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Crossroad between linear and nonlinear transcription concepts in the discovery of next-generation sequencing systems-based anticancer therapies

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The unprecedented potential of standard and new next-generation sequencing applications and methods to explore cancer genome evolution and tumor heterogeneity as well as transcription networks in time and space shapes the development of next-generation therapeutics. However, biomedical and pharmaceutical research for overcoming heterogeneity-based therapeutic resistance is at an important crossroads. Focus on linear transcription-based drug development targeting dynamics of simple intrapatient structured genome diversity represents a realistic medium-term goal. By contrast, the discovery of nonlinear transcription drugs for targeting structural and functional genome and transcriptome heterogeneity represents a long-term rational strategy. This review compares effectiveness, challenges and expectations between linear and nonlinear drugs targeting simple intrapatient variation and aberrant transcriptional biocircuits, respectively.

Introduction

State-of-the-art

Overcoming resistance to available linear transcription-based drugs [1], which is associated with high rates of metastasis and death [2], represents a top priority of the emerging genome network medicine [3]. However, shifting from linear transcription dogma [4] to nonlinear transcription-based discovery of next-generation drugs [5] is at present an unrealistic clinical approach, representing a big challenge for genome and network science [6,7]. Despite advances in multimodal treatment, including surgery, radiotherapy, chemotherapy and targeted drugs (http://www.nccn.org/professionals/physician_gls/f_guidelines.asp#site), identification and establishment of eight hallmarks of cancer [8] and cancer genomics, progress in the post-genomic era remains slow, which is outlined in two critical reviews by Klein [9] and Vogelstein *et al.* [10]. Excellent basic science research in the dynamics of genomic clone evolution-based tumor heterogeneity in time and space and transcriptional biocircuits could be translated into the clinic shaping the future of genome network medicine (GNM) [3].

Time factor dynamics

The time factor is the most crucial variable for understanding cancer genome and tumor evolution-based development of heterogeneity, therapeutic resistance and metastasis. The latest evidence on dynamics of noncoding genome functionality [11], transcriptional networks [12,13], cancer genomic clone evolution [14] and emerging heterogeneity at the microscopic level is reflected at tumor growth and metastasis [2,15]. Genomic clone evolution-based heterogeneity in time and space, either at delaying diagnosis before treatment or in response to systemic therapy, can be associated with therapeutic resistance and metastasis [14]. The comprehensive analysis of intratumor heterogeneity and circulating genomic clone heterogeneity (cGCs) can reveal the intrapatient (IP) genomic diversity [16,17] that is crucial for predicting and preventing therapeutic resistance.

New NGS application

Changing clinical NGS strategy

NGS has revolutionized biomedical research [18] – but only recently. The latest NGS applications allow accurate evaluation of spatiotemporal tumor evolution and heterogeneity-based prediction and prevention of therapeutic resistance-based metastasis.

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Simple and complex NGS applications including multiregional biopsies [19] in combination with circulating tumor DNA (ctDNA) sequencing analyses [20] and single-cell genome technique [16,21] provide the unprecedented potential not only for the identification of next-generation biomarkers but they also shape a new horizon in the development of two therapeutic strategies. The first realistic approach is based on simple linear transcription drugs and it is expected to increase the number of these targeted agents dramatically. The second nonlinear transcription-based strategy represents a future goal based on recent ENCODE project evidence [13] of dynamics of transcriptional biocircuits. This review discusses and compares the potential, challenges and future expectation of IP-based linear drugs and comprehensive nonlinear transcription agents.

Modern targeted linear drugs

Targeted therapy has revolutionized systemic treatment by targeting almost exclusively cancer cells with specific genetic alterations without affecting normal cells. Pre-treatment single-biopsy-based genetic testing is used as a biomarker provider to identify individual patients with a mutated or amplified gene. This interpatient-based personalized cancer treatment by using targeted drugs to inhibit the corresponding deregulated signaling pathway represents an important advance in modern oncology [22]. In a recent comprehensive review, Rask-Andersen *et al.* [1] reported that all drugs that had been developed up until 2010 have been based on simple single-gene linear transcription dogma [4]. On the basis of this dogma, the list of FDA-approved currently available drugs has rapidly grown to 64 agents (http://www.fda.gov/Drugs/InformationOnDrugs/ApprovedDrugs/ucm279174.htm). Table 1 summarizes the FDA-approved targeted drugs after 2010.

One of the most successful paradigms of translational research has been the monoclonal antibody trastuzumab (Herceptin®, Genentech) targeting the human epidermal growth factor receptor 2 (HER2) signaling pathway for breast cancer and gastric cancer [23]. A further development in this traditional single-pathway research field is the trastuzumab–emtansine conjugate drug T-DM1 that has prolonged overall survival by 6 months, compared with trastuzumab plus classical chemotherapy in metastatic HER2-positive breast cancer [24,25]. More recently, a long-term anticipated drug for HER2-negative patients accounting for 75% of all breast cancers has become a reality. Palbociclib (IBRANCE®, Pfizer), an inhibitor of cyclin-dependent kinase (CDK)4 and CDK6, has recently been approved by the FDA for use in combination with letrozole for the treatment of postmenopausal women with estrogen receptor (ER)-positive, HER2-negative metastatic breast cancer [26].

Limitation of linear drugs

Despite these advances with targeted drugs (Table 1), there has been a current general consensus that all these single-biopsy linear transcription-based agents are characterized by temporary and moderate antitumor effectiveness. A new horizon in overcoming this therapeutic resistance represents a new roadmap in understanding cancer dynamics of cancer genome evolution and tumor heterogeneity in time and space.

