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Transcriptomics and solid tumors: The next frontier in precision cancer medicine

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ABSTRACT

Transcriptomics, which encompasses assessments of alternative splicing and alternative polyadenylation, identification of fusion transcripts, explorations of noncoding RNAs, transcript annotation, and discovery of novel transcripts, is a valuable tool for understanding cancer mechanisms and identifying biomarkers. Recent advances in high-throughput technologies have enabled large-scale gene expression profiling. Importantly, RNA expression profiling of tumor tissue has been successfully used to determine clinically actionable molecular alterations. The WINTHER precision medicine clinical trial was the first prospective trial in diverse solid malignancies that assessed both genomics and transcriptomics to match treatments to specific molecular alterations. The use of transcriptome analysis in WINTHER and other trials increased the number of targetable -omic changes compared to genomic profiling alone. Other applications of transcriptomics involve the evaluation of tumor and circulating noncoding RNAs as predictive and prognostic biomarkers, the improvement of risk stratification by the use of prognostic and predictive multigene assays, the identification of fusion transcripts that drive tumors, and an improved understanding of the impact of DNA changes as some genomic alterations are silenced at the RNA level. Finally, RNA sequencing and gene expression analysis have been incorporated into clinical trials to identify markers predicting response to immunotherapy. Many issues regarding the complexity of the analysis, its reproducibility and variability, and the interpretation of the results still need to be addressed. The integration of transcriptomics with genomics, proteomics, epigenetics, and tumor immune profiling will improve biomarker discovery and our understanding of disease mechanisms and, thereby, accelerate the implementation of precision oncology.

1. Background

Recent advances in technology have improved our understanding of carcinogenesis and led to the discovery of novel therapeutic targets. Precision oncology combines data from tumor genomic profiling, cell-free DNA assays, proteomic and immune profile analyses, and assessments of other markers to individualize treatment according to unique patient and tumor characteristics [1,2]. Artificial intelligence and innovative clinical trial designs, including adaptive and N-of-1 trials, hold promise to accelerate the collection, analysis, and application of data on predictive biomarkers and novel targeted agents [3,4]. Until recently, precision oncology focused mainly on genomic profiling of tumors [5,6]. Advances in next-generation sequencing (NGS) technologies have enabled the time- and cost-efficient incorporation of genomics into daily clinical practice. Several precision oncology trials have demonstrated the clinical significance of genomics in identifying molecular alterations that are successfully targeted by novel treatments [3,4,6–9]. Comprehensive gene panels are currently being used to identify

Abbreviations: cDNA, complementary DNA; CNV, copy-number variation; CUP, cancer of unknown primary; EGOG, Eastern Cooperative Oncology Group; GTEx, Genotype-Tissue Expression; INFORM, Individualized Therapy for Relapsed Malignancies in Childhood; mRNA, messenger RNA; NGS, next-generation sequencing; PIPseq, Precision in Pediatric Sequencing; RNAi, RNA interference; RNA-seq, RNA sequencing; rRNA, ribosomal RNA; RT-qPCR, reverse transcriptase quantitative polymerase chain reaction; siRNA, short interfering RNA; tRNA, transfer RNA; WIN, Worldwide Innovative Network.

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molecular therapeutic targets and prognostic and predictive biomarkers, and prospective clinical trials are assessing the value of molecular testing in treatment selection across tumor types [10–19].

Despite this significant progress in the implementation of precision oncology, several challenges need to be addressed in clinical research and practice. First, it is critical to enhance our knowledge of tumor biology, the mechanisms of carcinogenesis, and driver alterations to improve our ability to identify robust prognostic and predictive biomarkers. Additionally, to date, only a few molecular alterations have been successfully targeted by novel agents [20–23]. Many of these alterations are rare, and a large number of patients need to be screened to identify a single potential therapeutic target [24]. Indeed, the proportion of patients who are matched to therapy in precision oncology trials generally ranges from 5% to 50 % and often depends on whether the study is conducted in a specialized clinic with access to novel agents, off-label drug use, timely molecular profiling, and the expertise of clinical trial leaders in genomics [3–7,24–31]. Therefore, our understanding of cancer complexity dictates that additional precision oncology methodologies need to be incorporated to enhance patient-treatment matching and prevent the development of treatment resistance.

