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Teaser Understanding and identifying the dynamic emergence of the druggable mutational and molecular landscape in response to therapy following Darwinian evolution creates the foundation of precision oncology to overcome therapeutic resistance.

Spatiotemporal diversification of intrapatient genomic clones and early drug development concepts realize the roadmap of precision cancer medicine

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The unmet clinical needs of high relapse and cancer-related death rates are reflected by the poor understanding of the genome-wide mutational landscape and molecular mechanisms orchestrating therapeutic resistance. Emerging potential solutions to this challenge include the exploration of cancer genome dynamic evolution in time and space. Breakthrough next-generation sequencing (NGS) applications including multiregional NGS for intratumor heterogeneity identification, repeated cell-free DNA/circulating tumor DNA-NGS for detecting circulating genomic subclones and their comparison to reveal intrapatient heterogeneity (IPH) could identify the dynamic emergence of resistant subclones in the neoadjuvant, adjuvant and metastatic setting. Based on genome-phenotype map, and potential promising findings, rigorous evaluation of IPH spatiotemporal evolution and early drug development concepts in innovative clinical trials could dramatically speed up the translational process to achieve clinical precision oncology.

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Introduction

Despite advances in diagnostics and therapeutics including single mutated or amplified gene-based targeted therapy, medicine remains an inexact science [1]. Integration of next-generation sequencing (NGS) technologies [2] and the computational systems biology [3] approach in the ENCODE [4] project have provided evidence on noncoding genome functionality affecting multigene expression profiling, genome and transcriptome architecture, as well as cell- and organ-specific disease-associated coding and noncoding variation [4–6]. These advances enable shifting from empirical to highly complex future precision medicine [1,7]. The new evidence on architecture and dynamics of transcriptional regulatory networks orchestrating crucial biological processes [5,8] could affect oncological outcomes. Designing the future framework of highly effective precision drug therapy, cancer research is at a critical crossroads. Should we continue to develop new drugs [9] based on the simple, linear transcription dogma [10] or is it time to focus progressively on next-generation nonlinear drugs disrupting aberrant transcriptional biocircuits [11]?

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Especially in cancer, the unprecedented power of NGS platforms [12] and evidence on validity of analytical NGS systems to detect tumor genomic heterogeneity that is crucial for the clinic have revolutionized patient-centric research [13,14]. A wide spectrum of NGS applications, including targeted NGS (tNGS), is increasingly used for a panel of known genes to guide therapeutic decision-making [15] and clinical trial designs, such as umbrella and basket studies [16]. Beyond tNGS, clinical implications could also be provided by large-scale whole-exome sequencing (WES) [17] and whole-genome sequencing (WGS) [18] studies through the identification of novel cancer driver genes and druggable mutations. Although tNGS, WES and WGS represent substantial translational research progress toward personalized cancer medicine [15–19], there are considerable constraints to overcome spatiotemporal tumor-evolution-based therapeutic resistance.

Metastasis is the cause of mortality in the vast majority of cancer patients [20] but origin and principles driving spread of cancer cells to distant organs remain poorly understood. Although there has been strong evidence on dynamics of genomic clone evolution [21], it is controversial whether metastasis arises from intratumor heterogeneity (ITH) [22–24] rather than rare subclones [25,26] or polyclonal seeding [27,28] within the primary tumor. Further innovative methods, including serial circulating cell-free DNA (cfDNA) or tumor-free DNA (ctDNA) in plasma followed by NGS (cfDNA/ctDNA-NGS) [29,30], and comparison of genomic alterations (GAs) between ITH and ctDNA-NGS in the same individual patient, can reveal comprehensive intrapatient heterogeneity (IPH) [31,32]. Despite these research endeavors to identify the emergence of subclonal heterogeneity to overcome therapeutic resistance, appropriate translational strategies are still in their infancy.

Based on the principles of the genome–phenotype relationship and genomic clone evolution, this review concentrates on advances and challenges of ITH, serial cfDNA/ctDNA-NGS analyses and comprehensive IPH. Great challenges and potential solutions in translating early drug development strategies, accurate therapeutic response prediction, patient monitoring and possible prevention of metastatic relapse into innovative clinical trial designs are discussed in this review, intending to reach precision oncology. The design of this article is delineated in Fig. 1.

Modern interpatient heterogeneity-based treatment

Clinical models including traditional clinicopathological features and genetic screening guide different therapeutic strategies among patients with the same cancer type. Standardization and analysis of clinical data, histological type, tumor node and metastasis (TNM) staging [33], imaging findings [CT, MRI, endoscopic ultrasound (EUS)] and sequencing of a panel of genes determine the interpatient heterogeneity-based treatment approach. The therapeutic options range from minimally invasive treatment, such as endoscopic mucosal resection (EMR) or submucosal resection (ESR) for early gastrointestinal (GI) tract cancer, to open or laparoscopic/robotic complete tumor resection (R0 resection) [33] or systemic therapy – only for localized or locally advanced non-metastatic disease (M0 stage). Adjuvant or neoadjuvant treatment has been standardized for most tumors, including radiotherapy, chemotherapy and, in a few cancer types, targeted therapy. By contrast, systemic chemotherapy and/or targeted therapy represents the primary therapeutic option for most cancer types in the metastatic setting (M1 stage) (https://www.nccn.org/).

For example, EMR/ESR with excellent quality of life (QoL) has been suggested for small early-mucosal intestinal-type gastric cancer; whereas, in more locally advanced stages (M0), gastrectomy with extended (D2) lymph node dissection, before or after neoadjuvant therapy, is the routine clinical practice. Systemic chemotherapy plus trastuzumab for HER2-positive patients has been recommended in the metastatic setting [34]. Apart from HER2 amplification, the *CDH1* gene also has important clinical implications. Therapeutic total gastrectomy (TG) irrespective of tumor location for patients with inherited *CDH1* mutations or prophylactic TG for healthy individuals with these germline mutations has become a standard for therapeutic and preventive hereditary cancer medicine [35,36].

In the breast cancer adjuvant setting, a clinical model using a combination of traditional clinicopathologic (age, tumor size, node status, histological grade) and molecular genetic (Estrogen Receptor/Progesterone Receptor (ER/PR), human epidermal growth factor receptor 2 (HER2), breast cancer 1 (*BRCA1*), breast cancer 2 (*BRCA2*)) characteristics guide decisions for systemic chemotherapy, anti-estrogens (ER/PR-positive) and trastuzumab (HER2-positive). Further developments in the treatment of metastatic disease include trastuzumab–emtansine conjugate (T-DM1, KADCYLA®, Genetech) [37] for HER2-positive and lately palbociclib (IMBRANCE®, Pfizer) for postmenopausal ER-positive, HER2-negative women [38]. For women with hereditary breast–ovarian cancer syndrome (BRCA1/2 mutation carriers) a specific, guidelines-based algorithm has been suggested in the prophylactic or therapeutic setting (https://www.nccn.org/). Several Phase III randomized control trials (RCTs) are underway including MARIANNE and KATHERINE for T-DM1, and PALOMA-2,-3,-4, PEARL and PENELOPE-B for palbociclib, to expand indications and improve clinical outcomes.

Lung adenocarcinoma is a major health problem with an estimated number of 1.8 million new cases and 1.6 million deaths annually [39]. Recently, the TKIs crizotinib and ceritinib targeting anaplastic lymphoma kinase (ALK)-positive non-small-cell lung cancer (NSCLC) have been approved in the treatment of metastatic NSCLC (http://www.fda.gov/). Another big challenge, besides lung cancer, is the hepatobilliary and pancreatic (HBP) adenocarcinomas. Despite standardization of surgery and adjuvant treatment, relapse rates range between 54% after hepatectomy for hepatocellular carcinoma (HCC) [40] and 81% after pancreatic resection and adjuvant chemotherapy with gemcitabine in pancreatic cancer [41].

Limitations of current therapy personalization

Despite current guidelines-based treatment strategies, progress in understanding tumorigenesis and metastasis is slow [42,43], explaining high recurrence and cancer-related death rates. Screening and early-stage diagnosis of some cancer types such as breast, colorectal and gastric cancer are associated with excellent prognosis after treatment [44], suggesting tumor homogeneity and low metastatic capacity. By contrast, survival rates for other cancer types including HBP and lung cancer are poor even for localized disease. Furthermore, relapse and death rates are alarmingly high for nearly all major cancer types in the advanced and metastatic