Therapeutic resistance and tumor heterogeneity

Explaining primary and secondary therapeutic resistance, the roadmap of tumor-evolution-based heterogeneity has become a top priority in biomedical and pharmaceutical research. Following Darwinian principles, the emergence of mutation in response to therapy develops genomic clones and cancer cells resistant to primary therapy. Shifting from simple interpatient diversity, genomic difference between patients with the same tumor stage and cancer type, to much more complex dynamics of IP diversity [27] in time and space we could approach next-generation personalized cancer medicine (NGPCM).

Currently, cancer is defined as a disease of the genome. Intrapatient diversity is termed here as the total set of cancer-associated genome changes in an individual patient. How could dynamics of intrapatient diversity evolution be discovered? Intrapatient genomic diversity includes intratumor heterogeneity, cGC and occult micrometastasis in patient without distant metastasis (stage M0) or with metastasis (M1) in the metastatic setting. Simple and complex NGS application and methods have been recently developed to identify dynamics of intrapatient diversity. For example, intratumor heterogeneity of primary tumors can be revealed with multiregional biopsy-based NGS [20,28]. Yates *et al.* [29] performed multiregional whole genome sequencing (WGS) in 50 primary breast cancers. The diversification of subclonal structure and tumor evolution can explain resistance to chemotherapy. The authors reported the importance of subclonal diversity in predicting therapeutic resistance and its evaluation in clinical trials of primary breast cancer.

Dynamics of circulating genomic clones

Noninvasive techniques have recently been reported for studying clonal evolution in response to therapy. Murtaza *et al.* [20] recently reported the establishment of cell-free ctDNA followed by whole exome sequencing (ctDNA–WES) proof-of-principle that could predict therapeutic resistance and recurrence by identifying the emergence of mutations with repeated ctDNA–WES. Therefore, serial ctDNA–NGS in the follow-up could be used for biomarkers to predict secondary therapeutic resistance and prevent tumor relapse [30–32]. Intrapatient genomic and cellular variation requires the comparison between intratumor and cGC in an individual patient to reveal whether clonal diversity undetectable in the primary tumor or the emergence of mutation in cGC is the cause for therapeutic resistance-based recurrence.

A further complexity in evaluating tumor heterogeneity can be the genomic difference even between individual cancer cells [33]. A long-held dream for accurate assessment of cellular diversity appears now a realistic goal [27] using single-cell techniques and the latest sequencing technologies. Wang *et al.* [21] developed and reported a whole-genome single-cell sequencing innovative technique, termed Nuc-seq. This is an exciting future perspective but at the present time it remains in the preclinical stage. Intratumor heterogeneity-based therapeutic resistance prediction can be much more complex if we take into account the diverse and interacting evolving clones reflecting a tumor ecosystem [17,27,34].

Next-generation personalized cancer therapeutics

Given the extensive genomic structural variation and tumor heterogeneity, completion of cancer driver genes for its cancer type is a basic research goal fundamental to achieve personalized cancer medicine (PCM). Classification of these genes [35] into eight

TABLE 1

New targeted anticancer drugs approved by the FDA after 2010 until November 2015.

Type of cancer	Drug	FDA approved	Therapeutic target
Cutaneous melanoma	Ipilimumab	October 2015	Human cytotoxic T-lymphocyte antigen 4 (CTLA-4)
Metastatic squamous non-small-cell lung cancer (NSCLC)	Nivolumab	March 2015	Programmed death-ligand 1 (PD-L1) and Programmed death-ligand 2 (PD-L2)
Metastatic squamous NSCLC	Pembrolizumab	2015	Programmed death receptor-1 (PD-1)
Unresectable or metastatic melanoma	Pembrolizumab	September 2014	PD-1
	Nivolumab	December 2014	PD-L1 and PD-L2
Metastatic colorectal cancer	Trifluridine/ tipiracil combination	September 2015	Anti-vascular endothelial growth factor (VEGF) biologic product, and an anti-epidermal growth factor receptor (EGFR) monoclonal antibody
Locally advanced basal cell carcinoma (BCC)	Sonidegib	July 2015	Inhibits the Hedgehog (Hh) signaling pathway
• Metastatic colorectal cancer (mCRC)	Ramucirumab	• April 2015	Antibody that binds to human VEGF-R2
• Metastatic NSCLC		• December 2014	
• Advanced gastric or GEJ adenocarcinoma refractory		• April 2014	
Breast cancer	Palbociclib	February 2015	Cyclin-dependent kinase (CDK) 4 and 6, in HER2-negative patients
Multiple myeloma	Panobinostat	February 2015	Histone deacetylase inhibitor
Thyroid cancer	Lenvatinib	February 2015	Kinase inhibitor
Chronic lymphocytic leukemia	Ibrutinib	February 2014	Kinase inhibitor
Relapsed or refractory B cell precursor acute lymphoblastic leukemia (R/R) ALL	Blinatumomab	December 2014	Bispecific CD19-directed CD3T cell engager
Ovarian cancer	Olaparib	December 2014	BRCA1/2
Chronic lymphocytic leukemia (CLL)	Idelalisib	July 2014	Kinase inhibitor
Relapsed or refractory peripheral T cell lymphoma (PTCL)	Belinostat	July 2014	Histone deacetylase inhibitor
Anaplastic lymphoma kinase (ALK)-positive metastatic NSCLC	Ceritinib	April 2014	Kinase inhibitor
CLL	Ofatumumab	April 2014	CD20-directed cytolytic monoclonal antibody
Mantle cell lymphoma (MCL) CLL	Ibrutinib	February 2014	Kinase inhibitor
Melanoma	Debrafenib/ trametinib combination	January 2014	BRAF
CLL	Obinutuzumab	November 2013	CD20-directed cytolytic monoclonal antibody
NSCLC	Afatinib	July 2013	EGFR/ERBB2
Melanoma	Trametinib	May 2013	Mitogen-activated protein kinase kinase (MEK)1
Melanoma	Debrafenib	May 2013	BRAF
Metastatic basal cell carcinoma, or with locally advanced basal cell carcinoma	Vismodegib	January 2012	Hedgehog pathway inhibitor
Advanced renal cell carcinoma	Axitinib	January 2012	Kinase inhibitor
NSCLC	Crizotinib	2011	Anaplastic lymphoma kinase (ALK)
Hodgkin lymphoma, anaplastic large cell lymphoma (ALCL)	Brentuximab vedotin	August 2011	CD30-directed antibody–drug conjugate
Melanoma	Vemurafenib	August 2011	BRAF
Melanoma	Ipilimumab	March 2011	Human CTLA-4

