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Drug resistance: origins, evolution and characterization of genomic clones and the tumor ecosystem to optimize precise individualized therapy

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Progress in understanding and overcoming fatal intrinsic and acquired resistance is slow, with only a few exceptions. Despite advances in modern genome and transcriptome analysis, the controversy of the three different theories on drug resistance and tumor progression, namely dynamic intratumor heterogeneity, pre-existing minor genomic clones and tumor ecosystem, is unresolved. Moreover, evidence on transcriptional heterogeneity suggests the necessity of a drug bank for individualized, precise drug-sensitivity prediction. We propose a cancer type- and stage-specific clinicogenomic and tumor ecosystemic concept toward cancer precision medicine, focusing on early therapeutic resistance and relapse.

Introduction

Despite integration of breakthrough technological systems into basic research to understand complex genome and transcriptome functionality [1–3], intrinsic and acquired therapeutic resistance, relapse and cancer-related death rates remain high [4]. Static and spatiotemporal genome and transcriptome analyses have provided strong evidence on genetic [5], genomic and transcriptional heterogeneity [3,6], suggesting an urgent need to shift from inexact medical science [7] to precise individualized prediction of drug sensitivity [8–13].

The origins and evolution of resistance to available systemic therapies remain unclear. Currently, three prevalent concepts predominate: dynamic diversification of genomic clones with subsequent emergence of intratumor heterogeneity (ITH) [14], pre-existing minor clones (PMC) [15] and interclonal cooperativity within the tumor as an ecosystem [16,17]. Accumulating evidence from multiregional (MR) next-generation sequencing (NGS) and serial circulating cell-free DNA NGS (cfDNA-NGS) studies suggests the need for patient-centric genomic trials to establish the clinical utility of dynamic ITH and circulating genomic subclones (cGSs) as predictive biomarkers guiding individualized targeted therapy [18]. By contrast, further basic and translational research, including single-cell DNA/RNA sequencing, CRISPR-Cas9 and mathematical models, is required to explore the potential clinical implications of PMCs and the tumor ecosystem to address the unmet needs of early and late resistance [15,19]. Based on a comprehensive critical analysis of available data on genome and transcriptome analysis and considering phenotypic events (resistance, relapse, death), this review proposes a breakthrough clinicogenomic concept on the individualized assessment of resistance origins and evolution to guide the optimization of systemic therapy and improve early resistance and relapse.

Clinical evidence on metastasis and relapse

Despite the war against cancer, malignancy remains the second leading cause of death [4]. Metastasis at diagnosis (M1 stage) and tumor relapse are currently not amenable to therapy and are associated with grim survival outcomes for most cancers. Although intensive research efforts have been directed toward dissecting tumorigenesis, metastasis and therapeutic resistance over

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the past 40 years, the exact molecular mechanisms underlying these processes remain poorly understood. Since the mid-1970s two contradictory theories have been proposed: gradual evolution of tumor cell populations [20] and pre-existence of highly metastatic parental tumor cell variants [21].

Advances and standardization in surgery, radiotherapy and systemic therapy, including chemotherapy and targeted treatment, have reduced locoregional and metastatic recurrence rates, improving overall survival [4]. However, oncological outcomes are considerably variable, depending on cancer type and tumor stage. Based on high-quality data from randomized controlled trials (RCTs) on disease-free survival (DFS) and overall survival (OS) rates, we have recently proposed a tumor aggressiveness classification [18]. For instance, HER2-positive breast cancer is considered a low-aggressiveness malignancy, featuring <10% resistance to dual anti-HER2 targeting plus chemotherapy in the adjuvant setting [22,23], whereas the highly aggressive pancreatic ductal adenocarcinoma is associated with <20% 5-year relapse-free survival [24]. These data suggest that, although intrinsic drug resistance has been successfully addressed for cancers with low metastatic potential, it still poses a major challenge for intermediate- and high-aggressiveness tumors. Thus, the limited efficacy of systemic adjuvant or neoadjuvant treatment to control phenotypic events, such as early resistance and relapse, highlights the necessity to precisely identify molecular characteristics governing early therapeutic failure or acquired resistance and late relapse in more- or less-aggressive tumors, respectively. Considering the fundamental principle of the genotype–phenotype map [25], the identification of the comprehensive intraindividual mutational landscape could realize the ultimate goal of improving poor oncological outcomes.

Breakthrough genome sequencing and editing technologies: exploring drug resistance

Taking the unmet clinical needs into consideration, with special regard to highly aggressive cancer types, the clarification of the origins of therapeutic resistance and tumor relapse has gathered immense scientific interest over the past 40 years. In 1976 and shortly after the establishment of the monoclonal nature of cancer, Peter Nowell published a landmark perspective on dynamic tumor evolution and progression, mostly based on cytogenetic research [20]. This theory conceptualizes tumorigenesis as a stepwise variation arising from genetic instability, where subclones are sequentially selected according to the Darwinian principles, gradually producing clonal divergence, potentially responsible for cancer relapse or metastasis. The author states that emerging cellular heterogeneity could include mutations ranging from point mutations to large chromosomal alterations, and makes a groundbreaking for the time suggestion for individualized treatment [20]. By contrast, Fidler and Kripke described an alternative concept the following year through a melanoma-cell-line-derived xenograft-based experiment [21]. The authors showed that metastatic potential is not a product of clonal evolution but rather a property of a minor pre-existing cell subpopulation within the parental primary tumor, concluding that therapeutic efforts should be concentrated against these aggressive subclones instead of the bulk of the tumor, visualizing a premature notion of targeted treatment [21]. These two fundamental concepts of tumor progression have often been revisited since, before and following the advent of NGS technologies [26–28]. However, the validity, speed, continuously lowering cost and widespread availability of NGS platforms have enabled a much more detailed characterization of tumorigenesis, as demonstrated by highly innovative recent studies pairing single-cell genome and transcriptome analyses with genome-editing technologies [3]. The two contrasting theories have been delineated in Fig. 1 in a cancer type- and stage-specific framework.