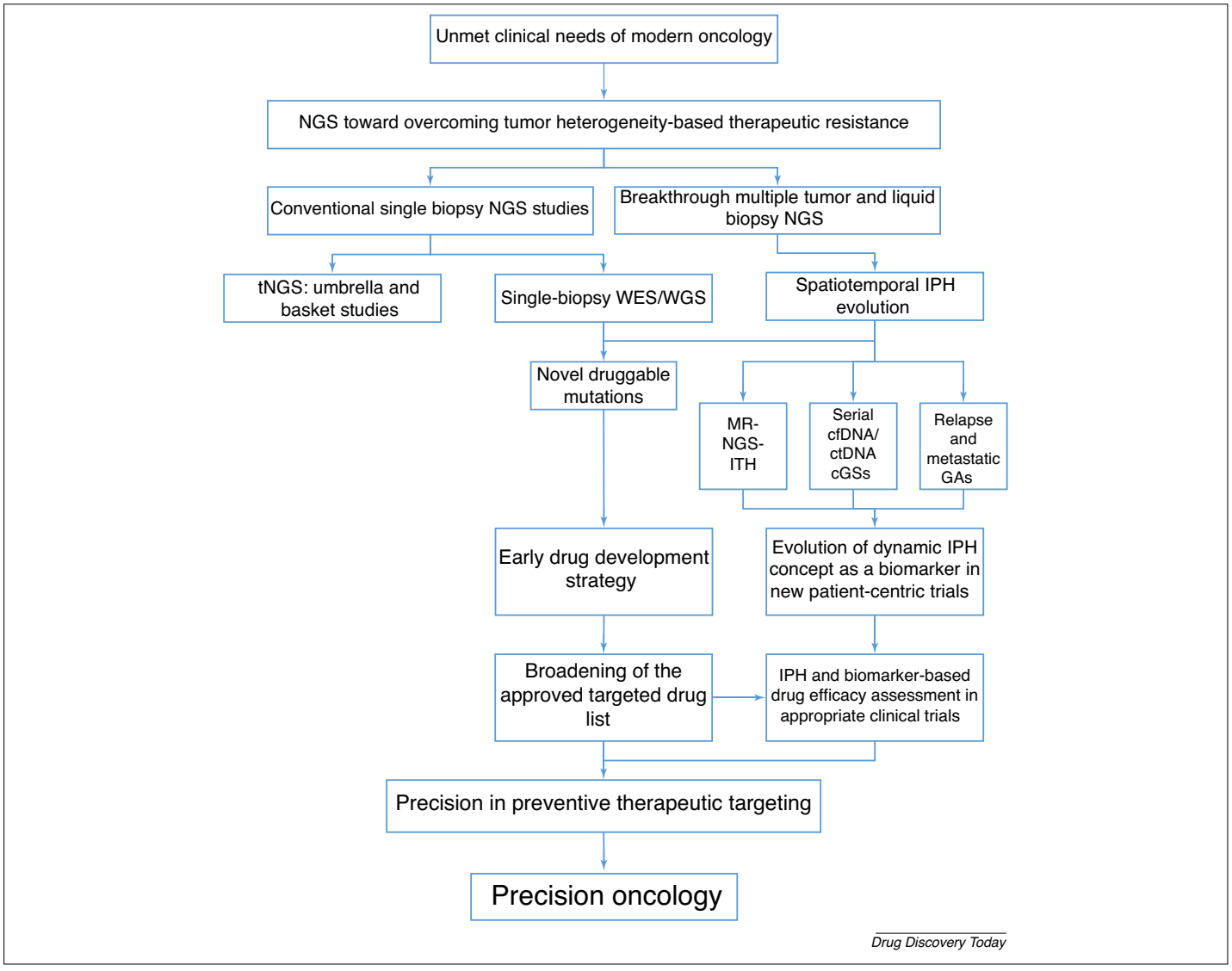

FIGURE 1

Overview and introduction. Abbreviations: cfDNA, cell-free DNA; cGS, circulating genomic clone; ctDNA, circulating tumor DNA; IPH, intrapatient heterogeneity; ITH, intratumor heterogeneity; MR-NGS, multiregional NGS; NGS, next-generation sequencing; tNGS, targeted NGS; WES, whole-exome sequencing; WGS, whole-genome sequencing.

setting, indicating high metastatic ability and therapeutic resistance [44].

Targeted drugs and the challenge of temporary efficacy

Despite recent advances on the rapidly rising number of targeted drugs for personalized therapeutic interventions, careful consideration of all clinical trials resulting in FDA approval [9,11] has revealed major constraints, such as modest and temporary antitumor efficacy of the tumor-guided agents. For example, T-DM1 has prolonged overall survival (OS) by 5.8 months in the EMILIA trial [45], but in the subsequent TH3RESA trial T-DM1 improved only progression-free survival (PFS) by 2.9 months without significant OS benefit [46]. In the adjuvant setting, the HERA trial has demonstrated a significant recurrence risk reduction of 50% with trastuzumab for HER2-positive breast cancer with a short median follow-up of only 2 years, whereas the recurrence rate with a follow-up of 8 years was as high as 23% [47]. In advanced or metastatic HER2-positive gastric cancer, trastuzumab plus chemotherapy significantly prolonged OS, but this benefit was limited to only 2.7 months in the ToGA trial [34]. However, for the FDA-approved crizotinib a PFS benefit of only 3.9 months was observed as compared with chemotherapy, without any OS prolongation [48].

In contrast to the reported PFS and OS benefit with some targeted drugs, multiple Phase III RCTs have provided negative results. For instance, no PFS or OS advantage was demonstrated for cetuximab in the adjuvant setting for wild-type colorectal cancer [49], for linifanib in advanced hepatocellular carcinoma (HCC) [50] and for cetuximab [51] as well as tipifarnib [52] for advanced pancreatic ductal adenocarcinoma (PDA). In summary, targeted drugs represent a progress toward the personalization of therapy, but modest and temporary efficacy, as well as the many negative large-scale Phase III RCTs suggest an urgent need to shift from current empirical medicine to precision oncology based on the

comprehensive GA and molecular landscape underlying *de novo* and acquired therapeutic resistance.

Genome sequencing technologies and clinical implications

Over the past decade, DNA sequencing technologies have progressively revolutionized biomedical research [2,12,53]. Early after the availability in the market of second-generation NGS technologies in 2005, rapid advances in sequencing platforms have led to the development of Illumina Hi-seq 2000, 4000, X five and X Ten, as well as lately to Oxford Nanopore and Qiagen GeneReader. For the first time, NGS technologies coupled with network methods were applied in the ENCODE project [6]. In the post-ENCODE era, scientific thought on the transcription dogma [10] and 'junk' noncoding DNA has radically changed. The modENCODE [54] and ENCODE [4] projects shape a new roadmap for understanding the human genome in health and disease. These technological and innovative methodological advances build the foundation for reaching genomics-based precision personalized medicine [55–57].

Since 2010, there has been an explosion in NGS analysis of patient-derived samples, beginning from small NGS studies [58] to large-scale WES [17] and WGS [18] analyses. Moreover, two international large projects, The Cancer Genome Atlas (TCGA) (http://cancergenome.nih.gov) and the International Cancer Genome Consortium (ICGC) (http://icgc.org), aiming at cancer driver genes' catalogue completion and discovery of novel actionable mutations, have begun, in 2006 and 2010, respectively. Computational algorithms within these two projects have been developed for distinguishing between passengers (neutral) and cancer driver mutations (causative) [59] as well as the functionality of genome-wide sequence variation [60]. However, given that less than 10% of the transcriptome has been understood [4,5], it is too early for computational-based identification of functional variants.

Over the past years, DNA extraction for NGS analysis from fresh-frozen clinical samples had been the standard approach. However, this is costly, time-consuming and gives no possibility for exploiting archived tissues. Lately, technical developments have enabled DNA extraction for tNGS, WES and WGS analyses from formalin-fixed paraffin-embedded (FFPE) tissues [61,62] to overcome these practical difficulties. This progress is changing the biobanking strategy, enhances clinical implications and emphasizes the limitations of the two older large international genome projects [63]. These advances shape crucial translational implications for several NGS applications, including tNGS and conventional NGS for individualized therapies and breakthrough technological genome systems that, through revealing spatiotemporal evolution of genomic clones, lead us to precision personalized treatment.

Targeted next-generation sequencing with umbrella and basket clinical trials

Substantial progress in the integration of tNGS into clinical care and new designs of patient-centric trials has been observed. Targeted NGS analysis is routinely used in public and private laboratories for decision-making on personalized targeted treatment. This approach, using single (or a panel of) genes, enables fast and low cost identification of mutated or amplified genes in individual patients. First-, second- and third-line therapies for the right patient at the right time recommended by the current guidelines include trastuzumab, lapatinib and trastuzumab–emtasine for HER2-positive and palbociclib for HER2-negative/ER-positive metastatic breast cancer. As for colon cancer, cetuximab for wild-type KRAS/NRAS and cetuximab or panitumumab for metastatic tumors with BRAF V600E mutation are recommended (https://www.nccn.org/). By matching the identified mutation with one or more agents from the catalogue of over 70 targeted drugs approved by the FDA (http://www.fda.gov/Drugs/InformationOnDrugs/ApprovedDrugs/ucm279174.htm), we can easily select patients for individualized therapy.

This progress in FFPE-based targeted NGS provides the opportunity for new designs of clinical trials. Umbrella studies can identify several subgroups of patients sharing a mutated or amplified gene for a specific cancer type. These studies provide the potential to either test the efficacy of existing drugs with biomarker-based selection of patients or to develop new agents targeting the identified GAs. For instance such a study for pancreatic cancer has identified subgroups of patients with *KRAS, HER2, BRCA2* and *ATM* genes [64] that could benefit from specific targeted therapy. Exploiting cancer-genome-based classification for diverse cancer types with the same GA [12] and the FDA-approved targeted drugs list, basket studies are being performed to identify patients with the same genetic variation and treat them with the same targeted agent, irrespective of their specific cancer type. Umbrella and basket strategies provide the potential for an early drug development approach [16].

Conventional NGS and discovery of novel druggable mutations

There has been an explosion in single biopsy-based NGS analysis over the past years aiming at the identification and potential completion of the cancer driver gene catalogue. We have recently reported WES and WGS studies and summarized the results into tables [11]. Many small or medium-scale studies have reported enthusiastic data for the identification of new genes involved in cancer and novel therapeutic targets. Potential clinical implications of these studies include the application of mutated genes as biomarkers and druggable GAs.

Constraints

Definitive evidence on extensive genetic and genomic heterogeneity [17,18,65] and spatiotemporal genomic clone evolution [21], as well as ITH [22–24] that requires multiregional biopsies, substantially limit the clinical expectations for overcoming therapeutic resistance with conventional single biopsy-based NGS analyses. Although this conventional NGS strategy can improve initial primary therapeutic response by identifying novel GAs and drugs targeting these genome sequence changes, there is no potential to predict and prevent acquired therapeutic resistance and the subsequent disease relapse.

Based on a recent large-scale WES study proving extensive genetic variation, Lawrence et al. recommend large-scale studies with $P < 0.01$ for valid identification of cancer driver genes. Therefore, it is questionable whether a cost-effective approach is meaningful for a total of 100 000 NGS analyses for 50 cancer types [17] required for valid discovery of actionable mutations. In contrast to the conventional single-biopsy NGS strategy, the concepts of spatiotemporal evolution with multiple solid and liquid biopsy

NGS analysis and early drug development provide much greater opportunities for overcoming primary and secondary therapeutic resistance.