All these targeted drugs approved by the FDA were assessed in November 2015 at the following link: http://www.fda.gov/Drugs/InformationOnDrugs/ApprovedDrugs/ucm279174.htm.

categories according to the corresponding hallmarks of cancer (deregulation of cell survival, growth, proliferation, apoptosis, angiogenesis, metabolism, invasion and metastasis) [8] can be useful in the clinic. For example, identifying intrapatient genes involved not directly in cell survival, apoptosis and metastasis but indirectly in pathways such as angiogenesis or metabolism could improve therapeutic decisions by selecting a combination of drugs inhibiting all these pathways. This strategy is suitable for a linear drugs approach rather than a nonlinear druggable targets discovery concept.

The evidence on dynamics of genomic clone evolution and tumor heterogeneity [14] can explain therapeutic resistance and recurrence in patients with completed tumor resection (R0) in the adjuvant setting (M0) or disease progression in the metastatic setting (M1). The IP diversity-based NGPCM shapes two future strategies in the discovery of next-generation drugs. The first therapeutic strategy on drug discovery continues to be based on linear transcription dogma [1] but it takes into account dynamics of simple IP diversity. This medium-term goal in the discovery of linear drugs is driven by simple biopsy-based NGS approach and novel NGS application combined with breakthrough methods to reveal simple IP dynamics in a spatiotemporal manner. The second, more-distant-future approach of nonlinear transcription-based drug discovery aims to disrupt transcriptional networks.

Linear personalized therapeutics

Breakthrough next-generation technologies provide two directions in the development of personalized novel linear drugs. The first one is based on the standard single biopsy and the second strategy is driven by dynamics of IP diversity.

Single-biopsy-based NGS in the discovery of new linear drugs

Single-biopsy NGS has been the standard approach in large studies. Many novel cancer driver genes, some of which might be of clinical utility for use as biomarkers or therapeutic targets, have been identified with WES (Table 2) [35–47]. Clinically more important, although more complex than WES, are WGS studies that have already discovered 78 novel genes and 19 potential WGS-based therapeutic targets (Table 3) [39,40,48–59].

Limitations

Despite a dramatic increase of novel NGS-based identification of cancer genes (Tables 2 and 3), including 117 in WES and 78 in WGS, there has been skepticism regarding the validation and clinical utility of these genes. Indeed, based on the largest WES study currently available on 4742 samples in 21 tumor types and extensive heterogeneity accessed, Lawrence *et al*. recommend larger studies of 600–5000 samples per tumor type, for achieving a statistical power in discovering novel robust biomarkers and therapeutic targets [35]. Although this NGS-based concept can lead to completion of the cancer genes list, it is questionable how many of these new discoveries could be translated into the clinic as biomarkers and/or therapeutic targets. Another important disadvantage of single-biopsy-based NGS is that this analysis does not take into account intratumor heterogeneity and cGC diversity in response to therapy. Recent advances in NGS application allowing the dynamics of IP diversity are discussed below.

Targeting dynamics of intrapatient heterogeneity

Breakthrough NGS applications in combination with novel methods, empowering the discovery of dynamics of IP diversity, provide a realistic approach in achieving two crucial translational goals: to use IP diversity first for biomarkers to predict therapeutic resistance; and to target IP structural genome diversity with available and new, developing linear drugs. Fig. 1 delineates a medium-term step-by-step strategy to simple IP structural genome diversity-based prediction and prevention of therapeutic resistance to linear drugs. This approach also provides the potential for dramatic increase of the linear drugs list.

Primary and secondary therapeutic resistance rates are currently high. Potential solutions for overcoming this failure provide the identification of intratumor and circulating genomic clone diversity (Table 4). Primary therapeutic resistance in R0M0 patients could be predicted by comparing multiregional-biopsy-based NGS for intratumor heterogeneity of the primary cancer with ctDNA–NGS-based identification of cGC diversity. A similar approach for M1 patients is shown in Fig. 1 by comparing WGS-based analysis in multiple solid and liquid biopsies between responder and non-responder patients for the identification of the genome set of changes between primary tumor, metastatic cancer(s) and cGCs. This simple IP genome diversity identification could predict therapeutic response. Moreover, it can lead to the discovery of multiple novel linear-drug-targeted dynamics of IP diversity. Collectively, a more effective targeting of IP diversity could result from a combination of targeted drugs available and should be developed from single-biopsy-based and IP-diversity-based agents.