Dynamic diversification of genomic clones and emergence of intratumor heterogeneity

Over the past few years and following an explosive increase of basic research and patient-derived sample NGS studies, the theory of Darwinian tumor evolution has gathered significant support. Table 1 [6,14,29–43] summarizes valid static and dynamic genomic and transcriptomic analyses demonstrating spatiotemporal clonal evolution over the disease course, with subsequent emergence of intratumor heterogeneity (ITH). Evidence on gradual clonal expansion and late emergence of driver mutations were first extracted from studies employing static multiregional (MR) NGS analysis of multiple distinct regions of the primary tumor at a single time point, through computational phylogenetic reconstruction [29–31]. A small, innovative multiregional whole-exome sequencing (WES) study on clear-cell renal cell carcinoma by Gerlinger *et al.* identified branched evolution and ITH as a universal event and driver mutations as subclonal, acquired during disease progression at a rate of ~75%, correlating this selective adaptation to targeted drug resistance and therapeutic failure [30]. These findings were validated by a recent study on >300 MR samples from 100 early non-small-cell lung cancer (NSCLC) patients by Jamal-Hanjani *et al.* [29]. Genomic instability and parallel evolution resulted in heterogeneous genomic landscapes and late acquisition of driver alterations in similar frequencies, as previously reported [30]. Remarkably, 17 of 100 tumors bore targetable mutations and 12 of these featured clonal and subclonal targets, whereas increased copy-number heterogeneity was an indicator of poor prognosis, marking a patient subgroup that could benefit from intensive monitoring and early, genome-based therapeutic interventions [29]. However, despite widespread availability and technical simplicity, tumoral genome analysis is limited to elucidate on the origins or ITH owing to the relatively small number of samples, because bulk genomic and transcriptional data could overly underestimate the extent and significance of ITH [44].

Attempting to resolve this issue, intensive genome research and technological advances have lately achieved the development of integrated NGS systems with the ability to detect ITH even among individual cells [45], through single-cell DNA/RNA sequencing [6,32–34]. This method promises to enhance our understanding of cancer biology and heterogeneity through sequencing of hundreds or thousands of individual cells to elaborately dissect the intratumoral genomic and transcriptomic landscapes [46]. For instance, Baslan *et al.* performed copy-number analysis of 332 tumor cells from two patients with estrogen receptor (ER)-positive breast cancer and pinpointed dynamic subclonal diversification as the source of ITH [33]. Unsurprisingly, Suzuki *et al.* later similarly demonstrated dynamic tumor evolution at the transcriptomic level [32]. Utilizing single-cell RNA sequencing (RNAseq) on 336

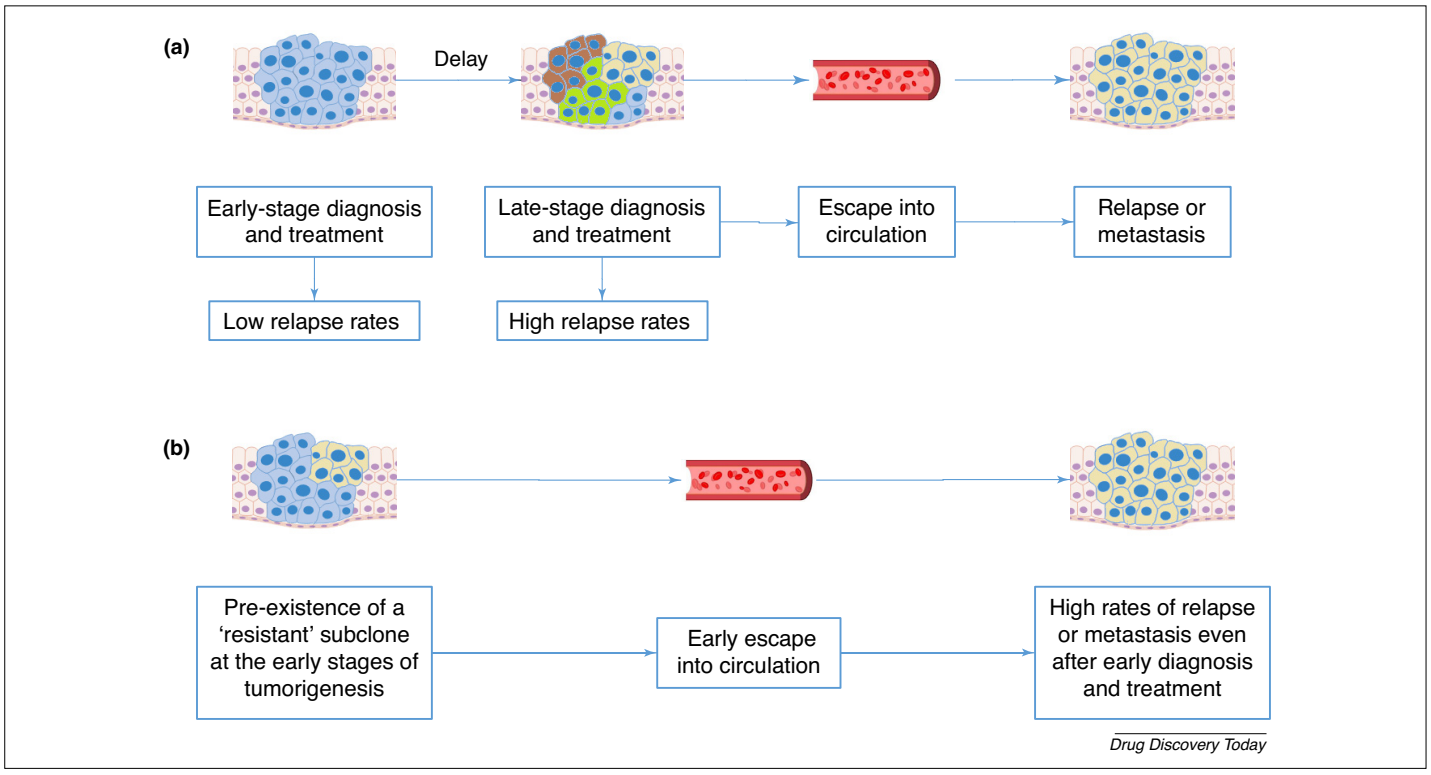

FIG. 1

Two major theories on the origins of metastasis and relapse. **(a)** Dynamic clonal evolution: hypothesis of tumor homogeneity at the early stages and emergence of intratumor heterogeneity as a late event leading to metastasis or relapse after treatment. **(b)** Pre-existing minor clones: a rare resistant cell subpopulation within the primary tumor is responsible for metastasis or relapse after treatment, even at the early stages of cancer.