Genome–phenotype map

Based on the fundamental biological process of the genotype–phenotype map [66], identification of occult genomic alterations could predict the phenotypic event of subsequent metastatic relapse several months before it can clinically be diagnosed with modern imaging technologies [67]. However, this roadmap to predict phenotypic events, such as relapse, from genome changes is highly complex owing to the nonlinear genome–phenotype relationship [66,67] and the dynamics of genomic clone evolution following Darwinian principles [21] and regulatory networks [8]. Exploiting this new knowledge on genome evolution in time and space for precise prediction, novel methods and breakthrough technological genome systems have been developed toward ITH, serial circulating genomic subclone (cGS) and comprehensive IPH identification.

Although the valid identification of all types of GAs is now feasible (point mutations, large CNAs, rearrangements), representing the foundation for precise relapse prediction, major challenges remain. Is a single cancer driver gene responsible for drug-resistance-related relapse as recently reported by Murtaza et al. [29] using the repeated ctDNA-NGS method or a combination of primary tumor clones with their interactions [27,28]? How could the dynamic emergence of subclones in response to therapy be revealed? If we could identify multiple and interacting subclones how could we effectively target these networks?

Despite the rational perspectives for cancer-genome-change-based recurrence prediction and early therapeutic intervention to disrupt progress from occult micrometastatic disease to clinical relapse (Fig. 2), multiple challenges represent a bottleneck in the realization of the researchers' and clinicians' dreams to prevent fatal relapse. Potential solutions to overcoming these hurdles include the concept of spatiotemporal tumor evolution and an early drug development strategy.

Dynamics of intrapatient heterogeneity

New methods and breakthrough NGS applications enable us to shift from interpatient to IPH identification. In this article, IPH is referred to the comprehensive set of an individual patient's GAs including ITH of the primary cancer, cGSs, occult micrometastasis and relapse, if it occurs, in the adjuvant setting (M0 stage) or additional genome changes in metastatic tumors (M1 stage) (Fig. 3).

Intratumor heterogeneity

Intratumor heterogeneity is referred to as the presence of genetic and genomic characteristics among different geographical areas within a patient's tumor. The ITH has been recognized as a crucial factor in understanding metastasis and raises new expectations for the prediction and prevention of therapeutic resistance and disease relapse [68].

Two contrary theories based on experimental and clinical models using mathematical approaches have been developed to explain the ability of some primary tumor cells to enter the circulation and colonize at distant organs. Most models and basic research on dynamics of genomic clone evolution and recent multiregional (MR) NGS (MR-NGS) analysis on patient-derived samples provide strong evidence on ITH [22–24]. By contrast, other models and clinical studies using HTS technologies that found genetic similarities between primary and secondary tumor(s) [58,69,70] support the preexistence of a small minority of a cell subpopulation that remains stable over the disease course and is responsible for metastasis [71,72] (Fig. 3). In two clinically relevant models, ClonTracer studies showed that the majority of resistant clones were part of small, preexisting subpopulations that selectively escaped under therapeutic challenge [73]. Irrespective of these two theories and contrary results, crucial for the clinic is

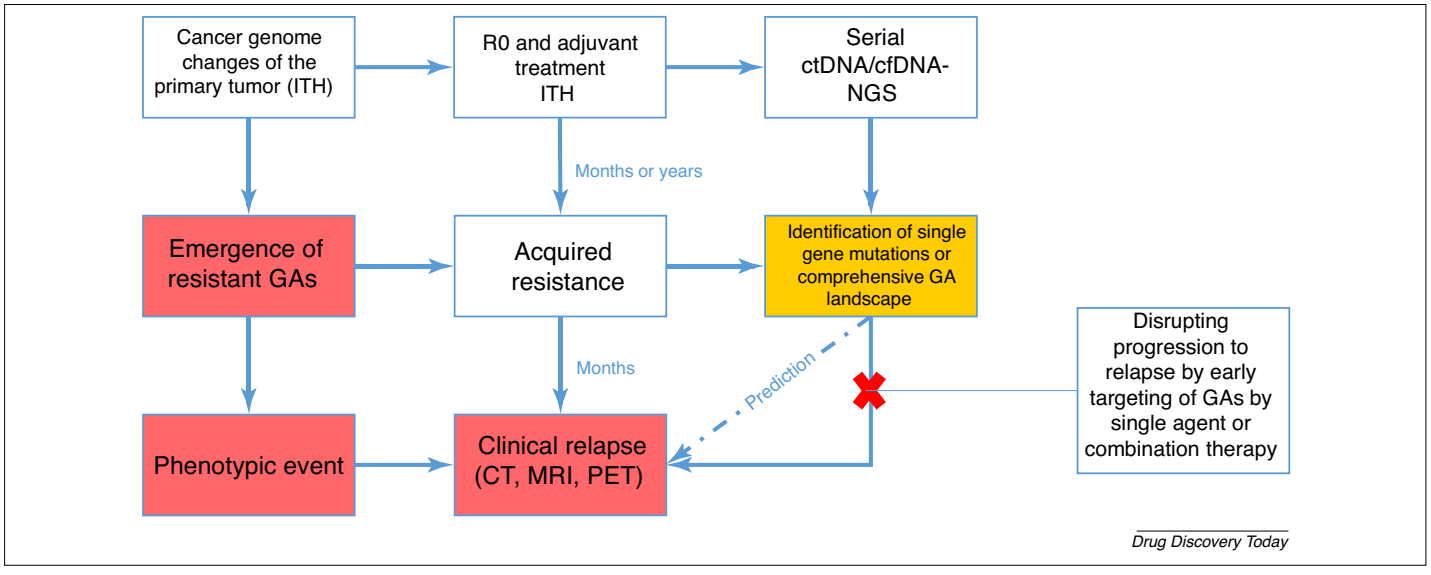

FIGURE 2

The fundamental principle of the genotype–phenotype map guides future precise prediction and, potentially, prevention of relapse-related deaths.
Abbreviations: cfDNA, cell-free DNA; ctDNA, circulating tumor DNA; GA, genomic alteration; ITH, intratumor heterogeneity; NGS, next-generation secuencing.

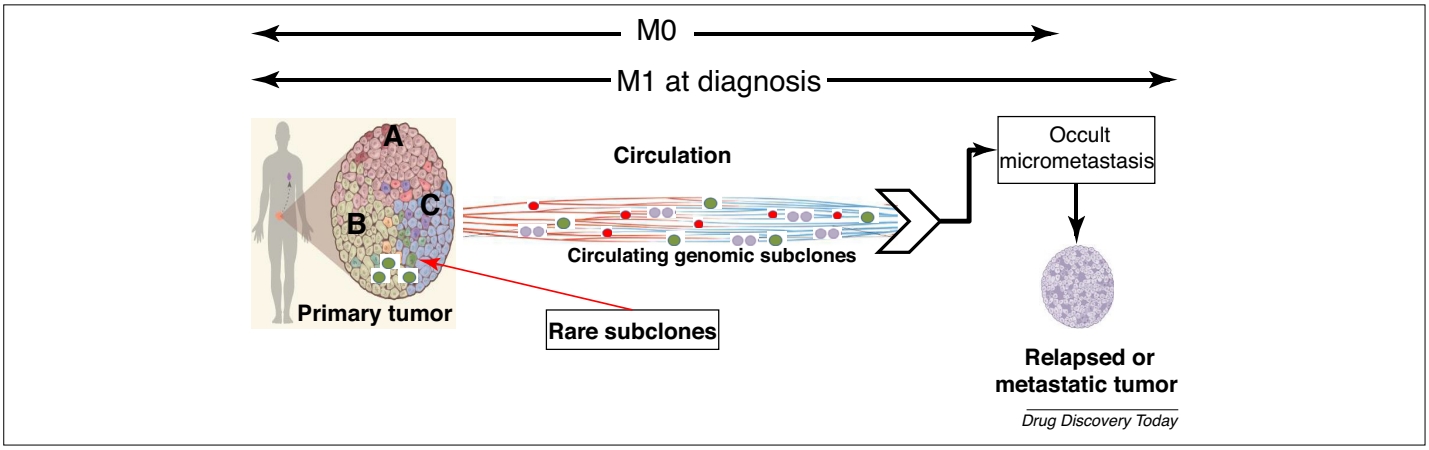

FIGURE 3

Metastasis or relapse arise from circulating cancer cells that are descendants of intratumor heterogeneity (ITH) or rare subclones within the primary cancer. M0, no distant metastasis; M1, distant metastasis.

the elimination of primary tumor cancer cells by surgery as well as cGSs and micrometastases with the initial adjuvant systemic treatment.

The assessment of ITH includes two options. The first simple concept investigates ITH at once and the second more-complex model but with potentially greater clinical implications focuses on the assessment of dynamic emergence of subclones before and in response to therapy (Table 1) [22–24,27,72,74–80]. The simple concept of multiregional biopsy-based NGS analysis can reveal ITH. Indeed, the suggestion that a tumor constitutes a mixture of cell subpopulations with different genetic characteristics merits accurate exploration for translation into patient care improvement [81]. At least eight MR-NGS studies have reported extensive ITH that considerably varies between 8–97% [22,74–80]. This wide intratumor diversity can not only explain high therapeutic resistance rates but also raises the possibility for developing more-effective therapeutic strategies (Table 1). Potential interaction between subclones represents another grand challenge in intratumor exploration and development of more-effective future therapies for targeting not only individual clones but also their interaction networks to reduce therapeutic resistance [82].