Complex dynamics of genomic clones diversity-based development of secondary resistance after initial response could be identified with simple serial ctDNA-based WES–WGS analysis [20,30–32]. However, comparison of the emergence of structural genome changes in cGCs with intratumor heterogeneity is essential to reveal whether rare subclonal cell populations or even individual cells within a primary tumor or cGCs diversity in response to therapy are responsible for acquired resistance. This simple intrapatient diversity identification every 3 or 6 months after treatment could precisely predict recurrence in R0M0 patients or metastasis progression in M1 patients before an oncological event (recurrence or metastasis progression or death) occurs. Further intrapatient structural genomic variation between groups with or without recurrence in R0M0 patients as well as with or without metastatic progression in M1 patients could provide additional important information on the molecular mechanism underlying acquired resistance. Once secondary resistance-based occult has been accurately predicted before it occurs, the next big challenge is the appropriate selection of effective linear drugs to prevent an oncological event. This effective new treatment will target the whole set of genome changes.

Rational design

The most rational way to prove whether the spatiotemporal IP genome structural diversity will provide clinical benefits is within current clinical trials for guidelines-based treated patients. The advantage of this translational concept is that the identification of IP structural diversity in time and space could subsequently be tested in randomized controlled trials (RCTs) to access its robustness as a biomarker to predict therapeutic resistance. Moreover, targeting this IP structural heterogeneity with available and novel drugs expected to be developed could prevent resistance-based recurrence or metastasis progression. For example, standard and new NGS applications have already identified a total number of 199 novel genes as well as 11 biomarkers and 19 therapeutic targets (Tables 2–4). However, it should be emphasized that the clinical implication of these NGS-based discoveries should be evaluated in well-designed clinical trials to prove the clinical utility of novel biomarkers and linear transcription-based drugs.

TABLE 2

Standard single-biopsy-based whole exome sequencing (WES) studies in different cancer types.

Type of cancer	Number of patients	Findings	Novel genes, clinical implications	Refs
The largest WES analyses to date				
21 tumor types	4742 in total (35 patients with rhabdoid tumor – 892 patients with breast cancer)	Identification of 33 genes that were not previously known to be significantly mutated in cancer, including genes related to proliferation, apoptosis, genome stability, chromatin regulation, immune evasion, RNA processing and protein homeostasis. Down-sampling analysis indicates that larger sample sizes will reveal many more genes mutated at clinically important frequencies. Estimates that near-saturation can be achieved with 600–5000 samples per tumor type, depending on background mutation frequency	33	[35]
WES studies in different cancer types				
Prostate cancer	150	New genomic aberrations in *PIK3CA/B, RSpondin, BRAF/RAF1, APC, β-catenin, ZBTB16/PLZF*	8	[36]
Pancreatic ductal adenocarcinoma	109	Identification of multiple novel mutated genes in PDA, with select genes harboring prognostic significance. *KRAS* mutations are observed in >90% of cases. *ARID1A* marker of poorer outcome. RBM10 mutation was associated with longer survival. BRAF and PIK3CA mutations expanding the spectrum of oncogenic drivers	5	[37]
Esophageal squamous cell carcinoma	104	*AJUBA, ZNF750* and *PTCH1* and the chromatin-remodeling genes *CREBBP* and *BAP1* in addition to known mutations	5	[38]
Breast cancer	103WES/22WGS[a]	Analysis of WES and WGS showed the significance of *CBFB* and translocation of *MAGI3–AKT3*. The mutations in *CBFB, RUNX1* and *GATA3* suggest the importance of understanding epithelial cell differentiation and its regulatory transcription factors in breast cancer pathogenesis	The use of ATP-competitive AKT inhibitors should be evaluated in clinical trials for the treatment of fusion-positive triple-negative breast cancers	[39]
Breast cancer	54 (and 15 WES)[b]	Mutations during clonal evolution occurred late in disease progression explaining tumor heterogeneity	NR	[40]
Testicular germ cell tumors	42	Identified over-representation of novel mutations in the tumor suppressor genes *CDC27* and *PRKRIR*	2	[41]
Small cell lung cancer	38	*TP53, RB1* and *PTEN* were identified as significant genes. *TMEM132D, SPTA1* and *VPS13B* could be also involved in *SCLC* development, with the products from their mutated alleles being potential therapeutic targets in SCLC patients	36	[42]
Controlateral breast cancer	25	For three patients, we identified shared somatic mutations indicating a common clonal origin demonstrating that the second tumor is a metastasis of the first cancer	0	[43]
Anaplastic thyroid carcinoma	22	Mutations in genes not previously associated with thyroid tumorigenesis were observed (*mTOR, NF1, NF2, MLH1, MLH3, MSH5, MSH6, ERBB2, EIF1AX* and *USH2A*), some of which could be targets for future therapeutic intervention	10	[44]
Metastatic melanoma	20	The first reported recurrent mutation causing a *P131L* mutation in the *RQCD1* (required for cell differentiation1 homolog) gene	1	[45]
Cervical adenocarcinomas	15	Identification of several frequently mutated genes including *FAT1, ARID1A, ERBB2* and *PIK3CA*	4	[46]
Glioblastoma	3	Thirteen genes were found to harbor variants [platforms found the genes *PTCH1* (patched 1) and *NF1* (neurofibromin1)]	13	[47]
Total	5427	NA	117[c]	NA

