screening coverage and accuracy, alternative parameters from basic clinical information, radiographic imaging, and traditional tumor markers should be in place. Among them, ctDNA has gained wide attention due to its non-invasiveness and high sensitivity, which basically meet the need of early screening\[42\].

### 2.2. Application value of ctDNA in early detection

Considering the genomic mutation in ctDNA, frequent driver mutations in genes such as \(EGR, KRA, PIK3CA\), and \(TP53\) and other less frequent mutations have been investigated in the Ion PGM and AmpliSeq Cancer Panel\[42\]. About 60.3% of early-stage NSCLC patients were found to have detectable ctDNA and its concordance with matched tumor DNA was 50.4%. ctDNA further exhibited a higher positive prediction value than traditional protein biomarkers in this study, which corroborated its potential as a promising early detection indicator\[12\]. Cohen \textit{et al.} integrated the mutation information in ctDNA and traditional protein biomarkers to elevate the efficiency of early-stage cancer evaluation and invented Cancer-SEEK method, which was applied in eight types of cancer with the sensitivity ranging from 69% to 98% at a nearly 100% specificity\[41\]. CAPP-Se, which is the first NGS-based method for ctDNA quantitation, was invented. This technique searches for exons harboring recurrent mutations in potential driver genes from the catalogue of somatic mutations in cancer and is modified according to whole-exome sequencing of NSCLC patients, to design the NCSLC selector. CAPP-Seq has a sensitivity of 100% in Stage II-IV NSCLC with a specificity of 96% and was able to detect ctDNA in half of Stage I patients\[43\]. The technique was further refined in the aspects of mutation spots selection, and a machine-learning method termed “lung cancer likelihood in plasma” (Lung-CLIP), which can robustly distinguish early stage NSCLC from inflammation or benign nodules, was designed. In patients with Stage I disease, Lung-CLIP has a sensitivity of 63% and a specificity of 80%\[43\].

Detection methods based on ctDNAs fragmentomics characters were also examined in NSCLC screening. Researchers developed an approach called DELFI by ctDNA genome-wide analysis. This method was based on cancer-featured fragmentomics and achieved an AUC of 0.87 in lung cancer after machine learning data analysis\[24\]. When further combined with clinical risk factors, carcinoembryonic antigen levels, and CT imaging, DELFI identified 91% Stage I/II and 96% Stage III/IV lung cancer patients at the specificity of 80%\[44\]. ctDNAs fragmentomics not only help to screen lung cancer among healthy people, but also reveal the tissue origin of ctDNA. Fragment patterns, such as size distribution, fragment patterns, location of nucleosomes, and open chromatin regions, have been proven as valid characters to ascertain the tissue source of ctDNA\[24,45-47\].

Aberrant ctDNA methylation that precedes genomic mutation is an ideal biomarker for individuals with low tumor burden, such as patients with early stage lung cancer\[48\]. A large-scale research in CCGA database found whole-genome bisulfite sequencing outperformed CNVs and SNVs in identifying patients with cancers. The detection pattern of targeted methylation meets the goal for population-level screening of more than 50 types of cancers and is able to locate the origin at high accuracy, suggesting that ctDNAs methylation is feasible in early detection\[48\]. Chen \textit{et al.} utilized nanoparticle-based DNA extraction (MOB) followed by qMSP to detect promoter methylation on eight candidate lung cancer-specific genes\[49\]. In a Chinese cohort, the three best methylation combination model, containing CDO1, SOX17, and HOXA7, attained the
Table 2. Overview of major detection assays of MRD