Dynamic emergence of ITH affects therapeutic resistance

Much higher clinical expectations are raised by the potential to assess the dynamic emergence of resistant subclones in response to therapy. For instance, Murugaesu et al. [23] have performed a MR-WES analysis in pretreatment endoscopic biopsies and post-neoadjuvant treatment (NAT) surgical specimens from eight patients with esophageal adenocarcinomas. This WES comparison before and after NAT revealed the emergence of resistant subclones following NAT [23]. This finding shapes a new approach for more-effective post-NAT treatment by designing new clinical trials with available or new agents targeting these subclones. In another study on 50 breast cancer patients, MR-WGS analysis was performed in pre-treatment core biopsies and surgical specimens after NAT [24]. Yates et al. [24] have demonstrated that potentially druggable mutations, which were identified in 26% of patients, were subclonal. Detection of subclones in this study was associated with three landmarks of cancer, including resistance to chemotherapy, invasion and metastasis. However, despite these highly promising results of these two small studies evaluating the emergence of resistant subclones in the short meantime before and after NAT, rationally designed clinical trials with larger numbers of patients following a predefined strict protocol are required to confirm the possible clinical utility of this subclonal heterogeneity-based approach.

Comparison of genetic and genomic characteristics between primary and relapsed or metastatic tumors has been previously reviewed [43]. More recently, Gundem et al. [27] analyzed multiple metastases arising from prostate tumors in ten patients by WGS. This study has provided strong evidence for polyclonal seeding, and metastasis-to-metastasis spread among various metastases in prostate cancer [27] (Table 1).

Single-cell genome technique

The ITH can represent not only different characteristics between various geographical areas but also even among individual cells [83]. Long-term research efforts to assess cellular characteristics with potential clinical implications are now beginning to become a pragmatic approach [84]. In the post-genomic medicine era, this single-cell sequencing (SCS) technique enables not only cellular heterogeneity but also noninvasive identification of cGSs [85]. A novel method termed nuc-seq using whole-genome single-cell sequencing was recently reported by Wang et al. [72]. Although this excellent model raises future clinical expectations for comprehensive intratumor diversity assessment, it is still in its research infancy.

Circulating genomic subclones: prognostic and predictive biomarkers

The noninvasive concept of NGS in circulating cfDNA from maternal plasma in prenatal diagnosis [86] has contributed to the substantial progress in cfDNA/ctDNA-NGS-based cancer research. Although principles and molecular mechanisms orchestrating cancer metastasis remain poorly understood, apart from the primary tumor, cells are also released into the circulation from relapsed or metastatic tumors (Fig. 3). Irrespective of the two contrary models, either of dynamically evolved subclones in ITH emergence or preexisting minimal disease clones within the

TABLE 1

Potential clinical utility of NGS systems to identify either simple static or dynamic emergence of intratumor heterogeneity

Cancer type	Number of patients and samples	Technology and methods	Findings	Potential clinical implications	Refs
Intratumor heterogeneity (ITH)					
HCC, ICC	1 pt with synchronous 2 PT HCC and 1 PT ICC and 2 recurrent tumors	MR-WES to all these tumors	Extensive mutational ITH	MR-NGS analysis of all IHT for each individual patient could potentially provide clinical benefits	[74]
HCC	10 pts, 43 lesions and 10 control samples	MR exome sequencing and WGS	• ITH varied between 8% to 97% among patients with HCC • Comparison of primary and intrahepatic metastasis showed substantial heterogeneity but high similarity (90%) with satellite nodules	This wide ITH suggests the need for assessing the clinical utility of multiple lesions and MR-NGS analysis in clinical trials	[75]
HCC	10 pts, 55 samples	MR-WES, CNA	• Extensive mutational and clonal ITH • GAs identified in 4 tumors can be targeted by existing pharmaceutical agents	ITH can affect therapeutic response but larger clinical trials with strict protocols are required	[76]
Prostate	3 pts, 12 samples	MR biopsy-based WES	Substantial genetic ITH	Potential use of MR biopsies-based WES as biomarker	[77]
Clear cell renal carcinomas	4 pts, 30 solid samples	MR biopsy-based WES from PT and MT	ITH in 67% of patients	ITH predictable of PTR, clinical trials required	[78]
Clear cell renal carcinomas	10 pts, 8–20 samples from PT for each pt	MR-WES (79 samples and comparison with 102 TCGA)	□□□ was identified in all cases Subclonal driver aberrations were found in 73–75% of pts	MR-seq can identify heterogeneous genomic landscapes of PT and subclonal evolution giving promising perspectives in overcoming therapeutic resistance	[22]
Non-small-cell lung cancer	7 pts with 25 distinct spatial samples	MR-WES and/or WGS before receiving adjuvant therapy	• Pronounced ITH in CNAs, translocations and mutations • A long period of tumor latency had preceded clinical detection	Further studies are required for overcoming therapeutic resistance	[79]
Lung	11 pts with resectable localized tumors on 48 tumor regions	MR-WES	Larger subclonal mutation fraction was associated with increased relapse risk	The preliminary data of this study suggest the need for further studies to provide evidence on clinical utility of WES-based ITH assessment	[80]
Subclonal evolution in response to therapy					
EAC	8 pts, 40samples	MR WES before and after NAC	Genomic subclonal evolution with high ITH and therapeutic resistance to NAC	MR-WES for ITH and subclonal evolution identification could shape new predictive and therapeutic horizons for EACs	[23]
Breast cancer	50 pts, 303 solid samples	MR biopsy-based WGS and targeted sequencing of the PT	In 13/50 (26%) cancers, potentially targetable mutations were subclonal. Subclonal structural genomic diversification	ITH can predict PTR but it requires clinical trial evaluation	[24]
Prostate	10 pts, 51 samples from PT and different metastatic sites	WGS of PT and MT samples	• Evidence for the existence of polyclonal seeding in human malignancy • The genomic evolution of metastatic prostate cancer arises from PT and occurs through acquisition of metastatic potential following castration resistance • This study supports the 'seed and soil' hypothesis in which rare subclones within the PT develop metastatic potential, rather than metastasis arising from ITH	• Further studies are required to clarify whether metastasis occurs from ITH of PT, rare subclonal evolution from PT, metastasis-to-metastasis spread or a combination of all • Such evidence will open new therapeutic avenues to prevent or control metastasis	[27]

TABLE 1 (*Continued*)

Cancer type	Number of patients and samples	Technology and methods	Findings	Potential clinical implications	Refs
Single-cell DNA technique					
Breast	2 pts	Single-cell genome WGS (nuc-seq)	• This method showed that aneuploid rearrangements occurred early in tumor evolution • Point mutations generate extensive clonal diversity whereas, by contrast, rearrangements remain highly stable	Further single-cell genome WGS studies could improve therapeutic interventions by elucidating on the controversy on metastatic spread and therapeutic resistance arising from rare subclones or ITH within the PT	[72]

Abbreviations: ctDNA, circulating tumor DNA; CAN, copy number alteration; EAC, esophangeal adenocarcinoma; GA, genomic alteration; HCC, hepatocellular carcinoma; ICC, intrahepatic cholangiocarcinoma; IHT, intrahepatic tumors; ITH, intratumor heterogeneity; MR, multi-regional; MT, metastatic tumor; NAC, neoadjuvant chemotherapy; NGS, next-generation sequencing; pt(s), patient(s); PTR, primary therapeutic resistance; PT, primary tumor; TCGA, The Cancer Genome Atlas; WES, whole-exome sequencing; WGS, whole-genome sequencing.

primary tumor, NGS of liquid biopsies for cGS identification provides potentially innovative solutions for the clinic [29,30].

Crucial for the clinic could be the patient-friendly noninvasive cGS identification associated with several advantages. First, cfDNA/ctDNA-based NGS could be used as a biomarker for patient monitoring after treatment, predicting and potentially preventing therapeutic resistance and disease relapse before it clinically occurs [29,30,87–89]. Second, in patients with difficulties in obtaining tissue for histological diagnosis, such as in pancreatic cancer, cGS identification provides an alternative to invasive diagnosis [90]. Third, in the relapse or metastatic setting, cfDNA/ctDNA-NGS could be used to avoid fine-needle aspiration (FNA)-associated

TABLE 2

Studies applying NGS analysis on circulating cell-free or tumor-free DNA

Cancer type	Number of patients and samples	Technology and methods	Findings	Novel discoveries and potential clinical implications	Refs
Liquid biopsies followed by tNGS					
Various types – CRC: 12, ovarian: 9, breast: 7, bladder: 3, lung: 2, etc.	39 pts, 159 plasma samples	tNGS in cfDNA for PI3K-AKT-mTOR pathway or MEK	Clonal response to targeted treatment was identified in tNGS of cfDNA	tNGS of cfDNA could be used for patients' monitoring after targeted treatment	[30]
Metastatic CRC	Prospective study of 53 pts, 159 samples	tNGS for a panel of 15 genes on ctDNA	• ctDNA was found in 98% of pts • In 48/52 patient-specific candidate tissue mutations were detected	Significant changes in ctDNA could predict CT response	[87]
PDA	259 pts	cfDNA from 259 pts tNGS on cfDNA in 48 pts	• Potentially targetable somatic mutations were identified in 14 of 48 patients (29.2%) • Potentially targetable amplifications were detected in CNAs	Somatic mutations and CNAs in targeted sequencing of plasma cfDNA could be used for diagnostic and therapeutic intents	[90]
Liquid biopsies followed by WES					
Breast, ovarian and lung	6 pts with advanced cancer (2 breast, 3 ovarian, 1 lung), 19 liquid biopsies	WES on ctDNA at various time points	• Establishment of ctDNA sequencing as proof of principle • Emergence of mutated genes in response to systemic therapy identified by serial ctDNA sequencing	WES of plasma ctDNA can be used as a biomarker to predict therapeutic resistance	[29]
Liquid biopsies followed by WGS					
CRC	1 patient with resistance to chemotherapy and cetuximab	WGS on ctDNA	KRAS mutations and MET locus rearrangements were detected in ctDNA but not in pre-treatment tumor samples	Emergence of mutations, amplifications and rearrangements was associated with resistance to targeted therapy	[88]
CRC and breast	10 pts (7 CRC, 3 breast cancer) and 10 healthy controls	WGS on ctDNA	Chromosomal CNAs and rearrangements in all patients but not in healthy controls	Potential noninvasive identification of structural genome changes	[91]
Prostate	9 pts (5 pts with castration-resistant and 4 pts with castration-sensitive prostate cancer), 25 controls, 13 plasma samples	WGS on ctDNA	• Multiple CNAs in plasma samples • In an index case, MR-NGS demonstrated ITH in the PT and stable novel chromosomal rearrangements were found in serial ctDNA, 13 years after resection, consistent with one metastatic clone	WGS is feasible in plasma DNA analyses and could potentially predict metastatic relapse	[89]

Abbreviations: cfDNA, cell free DNA; ctDNA, circulating tumor DNA; CRC, colorectal cancer; CAN, copy number alteration; ITH, intratumor heterogeneity; MR, multi-regional; NGS, next-generation sequencing; pts, patients; PDA, pancreatic ductal adenocarcinoma; tNGS, targeted NGS; WES, whole-exome sequencing; WGS, whole-genome sequencing.