single-cells derived from seven lung cancer cell lines, the authors detected high levels of transcriptional heterogeneity at single-cell resolution, which they associated with exposure to the targeted drug vandetanib. Most importantly, it is suggested that transcriptional divergence could act as an orchestrator of therapeutic resistance by providing an immense reservoir of gene-expression programs [32]. Moreover, one of the largest single-cell analyses to date by Tirosh and colleagues [6], on >4500 patient-derived melanoma cells, has confirmed genomic and transcriptional heterogeneity between and within tumors. This exceptional study is testament to spatiotemporal clonal evolution and identified clonal diversification, as well as intricate interplay between cancer and nonmalignant cells, as potential sources of therapeutic failure, highlighting the complexity of cancer as an ecosystem [6]. Nevertheless, static genome and transcriptome analysis, including MR-NGS and single-cell NGS, relies on computational reconstruction of phylogenetic trees to dissect the evolutionary history of cancer and, thus, requires validation by spatiotemporal studies on samples acquired over the course of disease and throughout systemic therapy to directly observe subclonal diversification in response to treatment.

Subsequently, studies on samples from distinct time points have emerged and can be distinguished in two major categories. The first includes analyses of single biopsies taken before and after systemic therapy [35,36]. By analyzing pre- and post-neoadjuvant treatment esophageal cancer samples by WES, Findlay *et al*. uncovered profound genomic shifts in response to treatment, including mutations potentially driving therapeutic resistance [35]. Similar findings have also been reported for advanced or metastatic tumors under systemic treatment, such as chemotherapy-refractory urothelial cancer [36]. These data on substantial and dynamic intra-patient heterogeneity suggest the need for serial genomic profiling to accurately predict therapeutic failure, with putative implications for primary and secondary decision making. The second major subgroup comprises studies of spatiotemporal design analyzing MR tumor samples over the cancer lifetime [14,37,38]. An outstanding example is a pioneering study by Yates *et al*. on breast cancer [14]. By applying MR targeted or whole-genome sequencing on specimens before and after neoadjuvant therapy (NAT), this is one of the first analyses to effectively track dynamic clonal evolution. As a response to NAT, 28% of patients featured a new subclone with alterations not present in the baseline samples, including drivers of drug resistance. This underlines the capacity of spatiotemporal cancer genome analysis for future therapeutic implications [14]. Other studies have further supported dynamic tumor diversification for different cancer types, including esophageal [37] and colorectal cancer [38], correlating clonal evolution and subsequent ITH with poor response to treatment and metastasis.

Furthermore, apart from tumor sample analysis, NGS of plasma cfDNA or circulating tumor DNA (ctDNA) has recently gathered much attention, particularly owing to the compelling idea of a noninvasive, patient-friendly platform enabling easy and effective patient monitoring. Although static cfDNA-NGS analysis has already provided data on selective adaptation and acquired resistance to epidermal growth factor receptor (EGFR) blockade [39],

TABLE 1

Studies supporting dynamic diversification of genomic clones leading to intratumor heterogeneity

Cancer type	Number of patients	Sample type	Technology	Findings	Translational and potential clinical implications	Refs
Static multiregional NGS analysis of the primary tumor to identify intratumor heterogeneity (ITH)						
Early-stage NSCLC	100	327 multiregional samples	WES	• Heterogeneous driver alterations were identified as late evolutionary events in >75% of tumors • Genome doubling and chromosomal instability were associated with ITH and resulted in parallel evolution of driver somatic CNAs	Chromosome instability was identified as a prognostic marker associated with increased risk of recurrence or death	[29]
ccRCC	10	79 multiregional samples	WES	All tumors exhibited branched evolution and ITH, whereas 73–75% of driver mutations were subclonal, acquired during tumor progression	MR-NGS could identify subclonal tumor evolution with potential therapeutic implications	[30]
HCC	23	49 multiregional samples	WGS and RNAseq	Mutational divergence after treatment with sorafenib, TACE or RFA and tumor-in-tumor nodules indicate the existence of ITH due to clonal selection	WGS could improve therapeutic precision	[31]
Single-cell genome and transcriptome sequencing						
Melanoma	19	4645 single cells	RNAseq	• Intra- and inter-individual, spatial, functional and genomic heterogeneity were identified • Spatiotemporal evolution of single cells and their complex interplay could be responsible for drug resistance • A subpopulation resistant to targeted treatment was detected in all tumors	These findings could provide a novel tool for future translational applications	[6]
Lung	7 cell lines	336 single cells	RNAseq	• High levels of transcriptional heterogeneity were identified between individual cells • Targeted treatment resulted in dynamic transcriptional heterogeneity • Divergence in transcriptome regulation could be a driver of therapeutic resistance	Further single-cell transcriptome analyses could enable translational implications	[32]

TABLE 1 (*Continued*)