<table>
<thead>
<tr>
<th>Detection assay</th>
<th>Requirement of tissue</th>
<th>Representative methods</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
</table>
| Fixed MRD panel (tumor-naïve) | No                    | CAPP-Seq               | • Independent of tumor tissue  
• Short detection period  
• Stable monitoring system | • Cannot screen patients with rare mutations  
• Sensitivity is slightly lower than personalized panel |
| Personalized MRD panel (tumor-informed) | Yes  
(Signatera, ArcherDx) | TRACERx               | • Personalized designed panel  
• Better pertinence to individuals  
• Stable monitoring system | • Dependent of tissue sample  
• High cost  
• Long detection period |
| WGS                           | Yes                   | MRDetect              | • Taking multi-dimensional characters into account  
• Wide detection range for remediying the low density of ctDNA in bloodstream | • Lack of data to verify the result |
| Methylation                   | Yes                   | MEDAL[71]             | • Multi-locus analysis to improve detection sensitivity  
• Stable monitoring system  
• Short detection period | • Selection of cancer-specific differentially methylated regions needs refinement  
• Cutoff value remains to be set |

accuracy at 90% sensitivity and 71% specificity[49]. Due to the low abundance of ctDNA and degradation during bisulfite conversion, methylation detection was restricted to specific regions and had insurmountable technical barrier on its accuracy. Cell-free methylated DNA immunoprecipitation and high-throughput sequencing (cfMeDIP-seq) overcome this challenge by avoiding bisulfite conversion step and taking whole genome sequence into account[50]. CfMeDIP-seq's practicability was checked in a lung cancer cohort in a random forest prediction model, which demonstrated its competence with high sensitivity and specificity of 91.0% and 93.3%, respectively[51].

3. Post-operative MRD detection

MRD is a term first used in blood cancer, referring to the small number of cancer cells that remain in the body after treatment[52]. For patients who are MRD-positive, the number of remaining cancer cells may be so small that they cannot be detected by traditional tests, such as CT. MRD can be identified through highly sensitive and specific molecular diagnostic technique and a positive MRD suggests possibility of recurrence, even though the patients have no noticeable clinical signs of cancer[53]. When recurrence or metastasis is clinically verified by standard measures, it has already stored the large number of cancer cells in the tumor that harbor more than tens of millions of cells can hardly be wiped out by secondary lesion resection. A liquid biopsy for MRD would provide a minimally invasive measurement, which could identify patients at higher risk of early relapse so that necessary intercptions can be arranged in advance[54].

The approaches for MRD detection include tumor-informed assay, tumor-naïve assay, WGS, and methylation detection. Different from early screening of lung cancer, doctors are able to obtain pre-operative blood sample or tumor tissue and tumor-adjacent tissue which demonstrates individual tumor mutation landscape before MRD detection. According to the tumor mutation information and variants from previously obtained non-cancerous tissue, clinicians can design personalized monitor panel of MRD tracking on several tumor-specific mutations, to achieve better pertinence to individuals and gain higher accuracy in the absence of interference from irrelevant mutations. This approach is termed tumor-informed assay. While the strategy of tumor-naïve assay is taking a panel with mostly seen tumor mutations, unknown of personalized mutation information. This tumor-naïve assay is independent of tumor tissue and shortens detection period with stable monitoring system, which fits the industrialization circuit better[55]. However, without the mutation information of individuals, tumor-naïve assay cannot cover rare mutations and has a slightly lower sensitivity than tumor-informed assay. WGS and methylation detection methods are used relatively lesser in MRD. WGS takes multi-dimensional characters into account to remedy the low density of ctDNA by wide detection range. Methylation analysis targets multiple loci to detect minor ctDNA remaining in the patients. More studies are needed to verify the efficacies of these two methods. For methylation in MRD, cancer-specific differentially methylated regions (DMRs) need refinement and cutoff value remains to be set (Table 2).