TABLE 3

Tumor and liquid biopsy analysis applying NGS

Cancer type	No. of patients and samples	Technology and methods	Findings	Clinical importance	Refs
Breast	1 ER+/HER+ metastatic breast, 8 tumor and 9 plasma	Exome and targeted sequencing	Comparison between tumoral and liquid biopsy sequencing analyses can allow multifocal dynamic heterogeneity identification	These comparisons open new predictive and therapeutic horizons	[93]
Prostate	1 index case out of 9pts	Multiregional WGS analysis of primary tumor and comparison with plasma DNA 13 years after primary tumor resection	Identification of different copy number changes in each primary tumor sector suggesting multifocal disease	ctDNA-WGS revealed chromosomal rearrangements, stable in serial plasma analyses over a 9-month period, consistent with the presence of one metastatic clone	[89]
HCC	4 pts HCC+ 1 breast ovarian	NGS in tumor specimens and shotgun MPS in plasma samples	Comparison of MR-NGS with ctDNA sequencing showed that liquid biopsies can reveal tumoral heterogeneity	Shotgun MPS of liquid biopsies could represent a diagnostic and monitoring tool	[94]
ER+/HER2− metastatic breast cancer	1 pt with 5 biopsies from primary tumor and liver metastasis, 4 plasma samples	MPS and ctDNA	Targeted plasma ctDNA-MPS analysis and comparison with primary and metastatic tumor represents the comprehensive set of genetic alterations at different time-points	Serial ctDNA-MPS could be used for patient monitoring and predictive biomarker	[95]
High-grade serous ovarian cancer	6 pts, 31 tumor samples and 4 pts with 26 plasma samples	Exome sequencing, CNA, targeted amplicon deep sequencing, gene expression profiling and ctDNA-deep sequencing in 4 pts	• Extensive genomic heterogeneity of the PT before treatment • Mutations in the ancestral clone and in subclones could be detected with ctDNA sequencing	Comparison of ITH and subclonal mutations in ctDNA in future studies is required for overcoming therapeutic resistance	[96]
Metastatic melanoma	12 pts	• Pyrosequencing, melting curve analysis or Sanger sequencing in *BRAF*, *cKIT*, *NRAS* and *TERT* • ctDNA plasma levels were detected with BEAMing technologies; in 1 pt PCR and NGS	• Mutations in 4/5 pts in ctDNA were identical to PT • Plasma levels of ctDNA were correlated with clinical and radiologic outcomes	Prospective large studies are required to assess prognostic and predictive value of ctDNA	[97]
Pancreatic, biliary carcinomas	18 pts PDA, 8 pts biliary cancer	Prospective tNGS (54 genes) in cfDNA using NGS	• Comparison of sequencing data in tumor and cfDNA showed high mutational similarity (90.3%) • Diagnostic accuracy of cfDNA sequencing was 97.7%, with 92.3% average sensitivity • Changes in cfDNA correlated well with tumor marker dynamics in serial sampling	cfDNA could be used as a feasible and accurate diagnostic approach to prevent FNA complications, but requires investigation in clinical trials	[98]
HCC	41 pts	Targeted sequencing of 3 genes (*TERT*, *CTNNB1* and *TP53)* using NGS of plasma ctDNA and matched tumor DNA samples	• Mutations in ctDNA were associated with vascular invasion and poor outcome • Comparison of targeted sequencing in the 3 genes showed the same mutation in ctDNA and tumor, but in 1 patient a tumor-associated mutation was found in ctDNA and not in the PT	• Sequencing of plasma ctDNA could be used as a biomarker to predict oncological outcomes • However, clinical trials are required to confirm the clinical utility of this concept	[99]
Ovarian	47 FFPE tumor specimens, 69 plasma samples, 38 different individuals with advanced ovarian cancer	• Tagged-amplicon deep sequencing (TAm-Seq) in 47 FFPE tumor samples • Tam-Seq in plasma DNA of 38 pts	• Sensitivity and specificity of ctDNA-Tam-seq was >97% • TP53 mutations were identified in 67% of samples from 20/38 pts • In 1 pt, Tam-Seq of FFPE from the PT and 3 plasma samples collected serially at the time of relapse, showed the same TP53 (p.R273H), but not the PIK3CA (p.E545K), KRAS (p.G12V) or TP53 (p.R248W) mutations between tumoral and liquid biopsies	ctDNA Tam-Seq could be used for plasma DNA sequencing and patient monitoring	[100]

TABLE 3 (*Continued*)

Cancer type	No. of patients and samples	Technology and methods	Findings	Clinical importance	Refs
Early breast cancer	55 pts and multiple tumor and liquid samples	• Prospective cohort of 55 patients • Targeted sequencing of tumoral biopsies before and after NAC and, if feasible, in the RT as well as serial ctDNA • 55 serial ctDNA and ctDNA-tNGS in 5 patients	Serial targeted sequencing of ctDNA could identify minimal residual disease (MRD) and predict clinical relapse approximately 8 months before it clinically occurs	Larger prospective studies are required to confirm the clinical value of serial ctDNA in comparison with tumor NGS for predicting MRD-based relapse	[31]
Metastatic lung cancer	68 pts (from BioCAST/IFCT-1002 never-smokers cohort)	NGS analysis with targeted sequencing in 68 cases matched for tDNA and cfDNA in plasma prior to treatment	cfDNA plasma concentration was significantly associated with the number of metastatic sites 26 mutations were common in cfDNA and tDNA Sensitivity of the test (cfDNA vs tDNA) 58%, specificity 87%	Targeted sequencing of cfDNA is feasible and could be used as a biomarker for personalized treatment	[32]

Abbreviations: cfDNA, cell-free DNA; ctDNA, circulating tumor DNA; CAN, copy number alteration; IPH, intrapatient heterogeneity; MPS, massively parallel sequencing; MT, metastatic tumor; NGS, next-generation sequencing; pts, patients; PT, primary tumor; tNGS, targeted NGS; tDNA, tumor DNA; WGS, whole-genome sequencing.

complications for identifying subclones in these secondary tumors. Fourth, if the hypothesis of metastasis-to-metastasis spread through circulation is confirmed [27], then comprehensive cGS landscapes might provide an optimal basis for more-effective therapies of metastases. Fifth, this patient-friendly noninvasive liquid-biopsy-based method, if clinically validated, will become essential, because excision or FNA of recurrent or metastatic tumors after R0 resection is not recommended in most cases. These potentially major advantages of this method have resulted in the development of a cfDNA/ctDNA-NGS strategy. Seven non-invasive studies, including three tNGS, one WES and three WGS analyses, using peripheral blood samples for cfDNA/ctDNA detection followed by HTS technologies, have recently been reported (Table 2) [29,30,87–91].

In a recent study, Murtaza et al. [29] performed a serial ctDNA-NGS analysis in 19 plasma samples obtained from six patients with breast, ovarian and lung cancer. Quantification of allele fractions in plasma identified the emergence of mutations associated with acquired therapeutic resistance. In breast cancer, in one patient treated with chemotherapy, an activating mutation in phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*) was found. Following treatment with tamoxifen and trastuzumab and subsequent treatment with lapatinib in the second patient, a truncating mutation in mediator complex subunit 1 (*MED1*) and a splicing mutation in growth-arrest-specific 6 (*GAS6*) were found, respectively. These methods revealed a truncating mutation in retinoblastoma 1 (*RB1*) in one out of three ovarian cancers treated with chemotherapy whereas in lung cancer a resistant mutation in epidermal growth factor receptor (EGFR: T790M) was found following gefitinib treatment. These data establish a proof-of-principle that ctDNA exome sequencing can be used as a biomarker to predict acquired resistance, but additional larger studies are required.

More recently, Frenel et al. [30] reported a study of serial cfDNA-tNGS on 159 plasma samples from 39 patients with various cancer types. According to the authors, clonal response to targeted treatment was identified leading to the conclusion that tNGS of cfDNA could be applied for patient monitoring after therapeutic interventions. In another study of ten patients (seven with colorectal cancer and three with breast cancer) and ten healthy controls, WGS analysis of plasma ctDNA revealed that chromosomal copy number alterations (CNAs) and rearrangements were present only in cancer patients [91].

Apart from patient monitoring, identification of cGSs could be used for diagnostic and therapeutic purposes. The approach of cfDNA-tNGS for assessing mutations and CNAs has been reported by Takai and colleagues in 48 patients with pancreatic ductal adenocarcinoma [90]. Following this concept, the researchers suggest the prognostic, diagnostic and therapeutic clinical relevance of this approach. A study by Sausen et al. [92] was designed as a ctDNA-exome/targeted sequencing analysis of pancreatic cancer. The authors performed a WES study in 24 pancreatic tumors coupled with repeated liquid biopsies from these patients and concluded that ctDNA can be used as a biomarker to predict recurrence.