Cancer type	Number of patients	Sample type	Technology	Findings	Translational and potential clinical implications	Refs
ER + breast	2	332 single cells	CNA	Subclonal heterogeneity supports dynamic diversification over the disease course	Further large-scale basic research is required to understand heterogeneity between individual cells for future translational implications	[33]
1 ER + and 1 TNBC	2	179 single cells	CNA and SNV	Aneuploid rearrangements occurred early in tumor evolution and remained highly stable, whereas point mutations evolved gradually, producing extensive subclonality	Dynamic evolution of point mutations suggests the potential for robust biomarker and targeted drug development	[34]
Temporal, single-biopsy NGS studies						
Esophageal	30	Pre- and post-NAT PT samples	WES	Major changes in driver mutation presence or frequency were observed after neoadjuvant chemotherapy	Further studies on ITH before and after NAT are required to extract potential clinical implications	[35]
Urothelial	16	PT and progression after chemotherapy	WES	Chemotherapy-treated advanced urothelial carcinoma features extensive and dynamic clonal evolution, potentially associated with resistance	New studies on serial metastatic biopsies are needed to clarify potential clinical benefits	[36]
Spatiotemporal NGS analyses						
Breast	18 before and after NAT (50 total)	Pre- and post-NAT MR-NGS of the PT	MR-tNGS, MR-WGS	• In 5/18 pts, a subclone was identified in the post-NAT samples only, indicating spatiotemporal clonal evolution • Amplifications of *CDK6*, *FGFR2* and *MYC* and a deletion within *RUNX1* were identified as drivers of resistance	The finding that 28% of pts featured new subclones after NAT suggests the need for MR-NGS before and after NAT with potential diagnostic and therapeutic implications	[14]
Esophageal	8	40 pre- and post-NAT samples	MR-WES	• High mutational heterogeneity was identified before and after platinum NAT • High levels of ITH were associated with poor response to NAT	Dynamic ITH could predict therapeutic resistance, large-scale studies are required	[37]
Colorectal	5	35 MR-samples of PT and liver MT	MR-WES and CNA	• High levels of ITH were identified in the PTs and the MTs • Clonal evolution of the PT was responsible for metastasis	Dynamic clonal evolution could be a predictive biomarkers, following validation	[38]

TABLE 1 (*Continued*)

Cancer type	Number of patients	Sample type	Technology	Findings	Translational and potential clinical implications	Refs
Single liquid biopsies with cell-free DNA NGS						
Advanced colorectal	1397	Static cfDNA samples	tNGS	Following anti-*EGFR* targeted treatment, emerging *EGFR* mutations were identified in the setting of acquired resistance	Despite large size, patient-centric trials are required to confirm dynamic clonal evolution as a cause of emerging resistance	[39]
Serial detection of circulating genomic subclones (cGSs)						
Metastatic solid tumors	39	159 longitudinal samples during and after targeted treatment	tNGS	• 13/23 pts with at least one mutation in cfDNA at trial initiation received a matched targeted drug	• Serial cfDNA-tNGS could be used as a prognostic and predictive biomarker	[40]
				• Monitoring of mutation allele frequency in serial plasma samples demonstrated potential clonal responses to targeted therapy, associated with time to progression	• Larger studies combining serial cGS identification with MR-NGS of PTs and MTs are required for confirmation	
Advanced prostate	20	40 samples before and after treatment	tNGS and CNA	Dynamic clonal evolution in response to therapy was identified	Validation of dynamic clonal evolution in response to therapy could provide major therapeutic implications	[41]
Metastatic cancers	6	19 samples at various time points	WES	Dynamic emergence of acquired therapeutic resistance was observed	This study provides proof-of-principle for serial cfDNA-NGS as a predictive biomarker, requiring validation	[42]
Spatiotemporal analysis combining ITH and cGS detection						
Early NSCLC	100	MR-WES (327 samples), pre-op ctDNA, serial post-op ctDNA in 24 and RT WES in 4	WES	Profiling of plasma ctDNA identified patients at high risk of recurrence, through the detection of emerging subclones	Serial ctDNA-based liquid biopsies could enable prediction and early targeting of emerging subclones responsible for relapse	[43]

Abbreviations: cfDNA, cell-free DNA; cGS, circulating genomic clone; ctDNA, circulating tumor DNA; ccRCC, clear-cell renal cell carcinoma; CNA, copy-number alteration; HCC, hepatocellular carcinoma; ITH, intratumor heterogeneity; MT, metastatic tumor; MR, multiregional; NAT, neoadjuvant treatment; NGS, next-generation sequencing; NSCLC, non-small-cell lung cancer; PT, primary tumor; RFA, radiofrequency ablation; RNAseq, RNA sequencing; SNV, single nucleotide variant; tNGS, targeted NGS; TACE, transarterial chemoembolization; TNBC, triple-negative breast cancer; WES, whole-exome sequencing; WGS, whole-genome sequencing.

serial liquid biopsies are conceptually more appropriate to directly detect and elucidate the principles driving tumor evolution [40–42]. Following a breakthrough study by Murtaza *et al*. that yielded proof-of-concept for the predictive capacity of cfDNA-NGS in the identification of emerging resistant circulating subclones [42], other studies have reproduced these findings on different cancer types [40,41]. Serial monitoring by cfDNA-NGS throughout the course of cancer and over systemic therapy, including targeted drugs, found dynamic clonal diversification in response to therapy correlating to oncological events, such as secondary drug resistance and tumor progression [40,41]. However, these studies are limited by small sample size and lack of comparative analysis to matched tumor tissue.

Thus, a novel concept of patient-centric genomic trials first described by Biankin *et al*. in 2015 [47] aims to address these unmet needs by combining full clinicopathologic documentation and genome analysis in time and space [18]. In this context, Abbosh and colleagues recently published an early report on the first 100 patients from the highly promising TRACERx registered clinical trial (NCT03004755) [43]. This study couples WES of primary and relapsed NSCLC with serial ctDNA-WES before and after treatment. The authors report that this exceptional design could potentially readily identify high-risk patients by detecting emerging subclones responsible for relapse. Regardless of whether ctDNA profiling could complement or even replace CT-based follow-up, it could provide major therapeutic implications by enabling early targeting of aggressive subclones [43]. Therefore, the final results of the trials are eagerly anticipated, and further clinicogenomic trials are warranted to elaborate on the mechanisms underlying tumorigenesis and the hallmarks of cancer.