The major use of detecting MRD after surgery is to monitor patient’s prognostic performance and indicates recurrence ahead of traditional evidence. In a prospective study based on TRACERx research, Abbosh et al. followed up 24 NSCLC postoperative patients for a median of 775 days. Among those with recurrence, 93% patients were MRD-positive with at least two SNVs in plasma before or at the timepoint of relapse. Meanwhile, only one MRD-positive individual showed no clinical evidence of relapse. The detection of ctDNA followed the radiographic evidence of recurrence by a median of
70 days\cite{10}. CAPP-Seq targeting 128 frequent genes in lung cancer was applied to detect ctDNA in NSCLC patients in the study conducted in North America. The ctDNA levels in 20 patients showed no reduction after surgery in at least one post-operative timepoint and the patients experienced relapse without exception\cite{11}. The cohorts in Europe and North America all exhibited close relationship between MRD and relapse risk. Similar result was first reported in a study of East Asian cohort and the study further illustrated the significant difference of recurrence-free survival in patients who were either ctDNA-positive or ctDNA-negative at 3 days after resection surgery, thereby setting a precise timepoint of MRD detection to predict long-term relapse. About 85.7% (6 / 7) of MRD-positive patients experienced recurrence and MRD was 165 days earlier than clinical recurrence verified by post-operative CT\cite{12}.

ctDNA concentration in plasma is quite low after routine therapy. VAF of ctDNA after the surgery is <0.1% in plasma, which is lower than the detection threshold of most detection methods\cite{13}. Multi-omics are undoubtedly considered an improvement to the detection sensitivity. Chen et al. constructed an MRD prediction model based on the combination of mutation score and regional methylation ratio. Sixty-seven genetic variants were selected after filtration of clonal hematopoiesis with allele frequencies ranging from 0.03% to 6.00% and then a mutation score was constructed by a weighted sum of the allele fractions. Three hundred and fifteen DMRs were identified and fed into penalized Cox regression analysis to obtain a 12 DMR-featured methylation-based prognostic score (MPS). Mutation score and MPS were used together to construct a bi-omics prognosis score to distinguish the high recurrence group who had worse overall survival\cite{14}. Another model called MRDetect used WGS and tumor-informed methods to detect ctDNA at a lower threshold. MRDetect-SNV and MRDetect-CNV, which focus on different characters of ctDNA mutation, reached the detection limit of tumor fraction at 10^{-3} and 5 × 10^{-5}, respectively. Integrating these two MRDetect methods based on various features of ctDNA, the model achieved high clinical specificity of 96%, while maintaining high sensitivity of 67% in lung adenocarcinoma detection\cite{15}.

More characters of MRD are being conceived for more accurate and detailed depiction of relapse. Apart from the existence of MRD, ctDNA growth rate is another strong prognostic factor and can describe biological behavior of residue tumor cells. In post-operative patients with Stage III colorectal cancer, the increasing ctDNA growth rate was bimodal, categorized into fast-growth pattern and slow-growth pattern, in which ctDNA concentration increased by 143% and 25% per month. Further survival analysis showed striking result that slow-growth pattern group had statistically similar overall survival rate as ctDNA-negative group after initial therapy, which may change the therapy schedule in MRD-positive patients. Moreover, the growth rate was found stable after the surgery for a long time; with only two timepoint of ctDNA examination, the ctDNA growth velocity can be determined, which can be used to predict relapse\cite{16}. This research supports ctDNA as a biomarker of prognosis, but unfortunately no study has been performed to evaluate its application in lung cancer.

4. Treatment selection and response evaluation

Patients with locally advanced lung cancer are barely subject to resection surgery, but they receive adjuvant therapy to alleviate tumor burden. Certain people who are eligible for targeted therapies or immunotherapies survive longer according to NCCN guidelines\cite{17}. Adjuvant therapy can effectively improve the prognosis after the whole process of therapy, but each therapy is a double-edged sword. Misuse or overdose will bring about severe side effects and induce drug resistance. A growing line of evidence points out that post-operative ctDNA acts as a reliable biomarker for treatment selection and prognostic evaluation\cite{18}.