Despite the promising findings summarized in Table 2, challenges remain in translating cGS identification in the clinic owing to several technical and methodological flaws. The cfDNA/ctDNA-NGS system has not yet been standardized for wide use in clinical trials, and there is a lack of an appropriate protocol comparing cGSs with primary and relapsed or metastatic tumor GAs in these studies.

Comprehensive landscape of tumoral and circulating genomic subclones

Recent landmark studies on intratumor subclonal heterogeneity [24] and circulating tumor-free DNA variation [29] provide exciting perspectives in predicting intrinsic and acquired resistance to cytotoxic and targeted therapy. However, there has been skepticism on whether each one of these two strategies alone will result in substantial clinical success. Indeed, identification of ITH alone, without considering cGSs, faces serious limitations in predicting therapeutic resistance and, therefore, reduces therapeutic tumor responsibility. Similarly, serial cGS identification alone, without detection and combinational therapeutic targeting of ITH, limits cGS capacity for precise prediction of acquired resistance and subsequent relapse. Eleven studies have compared tNGS analysis of plasma cfDNA or ctDNA to matched tumor sequencing data (Table 3) [31,32,89,93–100]. The findings of these cfDNA/ctDNA-tNGS analyses suggest that matched tumoral and liquid biopsies

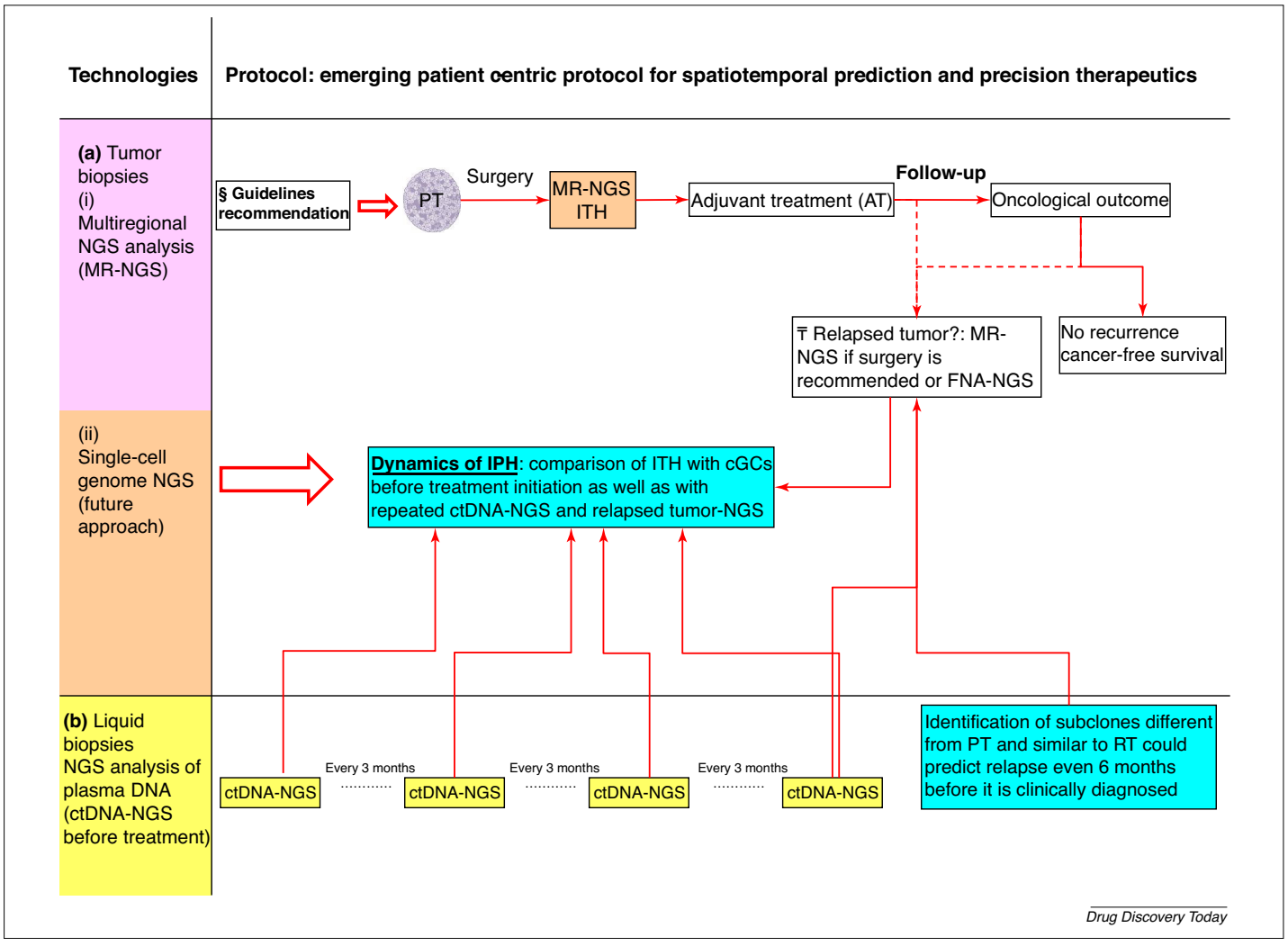

FIGURE 4

Spatiotemporal IPH evolution following systemic treatment in the adjuvant setting. Rigorous evaluation of the dynamic evolution of IPH concept by comparing primary tumor ITH, cGSs and relapsed tumor GAs among patients with (group A-IPH: ITH vs serial cGSs vs relapse GAs) or without (group B-IPH: ITH vs serial cGSs) recurrence in future clinical trials. Patients enrolled in these studies are strictly treated according to current guidelines, based on their clinicopathological and imaging features. Potential clinical validity of MR-NGS and single-cell genome NGS for ITH identification, serial cfDNA/ctDNA-NGS for cGSs and their comparison with GAs from relapsed tumor FNA or surgical specimen could be used as predictive biomarkers to direct therapy. Improving primary, systemic therapy targeting ITH and cGSs can reduce intrinsic resistance. Patient monitoring with serial GA in cGS identification could potentially prevent subsequent relapse by early targeting of GAs. Abbreviations: cfDNA, cell-free DNA; cGS, circulating genomic subclone; ctDNA, circulating tumor DNA; FNA, fine-needle aspiration; IPH, intrapatient heterogeneity; ITH, intratumor heterogeneity; MR-NGS, multiregional NGS; NGS, next-generation secuencing; PT, primary tumor; RT, relapsed tumor.

could be used as a biomarker to predict therapeutic resistance and subsequent recurrence, whereas at the same time early therapeutic targeting of the identified cGSs opens new avenues for improving disease-free survival or even preventing fatal relapse.

Despite overoptimistic reports, available studies combining NGS analysis of tumors and cGSs in individual patients are associated with a series of weaknesses. Small size, high levels of heterogeneity and lack of strictly prospective protocol that includes a complete comparison of MR-NGS-ITH, cGSs and relapsed or metastatic tumor GAs represent unmet translational needs to rigorously evaluate comprehensive IPH. For instance, five out of 11 studies analyzed only six or fewer patients and only one study has conducted MR-NGS-ITH. Yet, there is no published study that comprehensively compares primary cancer ITH with serial cfDNA/ctDNA-NGS and relapsed or metastatic tumor GAs.

Building precision oncology

Theoretically, therapeutic resistance and relapse rates could be reduced by exploring dynamic emergence of IPH. Current intrinsic tumor nonresponsiveness could be improved by comparing pretreatment cGSs to primary tumor ITH. This comprehensive analysis, after R0 resection and before adjuvant systemic treatment, could enable administration of more-effective drug combinations targeting the whole set of GAs in the primary tumor and cGSs potentially responsible for subsequent relapse. Patient monitoring with serial cfDNA/ctDNA-NGS over the disease course potentially reveals acquired resistance and recurrence several months before imaging diagnosis. Early and precise therapeutic intervention of cGS GAs at the stage of occult micrometastasis shapes a new horizon for dramatically improving relapse-free survival. Translational and clinical validity of spatiotemporal IPH evolution and precise therapeutic targeting of dynamically evolved GAs could