Abbreviations: PIK3CA/B, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; BRAF/RAF1, murine sarcoma viral oncogene homolog B; APC, Adenomatous polyposis coli; ZBTB16/PLZF, zinc finger and BTB domain containing 16/ Promyelocytic leukemia zinc finger ortholog; PDA, Pancreatic Ductal Adenocarcinoma; ARID1A, AT-rich interactive domain-containing protein 1A; RBM10, RNA Binding Motif Protein 10; AJUBA, Ajuba LIM protein; ZNF750, Zinc Finger Protein 750; PTCH1, Protein patched homolog 1; CREBBP, CREB Binding Protein; BAP1, BRCA1 associated protein-1; CBFB, Core-binding factor subunit beta; RUNX1, Runt-related transcription factor 1; GATA-3, Trans-acting T-cell-specific transcription factor; CDC27, Cell Division Cycle 27; PRKRIR, 52 kDa repressor of the inhibitor of the protein kinase; TP53, Tumor protein p53; RB1, retinoblastoma 1; PTEN, Phosphatase and tensin homolog; TMEM132D, Transmembrane Protein 132D; SPTA1, Spectrin alpha chain, erythrocyte; VPS13B, Vacuolar Protein Sorting 13 Homolog B; SCLC, small cell lung cancer; mTOR, mammalian target of rapamycin; NF1, Neurofibromatosis type 1; NF2, Neurofibromatosis type 2; MLH1, MutL homolog 1; MLH3, MutL Homolog 3; MSH5, MutS Homolog 5; MSH6, MutS Homolog 6; EIF1AX, Eukaryotic translation initiation factor 1A, X-chromosomal; USH2A, Usher syndrome 2A; RQCD1, Required for Cell Differentiation1 Homolog; NR, not reported; NA, not applicable.

[a] This study includes 22 WGS also described in Table 3.

[b] This study also includes 15 WGS also included in Table 3.

[c] Only 33 meet the criteria set by The Lawrence Recommendation.

TABLE 3

Standard single-biopsy-based whole genome sequencing (WGS) analyses in diverse cancer types.

Type of cancer	Number of patients	Number of novel genes identified	Future potential biomarkers and therapeutic targets	Refs
Pancreatic	100	2 (*KDM6A* and *PREX2*)	Potentially yes	[48]
Gastric	100	26 (*MUC6*, *CTNNA2*, *GLI3*, *RNF43* and others)	*RHOA:* potential druggable target *RNF43:* druggable target for potential WNT inhibitor	[49]
High grade serous ovarian cancer	92	1 (*ABCB1*)	*ABCB1*, *MDR1*	[50]
Colorectal	74	23 *TCF7L2*, *TET2* and *TET3* ERBB3	*R-spondin* gene fusions and *TCF7L2*, *TET2* and *TET3* potential therapeutic targets	[51]
Gastric	49	9	*SLIT/ROBO*	[52]
Breast	46	5 (RUNX1, CBFB, MYH9, MLL3 and SF3B1)	Could produce therapeutic advances	[53]
	22 WGS (103 WES)[a]	3 (*CBFB, RUNX1* and *GATA3*)	The use of ATP-competitive AKT inhibitors should be evaluated in clinical trials for the treatment of fusion-positive triple-negative breast cancers	[39]
	15 WGS (and 54 WES)[b]	NR	Mutations during clonal evolution occurred late in disease progression explaining tumor heterogeneity	[40]
Hepatocellular	27	None	No	[54]
Melanoma	25	1 *PREX2*	New insights into tumor biology, therapeutic resistance and developing treatment regimens	[55]
Renal cell cancer	22	1 *PI(3)K/AKT*	*PI(3)K/AKT* strong therapeutic target, potential value of MTOR and/or related pathway inhibitor drugs	[56]
	14	1 *TCEB1*, other newly identified recurrent mutational targets included *TET2*, *KEAP1* and *MTOR*	No	[57]
Esophageal squamous cell carcinoma	17	2 *ADAM29* and *FAM135B MIR548 K*	Potentially biomarkers *ADAM29 and FAM135B MIR548 K.* Novel therapeutic targets such as *PSMD2, RARRES1, SRC, GSK3B* and *SGK3*	[58]
Chronic lymphocytic leukemia	4	4 Notch 1 (*NOTCH1*), exportin 1 (*XPO1*), myeloid differentiation primary response gene 88 (*MYD88*) and kelch-like 6 (*KLHL6*)	*NOTCH1* and *MYD88* mutations are activating events and potential therapeutic targets	[59]
Total	607	78	NA	NA

Abbreviations: NGS, next-generation sequencing; WES, whole exome sequencing; WGS, whole genome sequencing, cGC, circulating genomic clone heterogeneity; NA, not applicable.
[a] The study on 22 WGS and 103 WES by Banerji *et al.* [39] has been included in Table 2. The conclusion of this study is that recurrent genomic fusion involving AKT3 suggests that the use of ATP-competitive AKT inhibitors should be evaluated in clinical trials for the treatment of fusion-positive triple-negative breast cancers, a subtype where limited therapeutic options exist beyond systemic cytotoxic chemotherapy.
[b] This study also includes 54 WES also included in Table 2.

Challenges of the simple IP concept

In addition to the time period required to use IP dynamics as a clinical concept of NGPCM, there is skepticism about the accuracy of the simple IP strategy as a biomarker to predict therapeutic resistance without taking into account the noncoding genome regulatory role and functional heterogeneity as well as the temporary and moderate effectiveness of all linear drugs – even those that will be discovered over the coming years.