In part of the data from DYNAMIC and TRACERx, it is suggested that the curative effect of adjuvant therapy is associated with ctDNA level\cite{19,20}. The therapeutic effect prediction ability of ctDNA has shown in patients receiving targeted therapy and immunotherapy. ENSURE study revealed that epidermal growth factor receptor (EGFR) mutation-positive NSCLC patients taking erlotinib had longer progression-free survival compared with patients receiving chemotherapy, and this result contributed to FDA’s recognition of EGFR mutation as the first mutation employed in liquid biopsy test to guide the selection of EGFR TKI\cite{21}. Consequently, the benefit of osimertinib was found in patients with EGFR Thr790Met-positive NSCLC, who reported 70% of objective response with toxicity effects of <5%\cite{22}. The therapeutic effect prediction ability of MRD was further demonstrated in patients receiving immunotherapy. Immune checkpoint inhibition (ICI), such as PD-(L)1 blockade, showed high clinical response rate accompanied by equivalent high level of toxic reaction in the treatment of advanced NSCLC. However, only a minority of the patients obtained long-term benefit from ICI. To identify the patients who obtained long-term benefit before or at the beginning of the therapy, pre-treatment peripheral T cell level and early on-treatment reaction of ctDNA were investigated and found to be associated with checkpoint blockade response, which served as ideal biomarkers. DIREct-On model combined with pre-treatment ctDNA, immune profiling, and early on-treatment ctDNA information can robustly predict the
probability of long-term clinical benefit with high accuracy among the individuals who received ICIs\textsuperscript{[64]}. With this prediction model, accurately forecast therapeutic effect of ICIs at an early stage by non-invasive methods became possible.

MRD not only serves as a prediction tool for the curative efficacy of adjuvant therapy, but also plays as a warning sign to escalate therapy in high-risk group or signifies the endpoint of treatment in patients with little chance to recur. In control research, ctDNA level was measured in 65 patients who had received standard chemoradiation therapy for locally advanced non-small-cell lung cancer and ctDNA-positive patients had shorter lifetime in general. Among the ctDNA-positive population, patients who received ICIs had better prognostic outcome than the ones without further therapies. However, in patients whose ctDNA was not detected after the previous chemoradiation therapy, subsequent ICIs granted little survival benefits and caused a severe drug-related pneumonia in one case\textsuperscript{[65]}. This result indicates that ctDNA is a terminal indicator of adjuvant therapy and could guide therapy arrangement. Further large-scale research is required to verify this notion, although the potential of ctDNA in monitoring therapeutic effects and acting as a reference to subsequent therapeutic schedule has already been revealed.

Although MRD has shown enormous potential as a prediction factor, more high-level evidence-based medical studies are needed to explore whether MRD could serve as a guide in therapy scheduling. Several clinical trials are ongoing (Table 3), such as MERMAID-1 and MERMAID-2, focusing on MRD as an indicator to provide second-line treatment after standard therapy or just as a threshold of granting adjuvant therapy so as to attain the best curative effect and decrease unnecessary toxic side effects in each patient\textsuperscript{[66-73]}.  