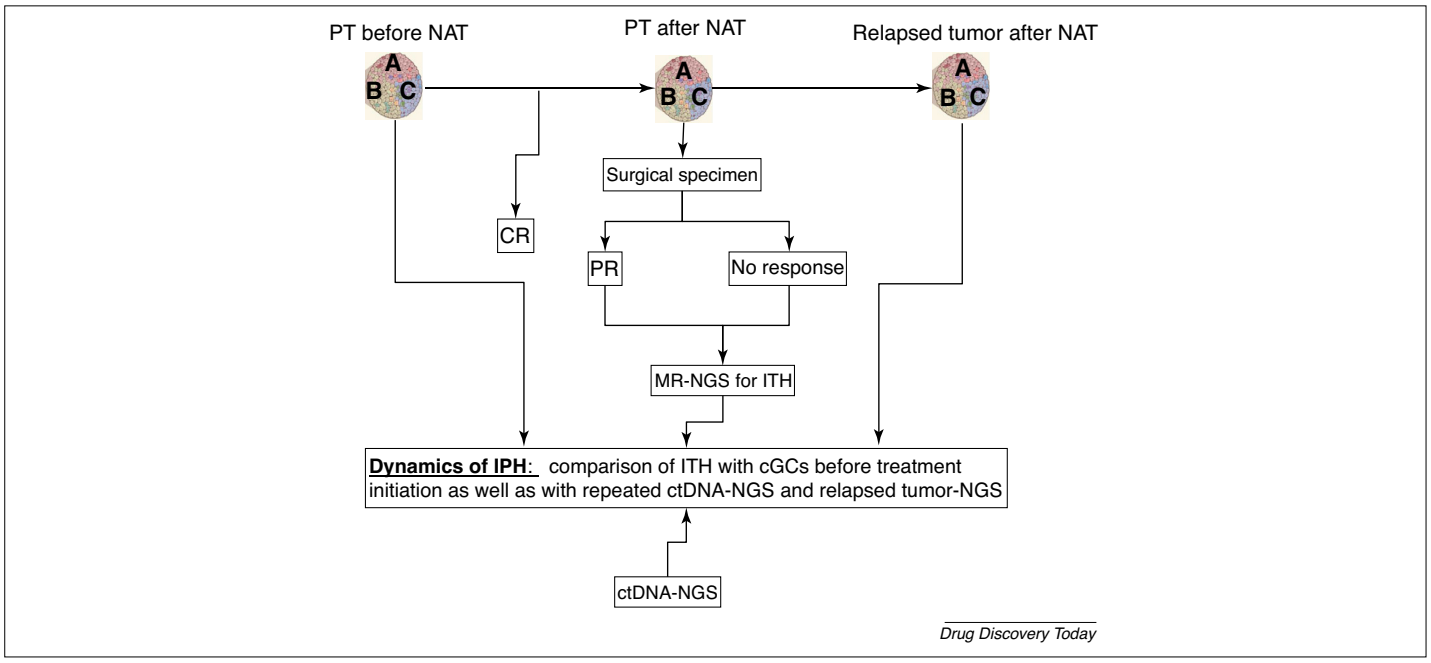

FIGURE 5

Dynamic emergence of IPH in the neoadjuvant setting. Assessment of IPH before and after NAT in innovatively designed patient-centric trials could be used as a predictive biomarker to guide neoadjuvant and potentially post-surgical chemotargeted therapy. A strict protocol based on the recommendations must be applied for NAT, as in locally advanced breast, esophangeal and rectal cancer. If such trials turn out positive they will reinforce precise therapeutic decision-making at four different time points: (i) NAT by comparing pre-surgical biopsy-based ITH to initial cGSs; (ii) post-surgical decisions based on MR-NGS versus cGSs; (iii) patient surveillance with serial cGSs over the disease course; and (iv) comparison of ITH, serial cGSs and relapsed tumor (if occurred) GAs. Abbreviations: cGS, circulating genomic subclone; CR, complete response; IPH, intrapatient heterogeneity; ITH, intratumor heterogeneity; MR-NGS, multiregional NGS; NGS, next-generation sequencing; NAT, neoadjuvant treatment; PR, partial response; PT, primary tumor.

most probably be achieved with innovatively designed future clinical trials.

The challenge of the future: clinical precision oncology

Innovative solutions have been developed for studying cancer genome evolution in time and space in the neoadjuvant, adjuvant and metastatic setting. Although MR-NGS for ITH identification is feasible, further refinements of technological genome systems, such as cfDNA/ctDNA-NGS and single-cell genome NGS, are required for integration into clinical trials. Rigorous evaluation of the dynamic emergence of IPH concept and early drug development strategy can provide not only clinical validity but also speed up the translational process to achieve clinical precision cancer medicine [57].

Validating IPH-based therapeutic response

For assessing the clinical value of ITH, cGS diversity and their comparison with relapsed tumor GAs (IPH) in the precise prediction and overcoming of therapeutic resistance and disease relapse, large-scale, prospective studies with long-term follow-up are required. Fig. 4 delineates a flowchart for such an optimal study protocol in the adjuvant setting that can potentially provide evidence for biomarker-based patient selection, leading to precision in predictive and therapeutic oncology. Possible positive results of these trials could provide the following clinical implications. First, primary decision-making for adjuvant treatment can be improved by targeting the comprehensive GAs identified by the comparison of primary cancer ITH with cfDNA/ctDNA-NGS-based cGSs. Second, repeated cGS identification can improve patient monitoring for predicting acquired therapeutic resistance and relapse before it occurs. By early targeting of these circulating druggable targets we might prolong time to recurrence or even prevent it. However, repeated cGSs at different time points should be compared with ITH and relapsed tumor to validate the predictive and therapeutic utility of this strategy. Further comparison of cGSs and ITH between patients with and without relapse could improve our understanding of acquired resistance-based recurrence.

Figs. 5 and 6 illustrate similar protocols to that given in Fig. 4, for the neoadjuvant and metastatic setting, respectively. The major strength of MR-NGS for ITH identification before and after NAT is that it reveals dynamic emergence of subclones in a relatively short time, allowing appropriate post-surgical adjuvant treatment to reduce recurrence rates. Specific cancer types such as breast, esophageal and rectal cancer are eligible for inclusion in such protocols following guideline-based neoadjuvant treatment (Fig. 5). In the metastatic setting (Fig. 6), comparison of MR-NGS ITH in primary and secondary tumors with cGSs could improve therapeutic decision making by selecting drug combinations targeting the comprehensive landscape of intrapatient GAs. Eligible patients for enrollment in such a protocol are those with primary colorectal cancer with resectable liver metastasis, based on current standard treatment. However, practical problems and challenges require simple and innovative solutions. First, bioinformatics of conventional and breakthrough system applications have not yet reached high-quality validity [13]. Second, technological

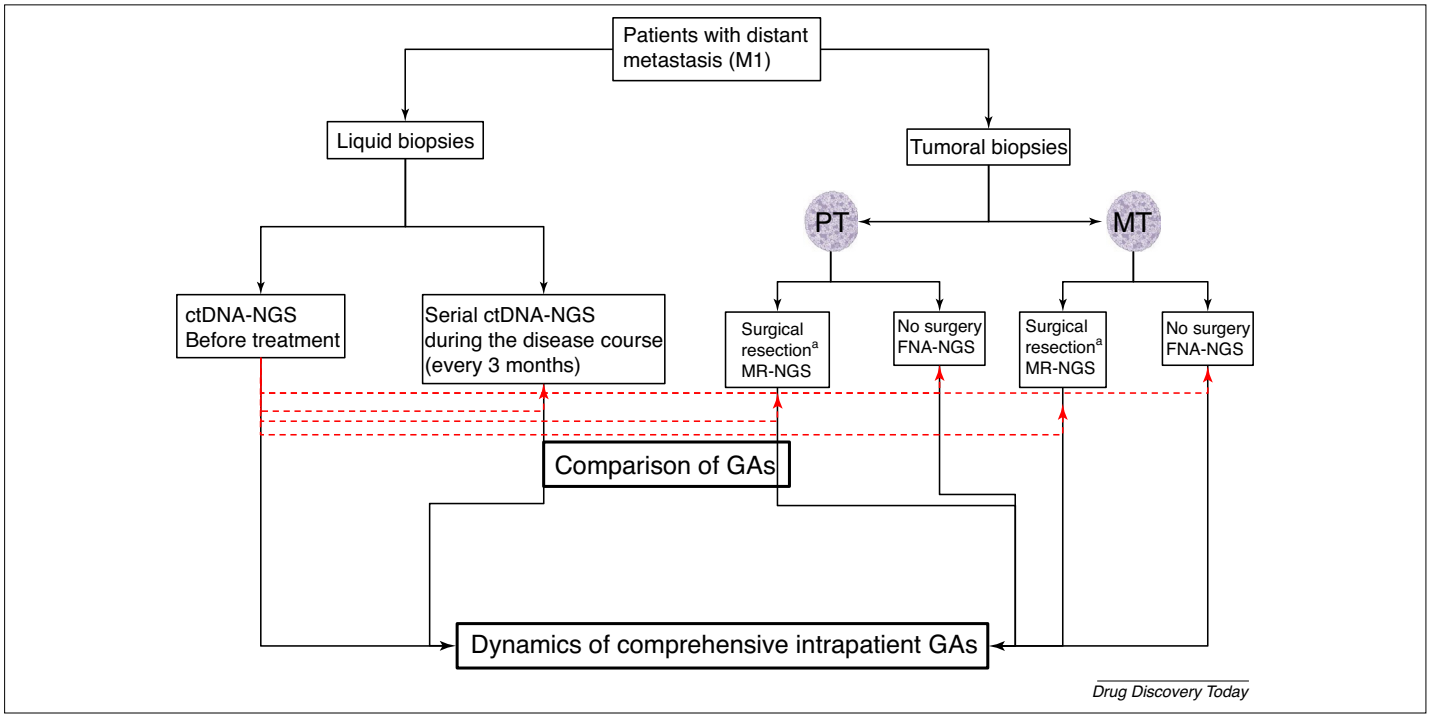

FIGURE 6

Exploration of the IPH strategy within clinical trials for patients with distant metastasis at diagnosis. Fig. 6 illustrates IPH application into clinicogenomic prospective studies that could provide clinical validity of ITH, cGSs and metastatic tumor GAs as a prognostic and predictive biomarker. Genomic analyses are performed on patients with similar clinicopathological features for a specific metastatic cancer type who are treated according to current guidelines. If the results for IPH as a drug efficacy predictor are positive, new avenues will open toward intrinsic resistance risk reduction, precise and early prediction of acquired resistance-based metastatic pregression, as well as the possibility for early therapeutic targeting of GAs in cGSs. Abbreviations: ctDNA, circulating tumor DNA; FNA, fine-needle aspiration; GA, genomic alteration; MT, metastatic tumor; MR-NGS, multiregional NGS; NGS, next-generation sequencing; PT, primary tumor. [a]Guideline-based treatment either for surgical resection of primary tumor and metastatic tumor (i.e., colorectal cancer, liver metastasis) or no indication for surgery.

improvements are required for the establishment of cfDNA/ctDNA-WES/WGS. Both these requirements should be met for the conduction of large-scale patient-centric trials.