Nonlinear drugs

Biomedical research on drug development is currently at a crossroads. Should current and future investigation on design of new drugs be shifted from simple linear transcription to a highly complicated nonlinear transcription-based therapeutics development? Table 5 summarizes different challenges and perspectives of linear versus nonlinear transcription drugs to predict and prevent therapeutic resistance in medium-term and long-term approaches. The fields of modern oncology and the pharmaceutical industry continue to be based on the traditional simple, single-gene-transcription dogma [2].

The list of targeted drugs receiving regulatory approval is rapidly growing and the design of NGS-based drugs to be developed in the near future continues to focus on linear drugs. However, clinical evidence from RCTs and meta-analysis

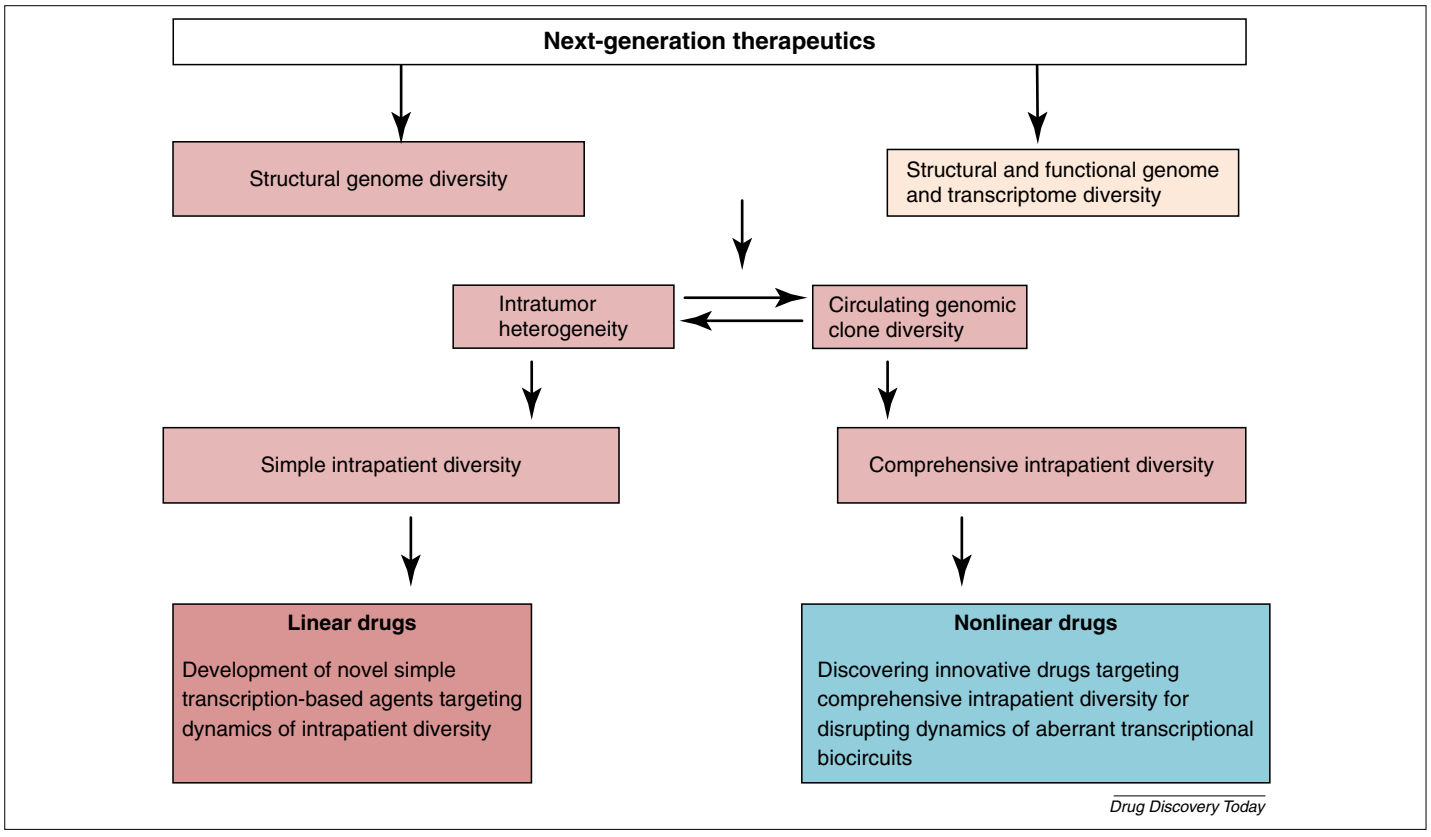

FIGURE 1

Design of future projects (and those underway) to empower the discovery of new linear and nonlinear drugs on the basis of standard and new next-generation sequencing (NGS) applications. Intratumor heterogeneity evaluation includes a medium-term approach with multiregional-biopsy-based whole exome sequencing (WES) – whole genome sequencing (WGS) and a long-term goal with WES–WGS–RNA-seq-ChIP-seq, as well as single-cell genome NGS. Circulating genomic clone diversity evaluation includes a medium-term approach for repeating ct-DNA-based WES–WGS in follow-up and long-term for ctDNA-based WES–WGS–RNA-seq-ChIP-seq. Accurate simple intrapatient heterogeneity can result from the comparison between intratumor and circulating genomic clone heterogeneity (cGC) diversity for each individual patient.

TABLE 4

Potential of multiple solid- and liquid-biopsy-based NGS to predict and reduce therapeutic resistance and relapse.