Table 3. Prospective MRD’s guidance on adjuvant/adjunctive therapy trials

<table>
<thead>
<tr>
<th>NCT/Ref.</th>
<th>Abbreviation</th>
<th>Phase</th>
<th>Inclusion population</th>
<th>Sample size</th>
<th>Testing time of MRD</th>
<th>Intervention</th>
<th>Primary outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCT04385368</td>
<td>MERMAID-1</td>
<td>3</td>
<td>Stage II–III NSCLC patients after completely resection</td>
<td>322</td>
<td>Post-operative</td>
<td>Placebo+SoC chemotherapy: Durvalumab+SoC chemotherapy</td>
<td>DFS in MRD+analysis set</td>
</tr>
<tr>
<td>NCT04642469</td>
<td>MERMAID-2</td>
<td>3</td>
<td>Stage II–III NSCLC patients after completely resection with ctDNA+</td>
<td>284</td>
<td>After curative-intent therapy within 96-week's surveillance</td>
<td>Placebo+Durvalumab</td>
<td>DFS in PD-L1 TC≥1%</td>
</tr>
<tr>
<td>NCT04367311</td>
<td>BTCRC-LUN19-396</td>
<td>2</td>
<td>Stage I–III NSCLC patients after completely resection with ctDNA+</td>
<td>100</td>
<td>Post-operative</td>
<td>NA Atezolizumab+Ctx</td>
<td>Percentage of patients with clearance of ctDNA</td>
</tr>
<tr>
<td>NCT04585477</td>
<td>-</td>
<td>2</td>
<td>Stage I–III NSCLC patients</td>
<td>80</td>
<td>After standard therapy</td>
<td>None Durvalumab</td>
<td>Number of patients with a≥3-fold drop in ctDNA level</td>
</tr>
<tr>
<td>NCT04585490</td>
<td>-</td>
<td>3</td>
<td>Locally advanced, unresectable stage III NSCLC patients</td>
<td>48</td>
<td>After CRT and two cycles of durvalumab</td>
<td>Durvalumab Duvalumab+Ctx</td>
<td>Change in ctDNA level following chemotherapy</td>
</tr>
<tr>
<td>NCT04267237</td>
<td>-</td>
<td>2</td>
<td>Stage II–III NSCLC after complete resection with ctDNA+</td>
<td>80</td>
<td>After the surgery and standard adjuvant chemotherapy</td>
<td>Atezolizumab RO7198457+Atezolizumab</td>
<td>DFS up to 62 months</td>
</tr>
<tr>
<td>NCT04611776</td>
<td>CATHAYA</td>
<td>2</td>
<td>Stage II–III NSCLC after complete resection with ctDNA+</td>
<td>160</td>
<td>After surgical resection, before adjuvant therapy</td>
<td>Placebo+platinum-doublet chemotherapy (Placebo maintenance) Atezolizumab+platinum-doublet chemotherapy (Atezolizumab maintenance)</td>
<td>ctDNA clearance rate at 6 months and DFS up to 159 months</td>
</tr>
<tr>
<td>NCT04354064</td>
<td>-</td>
<td>-</td>
<td>Pan cancer patients</td>
<td>3362</td>
<td>-</td>
<td>Observational</td>
<td>-</td>
</tr>
</tbody>
</table>

CRT: Cardiac resynchronization therapy; ctDNA: Circulating tumor DNA; Ctx: Chemotherapy combination with cyclophosphamide; DFS: Disease-free survival; MRD: Minimal residual disease; NSCLC: Non-small cell lung cancer.
Apart from prediction of therapeutic response, ctDNA can monitor the formation of drug resistance in real-time. Horn et al.[24] studied longitudinal ctDNA dynamic changes during ALK TKI procedure in 76 patients with ALK+NSCLC who were enrolled in the Phase I/II multicohort eXalt2 trial. Various baseline subset mutations at ALK were observed and dynamic changes of mutation proportion or types happened as the therapy went on. In a classical case, the patient who had prior medication history of crizotinib and ceritinib had ALK L1152V and G1269A baseline mutations, but these mutations disappeared when he experienced partial response during ensartinib treatment. At last, new ALK mutation E1210K was detected at the time of radiographic progression. This research suggests that ctDNA can monitor dynamic clonal evolution and guide selective pressures by therapy[40].

As for the future direction of this field, the timepoint to check ctDNA and modulate therapy schedule is definitely major research topics in MRD detection. Recent studies lack a unified standard and literature focusing on NSCLC in dynamic evolution during adjuvant therapy are scarce[38]. Balancing the benefit and toxicity to decide the most appropriate time to withdraw or escalate the therapy will undoubtedly achieve maximum benefits for patients of all stages[25].

5. Summary
ctDNA is a potential biomarker, which plays an instrumental role in the whole treatment process of NSCLC with high accuracy. It demonstrates wide utility in early detection to screen underlying cancer, in MRD detection to predict long-term prognosis, and in treatment arrangement to guide more precise and effective therapeutic schedule.

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Conflict of interest
No potential conflicts of interest were disclosed.

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