Pre-existence of resistant rare subclones

Nevertheless, and despite convincing evidence, the theory of gradual tumor evolution fails to explain the high relapse rates following resection and systemic therapy of aggressive cancer types, even at early stages, implying the early emergence of a minor, potentially nondetectable resistant cell subpopulation within the primary tumor orchestrating therapeutic failure. Table 2 [3,15,19,34,44,48–57] summarizes studies and findings supporting the second prominent theory of pre-existence, as well as other hybrid models of evolution coupling the two contradictory concepts. For instance, two static single-cell copy-number analyses on patient-derived breast cancer single cells propose a model of punctuated clonal evolution [48,49]. According to this model, genomic aberrations occur in short bursts of time, resulting in early ITH and expansion of a few dominant clones, preprogrammed to become invasive, metastatic or resistant at the earliest stages of tumor growth [58]. Accordingly, single-cell analysis highlighted copy-number alterations (CNAs) as early evolutionary events residing in extremely metastable minor nonclonal cell subpopulations, which remain highly stable in time and potentially drive progression and drug resistance [48]. Moreover, an intriguing single-cell RNA sequencing study highlighted the existence of very rare pre-resistant melanoma cells and proposed a potential future role for intratumor transcriptional heterogeneity as a predictive biomarker for resistance [50].

Multiple small genome analyses have returned data on pre-existing aggressive subclones as well. Schwarz *et al*. suggest that relapse of high-grade serous ovarian cancer (HGSOC) is caused by a prevalent pre-existing clone [51]. This clone represents a cellular minority of the primary tumor and is characterized by the early acquisition of resistant CNAs, as previously described, as well as by selective expansion during chemotherapy [51]. Castellarin *et al*. further supported this assumption by identifying relapsed mutations in matched HGSOC primary tumors before treatment [54]. Analysis of other cancer types, including breast, colorectal and prostate, has additionally produced similar findings. Apart from CNAs, chromosomal rearrangements have demonstrated substantial stability over the course of breast cancer owing to high levels of similarity between primary and corresponding metastatic lesions [52], whereas *MET* amplifications were identified as a novel mechanism of colorectal cancer resistance to anti-EGFR treatment, offering a selective advantage to a minor pre-existing subclone to expand under targeted therapy [53]. Moreover, Gundem *et al*. detected minor subclones with metastatic potential within the primary prostate cancer, supporting the 'seed and soil' hypothesis for cancer spread [55], first described by Stephen Paget in 1889 and often revisited since [59]. Interestingly, copy-number stability was once again observed in an NGS analysis of ctDNA-based liquid biopsies before and after systemic targeted and endocrine therapy with palbociclib–fulvestrant of advanced breast cancer [19]. On this basis, the authors proposed an innovative concept for resistance, where early and late cancer progression are caused by pre-existing and temporally evolving clones, respectively, coupling each prevalent theory with a distinct mechanism of therapeutic failure. However, the ability of ctDNA-NGS to identify rare subclones in peripheral blood samples is questioned [19].

Thus, in light of contrasting findings regarding tumor evolution, highly pioneering studies combining genome and transcriptome sequencing with genome editing systems have focused on the delineation of the mechanisms underlying drug resistance at the basic research level, mostly utilizing cancer cell lines [3,15,44,56,57]. Despite some studies supporting the pessimistic theory of pre-existence [44,57], most large-scale analyses support the coexistence of multiple distinct mechanisms of resistance to systemic therapy [3,15,56]. An exceptional, breakthrough study by Ben-David *et al*. represents one of the largest, cell-line-based single-cell DNA and RNA sequencing analyses, with the additional integration of CRISPR-Cas9 genome editing [3]. Through profiling >26 000 cell-line-derived single cells from multiple cancer types, the researchers concluded that genetic and transcriptional heterogeneity arises as a result of positive selection of pre-existing subclones, as well as *de novo*, reflecting highly variable drug responses at the single-cell level [3]. Hata *et al*. further supported the co-occurrence of both models on NSCLC cell lines and mouse xenografts, proposing distinct mechanisms for the early and late emergence of *EGFR* resistance, namely pre-existing or temporally evolving $EGFR^{T790M}$ mutant clones, respectively [15], reinforcing the findings of O'Leary *et al*. [19]. These findings underline three major standpoints. First, patient-centric studies, beyond basic research, are essential to extract translational or clinical implications [3]. Second, technological refinements and substantial improvement of accuracy are required to detect minor pre-existing subclones, because these cells can represent as little as 0.05% of the starting clonal population [44]. Third, NGS and genome-editing tools could be used in conjunction to effectively identify and

TABLE 2

Available data on the origins and evolution of tumorigenesis and drug resistance with emphasis of pre-existing minor clones

Cancer type	Number of patients	Sample type	Technology	Findings	Potential translational implications	Refs
Single-cell genome and transcriptome analysis						
TNBC	12	1000 single-cells	CNA analysis	• Minor subpopulations of nonclonal cells with high metastatic capacity were identified • CNAs were early evolutionary events that remain stable in time, potentially driving cancer progression and chemotherapy resistance • This study supports punctuated clonal evolution in short evolutionary bursts, as opposed to gradual tumor evolution	Punctuated copy number evolution could provide translational implications for TNBC	[48]
Breast and LM	2	200 single-cells	CNA analysis	• Punctuated clonal evolution was identified with late acquisition of metastatic potential • Three clonal cell subpopulations were detected	Further and larger single-cell DNA/RNA sequencing studies are required	[49]
1 ER + and 1 TNBC	2	179 single-cells	CNA and SNV analysis	Aneuploid rearrangements occurred early in tumor evolution and remained highly stable, whereas point mutations evolved gradually, producing extensive subclonality	This study supports mutation-specific origins of resistant clones	[34]
Melanoma	2	115 single-cells over targeted therapy	RNAseq	Very rare pre-resistant subpopulations of cells and extensive transcriptional heterogeneity were identified	Intratumor transcriptional heterogeneity could be used as a predictor of resistance	[50]
Spatial and temporal genome analysis						
High-grade serous ovarian	14	135 distinct tumor samples in time and space	MR-WGS for CNAs	• Data from copy-number analysis of relapse samples in 2/14 support the early evolutionary emergence of the prevalent recurrent clone • High clonal tumor heterogeneity was associated with worse overall and progression-free survival	Clinical relapse could arise from pre-existing tumor subclones, expanding during chemotherapy but validation is required	[51]
Breast	11	Matched PT and MT samples	WGS	Concordance for chromosomal rearrangements between matched PT and MT was 89% (61–100%), suggesting stability over the disease course	Further studies are required to confirm the pre-existence and stability of chromosomal rearrangements within the PT	[52]
Metastatic CRC	7	14 diverse tumor and 6 ctDNA samples over anti-EGFR therapy	dPCR, WES, WGS	• *EGFR*-targeting expanded a pre-existing resistant minor cell subpopulation with *MET* amplification • *MET* amplifications were identified as a novel mechanism of primary and secondary resistance	Potential correlation of pre-existing resistant clones to *MET* amplification encourage further evaluation of *MET* inhibition	[53]