Cancer type	No. of patients/ samples	Technologies and methods	Findings	Clinical importance	Refs/year
Multiregional biopsy-based NGS					
Breast	50/303	Multiregional biopsy-based WGS and targeted sequencing of the PT	In 13/50 (26%) cancers, potentially targetable mutations were subclonal. Subclonal structural genomic diversification	Intratumor heterogeneity (ITH) could predict primary therapeutic resistance (PTR) but it requires clinical trial evaluation	[29]/2015
Renal	4/30	Multiregional biopsy-based WES from PT and MT	ITH in 67% of patients	ITH predictable of PTR, clinical trials required	[19]/2012
ctDNA-targeted sequencing					
Pancreas	24/77	WES in 24 patients and ctDNA-targeted sequencing at various time-points	Chromatin-regulating genes *MLL, MLL2, MLL3, ARID1A* associated with improved survival. Detection of ctDNA was associated with predictable recurrence 6.5 months before it occurs	These *MLL* genes are prognostic significance and ctDNA could be used as biomarker to predict recurrence	[60]/2015
Breast, ovarian and lung	6/19	WES in ctDNA-targeted sequencing at various time-points	Establishment of ctDNA-targeted sequencing as proof of principle. Emergence of mutated genes in response to systemic therapy identified by serial ctDNA sequencing	Prediction and potential prevention of relapse several months before it occurs	[20]/2013

Abbreviations: ctDNA, circulating tumor DNA; MT, metastatic tumors; NGS, next-generation sequencing; PT, primary tumors; WES, whole exome sequencing; MLL, myeloid/lymphoid or mixed-lineage leukemia; ARID1A, AT-rich interaction domain 1A; ITH, Intratumor heterogeneity could predict primary therapeutic resistance (PTR).

TABLE 5

Differences, potential challenges and future perspectives of linear and non-linear transcriptional drugs.

Dynamics of therapeutic resistance	Linear transcription drugs	Non-linear transcription drugs
Basic platform for the discovery of new drugs	Simple, single-gene linear transcription dogma	Design of non-linear transcription agents on the basis of the ENCODE project[a]
Targeted drugs	More than 64 targeted agents approved by the FDA[b]	NA
Non-coding genome functionality	No consideration	Yes
Next-generation biomarkers	Simple intrapatient structural genomic diversity	Comprehensive intrapatient structural and functional genome and transcriptome heterogeneity
Overcoming primary therapeutic resistance	Multiregional biopsy-based WES/WGS (intratumor heterogeneity) and comparison with ct-DNA-based WES/WGS	Multiregional biopsy-based WES, WGS/RNAseq/ChiPseq (intratumor heterogeneity) and comparison with ct-DNA-based WES, WGS/RNAseq/ChiPseq
Overcoming secondary therapeutic resistance	Repeated ct-DNA-based WES/WGS	Repeated ctDNA-based WES, WGS/RNAseq/ChiPseq
Medium term	Single biopsy-based NGS for the development of new drugs[c] (Table 2) Multi-biopsy-based WES/WGS discovery of new linear drugs: • Primary therapeutic resistance reduction by comprehensive targeting of intratumor heterogeneity •Secondary resistance reduction by serial ct-DNA WES/WGS	• Progress in transcriptome mapping and transcriptional networks • Comparison of transcriptional biocircuits dynamics between health and cancer and between resistant and non-resistant patients
Long-term therapeutic goals	Targeting dynamics of intrapatient structural genome diversity with available and new linear drugs expected to be developed	Design of next-generation non-linear drugs targeting dynamics of comprehensive structural and functional intrapatient diversity and disrupting aberrant transcriptional biocircuits

Abbreviations: WES, whole exome sequencing; WGS, whole genome sequencing; ct-DNA, circulating-tumor DNA; NA, not applicable.

[a] Expected results from the ongoing ENCODE project, (2012) ENCODE project: first evidence of functionality of most non-coding DNA and transcriptional biocircuits regulating human normal genome – Genome Network Medicine.

[b] From the available list of 64 FDA-approved drugs.

[c] Seven druggable targets (Tables 2 and 3).

underline a moderate and temporary effectiveness of almost all linear drugs (Table 1) [8,9,15]. Although the medium-term perspective of dynamics of simple IP structural genomic variation and targeting with linear drugs shapes a realistic NGPCM approach and can improve treatment results over the coming years, there is skepticism regarding the magnitude of long-term survival in advanced disease. How could these moderate linear concept-based expectations be overcome in a long-term approach? The ongoing ENCODE project of most noncoding genome functionality, transcriptional networks [11,13] and other individual functional genomics projects in health could improve our understanding on whole genome and transcriptome regulation. However, this goal might require a very long time period. For example, approximately just ten transcription factors (TFs) have been identified by the ENCODE [11] project, whereas transcriptional regulation mapping and transcriptional networks regulating multigene expression profiling are still in their infancy [13]. How could this time period for understanding genome and transcriptome comprehensive deregulation in cancer be shortened? Fig. 1 shows how potential intelligent solutions might shorten the time required to achieve comprehensive IP genomic diversity-based NGPCM exploiting the medium-term project.

Necessity of the network approach

High-quality basic scientific major discoveries on biological system interactions [61] have revolutionized the research roadmap to reach the medicines of the future [7]. Using NGS and systems approaches, the ENCODE project in model organisms (modENCODE) [62] and, two years later, in humans [11,13], as well as other individual functional studies, has provided strong evidence on molecular interaction and transcriptional biocircuits regulating biological processes crucial for life [3,6,7,12,63,64]. This new knowledge on pragmatic biology is crucial for designing new and future diagnostic, predictive and therapeutic models for achieving highly accurate and effective NGPCM. But, is such a nonlinear strategy a realistic goal in the near future?