Drug discovery and early efficacy prediction

Definitive evidence on extensive interpatient genetic [17] and genomic [18] heterogeneity, as well as increasing support for dynamics of IPH development (Tables 1–3), highlight the need for substantially broadening the list of available targeted drugs approved by the FDA. Dynamics of genome-wide molecular mechanisms enabling cancer cells to escape drug effects remain poorly understood. This explains the multiple negative large-scale Phase III RCTs on targeted agents [49,50,101], the slow progress in the discovery of new approved drugs, the temporary efficacy of nearly all available agents and the isolated success of these therapies in the adjuvant setting, underlining the urgent need to shift from empirical approaches to precise therapeutic targeting. The concept of dynamic emergence of cancer ITH and cGSs enables us to understand high intrinsic and acquired resistance rates. Moreover, integration of IPH identification and conventional single-biopsy WES/WGS into large-scale studies facilitates the discovery of numerous novel valid druggable mutations. Once the catalogue of actionable GAs has grown, the next most clinically important step would be a drug discovery framework that could predict drug efficacy at an early stage of development. This early drug development strategy [16] could be achieved by enrolling patients with similar genomic characteristics and the same druggable mutation into small studies on experimental targeted pharmaceutical agents (Fig. 7) [102].

Oncotargets and clinical precision therapy

If validity of the IPH model integration into clinical trials is confirmed, considering guideline-based traditional clinicopathologic, imaging and treatment data among patients with relapse or metastatic progression, it will pave the way to accurate prediction of genome-based phenotypic events (relapse, metastatic progression, death). This spatiotemporal predictive strategy, coupled with the expected substantial broadening of the approved targeted drug list, will allow administration of drug combinations matched to each individual patient's set of GAs.

Initial decision making on neoadjuvant or adjuvant systemic treatment will be ensured by comparing and targeting ITH and cGSs, aiming to eliminate the disseminated cancer cells. Primary systemic treatment in the metastatic setting, including comprehensive targeting of primary cancer ITH, cGSs, as well as distant secondary tumor GAs, could substantially prolong time to progression and overall survival. Despite primary precision therapy, a patient's subgroup can experience acquired resistance and relapse. The serial cfDNA/ctDNA-NGS predictive strategy for patient monitoring could realize the researchers' dreams of precise prediction

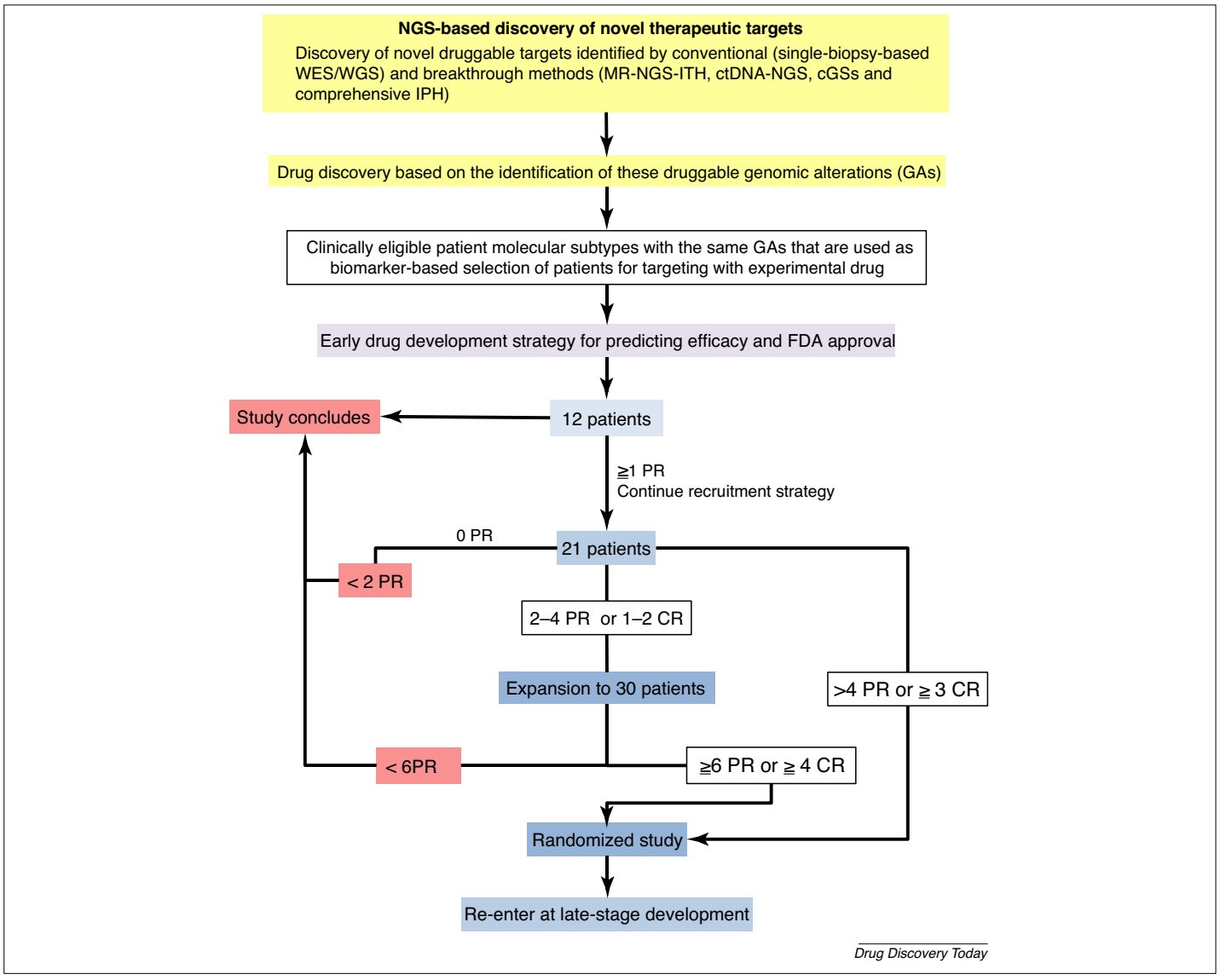

FIGURE 7

Integration of early drug development concept within small clinical trials with patient stratification according to specific oncotarget-directed experimental pharmacological agents. The potential for the discovery of novel oncotargets by conventional and breakthrough NGS applications highlights the necessity for predicting drug efficacy at an early stage of development. Early GA-based stratification of patients within small clinical trials could predict the final stage of development and FDA approval. A stepwise approach with interim analyses and trial population increases is applied. CR and PR are based on the RECIST 1.0 criteria [102]. Abbreviations: cGS, circulating genomic subclone; ctDNA, circulating tumor DNA; CR, complete response; GA, genomic alteration; IPH, intrapatient heterogeneity; ITH, intratumor heterogeneity; MR-NGS, multiregional NGS; NGS, next-generation sequencing; PR, partial response.

and early targeting of GAs in the circulation and micrometastases for disrupting fatal progression to relapse.

Challenges

Despite this expectation to reach precision personalized oncology, three major challenges are emerging. First, a practical problem in the conduction of these prospective studies in the adjuvant, neoadjuvant and metastatic setting is the possibility to obtain tumor samples from relapsed or metastatic tumors, necessary for genomic comparisons between ITH, cGSs and relapsed/metastatic tumors. Indeed, modern guidelines usually, with the exception of colorectal cancer with resectable liver metastasis, do not recommend surgical resection or FNA biopsies for subsequent GA identification. Therefore, such trials will require informed consent of the patient and institutional ethics committee permission.

Second, the undetectability of very-early-stage relapsed tumors after R0 resection and the dynamically evolved GAs at the micrometastasis in response to multidrug treatment against ITH and cGSs limit the expectations for accurate prediction and recurrence prevention. Third, in the post-ENCODE era, establishment of noncoding genome functionality and sequence variations affecting regulatory networks highlights the need to shift from the linear transcription dogma [10] to nonlinear transcriptional networks [4–6,8,103]. Therefore, it is not surprising that all available drugs and future drugs are being developed based on linear transcription are being associated with moderate and temporary

effectiveness [9,104]. By contrast, in a more distant horizon, next-generation drugs based on structural and functional genome and transcriptome changes, as well as on true nonlinear transcription, are expected to have much higher efficacy and durable antitumor activity [11]. However, multiple challenges, including organ-specific transcription factor (TF) identification, tracking sequence-specific TF-binding sites and understanding of transcriptional biocircuits in health will require tremendous long-term basic research efforts. Innovative drugs disrupting deregulated nonlinear transcriptional biocircuits [105,106] will overcome one of the greatest challenges faced by biomedical research in the fields of driver structural and functional genome and transcriptome changes, as well as the comprehensive set of dynamic regulatory networks.

The innovative clustered regularly interspersed short palindromic repeats (CRISPR)–Cas9 system, a powerful genome-editing tool [107,108], allows the generation of precision cancer mouse models, including mutations and rearrangements. In contrast to dynamic evolution of point mutations, large structural genome changes, such as CNAs and chromosomal rearrangements, appear to be stable over the disease progression [71,72]. The long-term dream of researchers to personalize genome editing in patients with large CNAs and rearrangements could be realized in the distant future with Cas-systems-based innovative progress. This strategy creates possibilities in understanding structural and functional genome heterogeneity, shaping the future of precision cancer medicine through genome-editing approaches [109,110].

Concluding remarks

Dynamic diversification of cancer genomes in time and space, before and after chemotargeted therapy, creates a new translational strategic framework. The spatiotemporal IPH evolution concept, including MR-NGS-ITH, serial cfDNA/ctDNA-NGS for cGS identification and their comparison with relapsed/metastatic tumor GAs, as well as the early drug development strategy following novel oncotarget discovery by NGS, hold major promises for developing IPH-based robust biomarkers and broadening the approved targeted drug list. However, refinement of technological genome systems and rigorous evaluation of rationally designed patient-centric clinical trials are required for the clinical validation of IPH and early drug efficacy prediction. Precision in spatiotemporal predictive therapeutic targeting of comprehensive IPH is expected to significantly reduce the alarmingly high rates of intrinsic and acquired resistance, relapse and cancer-related deaths.

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