TABLE 2 (*Continued*)

Cancer type	Number of patients	Sample type	Technology	Findings	Potential translational implications	Refs
HGSOC	3	9 ascitic tumor cell samples during standard treatment and relapse	WES	89% of relapse mutations were present in matched PTs before treatment, indicating that recurrent HGSOC arises from pre-existing tumor clones, selected during therapy	Further large-scale translational studies are required to clarify the potential of future optimized early therapeutic targeting to delay or prevent relapse	[54]
Prostate	10	51 PT and matched MT samples	MR-WGS	Phylogenetic reconstruction revealed that minor subclones within the primary tumor develop metastatic potential, supporting the 'seed and soil' hypothesis	Further translational research is required	[55]
Circulating tumor DNA analysis						
Advanced HR (+)/HER2(-) breast cancer	195	ctDNA before and after treatment	tNGS, WES	• Early and late progression were associated with pre-existing and temporally evolving clones, respectively, suggesting distinct mechanisms of resistance • Copy number profiles remain highly stable through treatment • ctDNA-NGS would be unlikely to detect many rare subclonal mutations	Further tumor stage-specific studies and technological refinements of cfDNA-NGS are required	[19]
Basic research supporting pre-existence alone or coupled with multiclonal evolution						
Multiple cancer types	106 cell lines	Cell lines and 26 465 individual cells	WGS, tNGS, single-cell RNAseq, CRISPR-Cas9	Genetic and transcriptional heterogeneity arises from pre-existing subclones and *de novo*, reflecting differential drug sensitivity	• Clinical models and translational research is required • Transcriptional heterogeneity suggests the need for extensive drug development	[3]
NSCLC	Multiple pre-established and patient-derived cell lines	Cell-lines and mouse xenografts	ddPCR, tNGS, ClonTracer, RNAseq	Early-resistant clones derive from pre-existing $EGFR^{T790M}$-containing cells, whereas late-emerging $EGFR^{T790M}$ clones derive from drug-tolerant cells, supporting both prevalent theories on cancer evolution	Novel therapeutic strategies targeting drug-tolerant cells could delay or even prevent the evolution of acquired resistance	[15]
NSCLC	HCC827 cell lines	Cell lines	tNGS, ClonTracer, RNAseq, qPCR	• Approximately 0.05% of the starting clonal population contributed to erlotinib resistance, whereas the vast majority of resistant clones were pre-existing and selected during treatment • The majority of pre-existing erlotinib-resistant clones can be eradicated by crizotinib treatment	• ClonTracer enables the detection of very rare pre-existing cancer-cell subclones (1 in 1 million), potentially overlooked by modern genomic analyses • These findings require validation by large single-cell RNAseq studies	[44]
CRC	DiFi and Lim1215 cell lines	Cell lines	Genotyping, tNGS, WES, BEAMing	Cetuximab resistance could derive from selection of a pre-existing *KRAS* amplified or mutant clone or as the result of *de novo* acquisition of a *KRAS* mutation	MEK inhibition could delay or reverse cetuximab resistance, but validation is required	[56]

TABLE 2 (*Continued*)

Cancer type	Number of patients	Sample type	Technology	Findings	Potential translational implications	Refs
NSCLC, CRC, breast	Various cancer models	Cell lines	CRISPR/Cas9, qPCR, tNGS	• Receptor inhibition could result in the selection of pre-existing resistant clonal subpopulations	• Combination therapy could prevent or delay resistance	[57]
				• Multiple resistant clones with distinct mechanisms of resistance can be present before therapy	• CRISPR-Cas9, coupled with genome analysis, could be used to develop and validate new therapeutic protocols	

Abbreviations: ctDNA, circulating tumor DNA; CRC, colorectal cancer; CNA, copy-number alteration; dPCR, digital PCR; HGSOC, high-grade serous ovarian cancer; LM, lymph node metastasis; MT, metastatic tumor; MR, multiregional; NGS, next-generation sequencing; NSCLC, non-small-cell lung cancer; qPCR, quantitative PCR; PT, primary tumor; RNAseq, RNA sequencing; SNV, single-nucleotide variant; tNGS, targeted NGS; TNBC, triple-negative breast cancer; WES, whole-exome sequencing; WGS, whole-genome sequencing.

evaluate novel therapeutic opportunities, including combinatorial targeted therapy, which could delay or even prevent acquired resistance [3,57].