The ongoing projects will provide critical information on improving our understanding on nonlinear transcriptional programs. By completing the list of cell- and tissue-specificity, TFs and regulatory variation will allow the TF-binding sequence-specific sites leading to nonlinear transcription mapping in health. Given that, at present, only 10% of TFs have been identified, this transcriptome mapping along with progress in understanding molecular networks will probably require a very long period. Moreover, even more time will be needed for complete cancer transcriptome mapping and subsequently health and cancer

transcriptome comparison-based development of nonlinear drugs. Alternative solutions to shorten this time are needed.

Alternative roadmap

>A smart alternative to shorten the required time is delineated in Fig. 1. The WGS-RNAseq-ChIPseq package-based comprehensive intrapatient diversity, including cancer regulatory variation [12,65] and cell-specific TF-binding sites in time and space, represents a reliable starting point for reaching transcriptional program mapping.

Nonlinear transcription druggable targets

This transcriptome-mapping-based comparison of groups of patients with or without recurrence or metastasis progression can open two innovative research directions. First, theoretical targeting of deregulated physical contact of cancer-cell-specific TFs with regulatory sequence variation in promoters and distant enhancer areas [66,67] with novel drugs could inhibit misregulating nonlinear transcription. Second, a revolutionary therapeutic strategic target could be the elimination or inactivation of cancer cells by disrupting intracellular and extracellular networks [68]. But this is a more distant future goal that will require breakthrough technologies and methods for clarifying how point mutations and large structural genome changes such as copy number alternations (CNAs) and genomic rearrangements affect and misregulate dynamics of transcriptional networks.

Cellular signaling networks

Exciting research on the comprehensive intracellular signaling pathway interaction network using a variety of living cell imaging with biosensors [69] and computational methods [70] for understanding intracellular signaling pathways, as well as protein–protein interactions and interactome [71], transcriptional biocircuits and gene–gene interplay [72], is crucial for understanding cell behavior and cancer cell deregulation. Exploring the potential discovery of nonlinear transcriptional druggable targets appears a more realistic future therapeutic goal than the high complexity of interactome [73] or gene–gene interaction networks in the pharmaceutical industry [72].

Future challenges

Although there are several optimistic reports on network-based therapeutics for breast cancer [74] and other tumor types [70], nonlinear transcription-based agents still remain a research dream. Future systematic work will be required for understanding transcription initiation by RNA polymerase II (pol-II) [75], identification of cancer-cell-type-specific TFs and mapping of TF-binding sequencing-specific variation. Given that most disease and cancer-associated variation identified by the Genome Wide Association Study (GWAS) lies outside genes [6], completion of the noncoding cancer regulatory variation as well as promoter–enhancer regulation appears essential [67]. Moreover, further research will be needed for the identification of long noncoding RNAs (lncRNAs) and epigenetic changes. Although substantial progress can be expected over the next decade in this field, the bigger challenges of the future will be how it might be possible to integrate all these big data into network modeling to understand and predict dynamics of transcriptional biocircuits. However, despite advances in network science, dynamics of network biology [76] and network medicine [7,77], regulatory network mechanisms underlying health and disease remain poorly understood. During the long period of life evolution, a tremendous number of disease-associated mutations have accumulated. This mutational landscape has emerged not only according to Darwinian theory but also as a result of nonadaptive origins, resulting in interactome complexity [73]. In the long evolutionary history, high network complexity has been developed as a regulatory network mechanism to overcome structural genome changes. The interplay of genome, transcriptome and RNA biosystems and the feedback mechanism between genome deficiency and nonlinear transcription further increase the complexity of transcriptional biocircuits and cellular signaling pathway networks representing an autoprotective mechanism to overcome cancer driver mutations. Therefore, there has been skepticism about whether these sophisticated or nearly chaotic networks [78] could be predicted.

Comparison of linear and nonlinear strategies

There has been uncertainty in the time period required for understanding and predicting transcriptional networks and the safety and effectiveness of drugs disrupting transcriptional networks remains unclear. Therefore, most hope for funding pragmatic nonlinear transcription-based development of nonlinear agents will probably result from the public sector. Despite limitations in funding highly complex basic genome and network science by the private sector with uncertainties regarding the period required to achieve genome network medicine in cancer [3], a rational precise research roadmap by the public sector has been started. With systematic work and innovation in genome technologies coupled with bioinformatics strategies, we hope that the time to achieve translation of transcriptional-biocircuits-based drugs into clinical NGPCM will be less than the 60 years that have been spent shifting from the linear transcription dogma to dynamics of transcriptional networks.

Concluding remarks

Designing the development of next-generation therapeutics, predicting and targeting dynamics of intrapatient genetic and genomic variation comprise the most rational roadmap to overcome therapeutic resistance and death. We are facing a crossroads. The first strategy of the traditional single gene/signaling pathway concept can dramatically increase the number of linear drugs targeting simple intrapatient structural genome diversity. This is a realistic approach potentially applicable in a medium-term perspective. The second highly complex roadmap to reach nonlinear drugs is just now beginning. Although it is based on truly organized transcriptional networks, much innovation will be required to understand interacting multiple gene/signaling pathway networks for discovering next-generation therapeutics blocking transcriptional biocircuit systems. Although this ENCODE-based transcriptional program functionality effort can revolutionize cancer therapy, it is a more distant future goal, attracting at present little investment by the pharmaceutical industry. This limited funding can be explained by the longer time required for financial benefit by the private sector.

Conflicts of interest

The authors declare no conflict of interest.

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