It is therefore apparent that both major long-standing theories have claimed significant support over the years, sustaining the ongoing controversy. In addition, mixed models of tumor evolution have been reported in the literature, combining the different concepts. For instance, experimental observations from bulk and single-cell NGS studies have implicated point mutations in the dynamic evolution of ITH [34], whereas large structural genome changes, including chromosomal rearrangements [34,52] and copy-number aberrations [19,48,51], have been identified as pre-existent and temporally stable [58]. Another hybrid model recently proposed by basic research [15], as well as a patient-centric liquid biopsy study [19], suggests that the timing of resistance reflects distinct evolutionary mechanisms. More specifically, early and late drug resistance were correlated with aggressive clones that either pre-existed or dynamically evolved from tolerant cells, respectively [15,19], providing a sound concept for the mechanisms underlying primary and secondary resistance, from a clinical viewpoint. However, both these models require validation through large, appropriately designed, single-cell studies.

Tumor ecosystem underlying drug resistance

The primary tumor is increasingly being considered as a complex ecosystem related to drug resistance, including intra- and extracellular interaction networks [17,60]. Interactions within individual cells include three major layers. The first is gene–gene interaction networks affecting susceptibility to cancer, as well as the genotype-to-phenotype map, including drug resistance and relapse [61–64]. The second is protein–protein interactions perturbed by up to two-thirds of disease-associated variants, including interplay between transcription factors [65,66]. Lastly, and the most important from a clinical point of view with potentially crucial therapeutic implications, the third field comprises a large-scale sophisticated regulatory network between transcription factors, transcription-factor-binding sites, functional noncoding mutations and target genes [67,68].

Furthermore, the complexity in understanding and predicting individualized drug sensitivity becomes even more sophisticated considering extracellular interplay [6,10,60]. This refers to an intricate and evolving active crosstalk between cancerous and non-malignant cells, stromal and immune [6,17]. For instance, Marusyk and colleagues demonstrated a mode of interclonal cooperation *in vivo*, termed non-cell-autonomous tumor growth [16]. According to this model, a tumor subclone of potentially lower fitness produces changes in the microenvironment stimulating the growth of all tumor cells [16]. Moreover, clearly identified clonal interplay between distinct breast cancer subclones was essential for the maintenance of ITH and tumor relapse after systemic therapy [69]. However, and in contrast to the vast majority of available data [3,6,16,17,69], a recent single-cell study by Roerink *et al.* supported cell-autonomous tumor growth with intratumor mutational and transcriptional heterogeneity, as well as differential drug response, being independent of the tumor microenvironment [45]. Currently, the three prevailing concepts: dynamic ITH, PMCs and interclonal interactions, do not take into

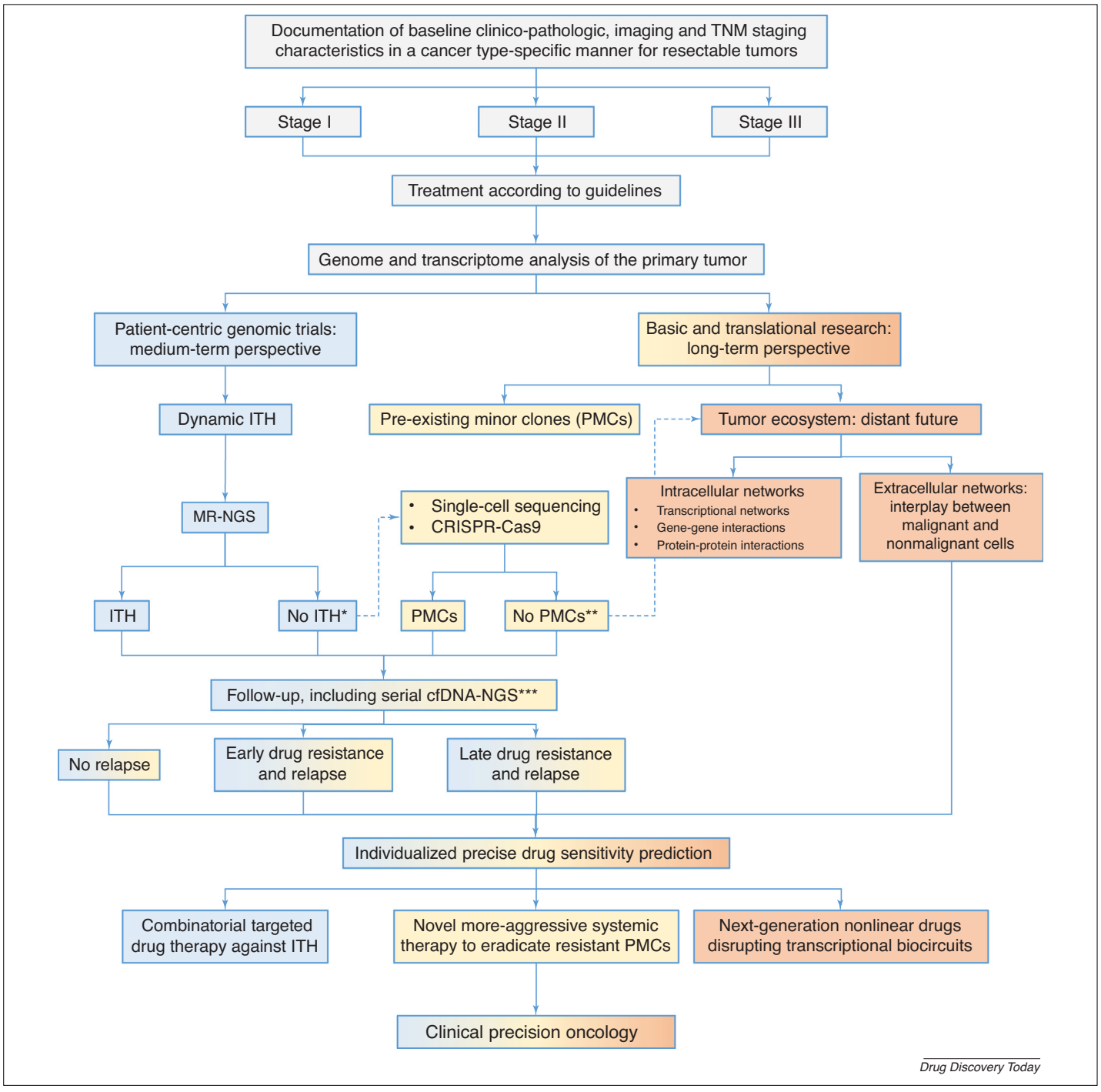

FIG. 2

Clinico-genomic and -transcriptomic model to overcome drug resistance and relapse. Stepwise algorithmic flowchart in a cancer type- and stage-specific framework to understand, predict and improve individualized drug response and oncological outcomes. **(a)** Spatiotemporal patient-centric genomic trials to identify dynamic ITH through MR-NGS. **(b)** Translational exploration of pre-existing minor subclones within the primary tumor through single-cell DNA and RNA sequencing. **(c)** Exploration of the tumor ecosystem, including intra- and extra-cellular interaction networks. Abbreviations: ITH, intratumor heterogeneity; MR-NGS, multiregional next-generation sequencing; PMC, pre-existing minor clone.

*For patients without ITH-based explanation of resistance, single-cell analysis is proposed.

**For patients without ITH- or PMC-based explanation of resistance, exploration of the tumor ecosystem could clarify the origins of resistance.

***Serial liquid biopsies of circulating cell-free DNA represent the most promising biomarker to predict acquired resistance and relapse.

account cancer type- and stage-specific clinical data. In this context, a rational approach to clarify this controversy lies in the development of a clinicogenomic model integrating high-quality clinical evidence.

Future outlook

Resistance to modern therapeutics is the predominant cause of relapse and death, remaining the greatest research challenge. Despite the recent integration of breakthrough technologies

and sophisticated methods to explore static and dynamic genome- and transcriptome-wide mechanisms underlying drug resistance, the long-term debate on dynamic clonal evolution versus pre-existing rare clones is still flaming. By contrast, an emerging concept visualizes the primary tumor as a whole ecosystem, driving early drug resistance. Furthermore, recent evidence on highly variable drug response, even among closely related individual cells within the primary tumor [3,45], substantially increases the complexity of individualized precise prediction of therapeutic resistance.

Clinicogenomic network model

Based on data extracted before [26–28] and after the advent of NGS technologies (Tables 1 and 2), as well as recent reports integrating breakthrough single-cell genome sequencing and editing technologies [3,6,70], coupled with definitive evidence on cancer type- and stage-specific early resistance and relapse [4,18], we propose a novel clinicogenomic and tumor ecosystemic model. This concept is based on the very low versus high relapse rates in the early stages of low- and high-aggressiveness tumors, respectively (e.g., breast versus pancreatic cancer) [22,24]. Clinical evidence is consistent with PMCs in aggressive cancers, whereas low and high relapse rates in early and advanced low-aggressiveness tumors suggest dynamic clonal evolution (Fig. 1).

The future perspective of individualized drug sensitivity prediction and precise therapeutic targeting of dynamic ITH, PMCs and cellular interaction networks are delineated in Fig. 2. Documentation of baseline clinicopathologic characteristics and stage-specific treatment according to modern guidelines reassure the validity of genome and transcriptome analysis. The three predominant concepts underlying therapeutic failure are evaluated in a step-wise algorithmic approach. First, the feasibility of spatiotemporal MR-NGS genomic trials within a medium-term evidence-based strategy could establish dynamic ITH as a predictive biomarker. Second, large-scale single-cell DNA/RNA sequencing translational studies could detect PMCs and guide novel therapeutics in a long-term timeframe. Third, exploration of intracellular networks, including transcriptional and noncoding RNA biocircuits [71,72], gene–gene interactions [73], protein–protein interactions [74] and the interactome [75], as well as extracellular cooperativity between cancerous and nonmalignant cells [76,77], will pave a distant future avenue toward the pharmaceutical controllability of temporal nonlinear networks [78].

Optimization of primary systemic therapy

Individualized characterization of the mechanisms underlying relapse will dictate distinct therapeutic strategies. A realistic goal in the foreseeable future lies in the combinational treatment with available and new to-be-developed oncotarget-guided drugs targeting dynamic ITH, aiming to reduce early resistance [79]. However, it should be noted that this therapeutic strategy is based on the central dogma of molecular biology [80]. Regarding accurate decision-making on primary systemic therapy, beyond tumoral MR-NGS, circulating minimal residual disease detected through pre- and post-surgery cfDNA genome [81] and methylome analysis [82] could contribute to individualized precise therapeutic targeting.

Furthermore, characterization of nonresponding PMCs by single-cell NGS could potentially guide the future development of novel therapies to eliminate resistant rare cell subpopulations. Ultimately, shifting from the current linear single-gene transcription dogma [83], which represents the foundation of modern drug development [84], to nonlinear regulatory networks [85] will enable the innovative discovery of drugs reprogramming network architecture. Thus, advances in the integration of single-cell RNA sequencing, CRISPR-Cas9 and computational models into the exploration of interaction networks will open a new path to understand and predict network dysregulation (Fig. 2). Deep exploration of nonlinear biocircuits represents the foundation for the development of next-generation therapies restoring comprehensive transcriptional deregulation [70,74,86–89].

Concluding remarks

The emerging clinicogenomic and regulatory network research framework of inter- and intra-individual genetic, genomic and transcriptional heterogeneity-based optimization of primary systemic therapy represents one of the greatest hopes to overcome fatal early resistance and relapse. Among the three prevailing theories, dynamic ITH could be validated within spatiotemporal genomic trials with a medium-term perspective. Regarding the remaining proportion of nonresponders to primary combinatorial treatment, two exciting future perspectives are being shaped. Detecting rare resistant subclones through large-scale single-cell NGS translational studies will facilitate the development of novel aggressive therapies to eliminate these pre-existing minor genomic clones. In the future, understanding temporal interacting biological systems acting as an integrated ecosystem will open a new avenue toward next-generation therapies disrupting aberrant regulatory networks.

Conflicts of interest

The authors report no conflicts of interest. The authors report no funding. The present manuscript has not been published previously, is not under consideration for publication elsewhere and its publication is approved by all authors.

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