Transcriptomic analyses have been included in precision oncology trials only recently and infrequently (Table 1) [7,32–34]. Transcriptomics refers to the study of all the RNA transcripts in a cell population, typically by using high-throughput technologies, namely microarrays and RNA sequencing (RNA-seq) [35]. In contrast to analysis of DNA sequencing data, the assessment of RNA status and measurement of transcripts can correlate gene expression with biologic activity and cellular status (Table 2) [32–34,36–64]. Gene expression, in turn, is influenced by genetic and epigenetic factors, such as DNA methylation and histone modifications. Early results of clinical trials suggest that transcriptomic analysis can increase the number of patients matched to drugs [7]. Therefore, transcriptomics is a potentially valuable, though underused, technique for unraveling the underlying mechanisms of cancer and moving towards the implementation of precision oncology.

2. History

Early methods to assess gene expression included Northern blotting, reverse transcriptase quantitative polymerase chain reaction (RT-qPCR), and sequencing of short nucleotide arrays (expressed sequence tags) that were generated from complementary DNAs (cDNAs). However, these methods were developed to evaluate limited numbers of transcripts and are inadequate for comprehensive RNA profiling. Subsequently, serial analysis of gene expression [65] and DNA microarrays enabled the analysis of large-scale gene expression arrays [57,66–68]. With microarrays, investigators can quickly assess the expression levels of thousands of genes simultaneously. Comprehensive profiling of tumor samples, normal tissues, and cancer cell lines yielded a large volume of transcriptomic data. Dedicated databases were developed to store gene expression datasets that were made publicly available to allow their use by other investigators [69,70]. Examples of the implementation of gene expression analysis in clinical practice include the commercially available MammaPrint and Oncotype DX assays, which are used to assess prognosis and/or select treatment in patients with breast cancer [51,71].

3. Transcriptome biology

3.1. Dynamic nature of the transcriptome

In multicellular organisms, the same genes, and thus the same genome, are found in almost every cell. Not every gene is transcriptionally active in every cell, however, and different patterns of gene expression appear in different types of cells. In addition, multiple RNA variants can be produced by a single gene owing to alternative splicing, RNA editing, or alternative transcription initiation and termination sites. The total transcriptional activity, that is, the full range of RNA molecules expressed, is reflected in the transcriptome of an organism. The transcriptome can be represented as the percentage of the genetic code that is transcribed into RNA molecules, which is estimated to be less than 5% of the genome in humans [72]. In contrast to the genome, the transcriptome changes in response to cellular cues. Indeed, an organism's transcriptome varies dynamically depending on many factors, including environmental conditions and developmental stage.

3.2. Epitranscriptomics

Epitranscriptomics, also known as RNA epigenetics, describes the diverse posttranscriptional modifications occurring in cellular RNA. This dynamic processing occurs during RNA maturation under the regulation of RNA-binding proteins. To date, more than 150 types of RNA modifications have been identified, including RNA methylation and editing [73]. While the exact role of these modifications is still under investigation, studies have shown that it extends from maintaining the structure of RNA to regulating critical cell systems and that disruptions in RNA processing are associated with various diseases, including cancer [74,75]. Research focusing on specific modifications has revealed associations between deregulation of RNA processing and

Table 1
Examples of precision medicine trials using transcriptome analysis: Design and outcomes.

Year First/Last authors	Trial name	Trial type	No. of pts consented	Proportion of pts. matched	Biomarker(s)	Outcome	Institute(s)	Comments
2019 [7] Rodon/ Kurzrock	WINTHER	Prospective, navigational	303	35 %	NGS, transcriptomics	Higher matching scores correlated with longer PFS ($P = 0.005$) and OS ($P = 0.03$)	5 countries (Spain, Israel, France, Canada, USA)	First trial in solid tumors to include transcriptomics
2018 [32] Weidenbusch/ Burdach	PROVABES	Prospective	20	45%	Gene expression profiling	Matched treatment was associated with improved OS ($P = 0.001$) and PFS ($P = 0.0011$)	Germany	Refractory pediatric sarcomas
2016 [33] Worst/ Fleischhack	INFORM	Prospective	57	18%	Whole-exome, RNA sequencing	Feasibility of use of comprehensive molecular analysis to guide treatment	20 centers, Germany	Pediatric solid and hematologic malignancies
2016 [34] Oberg/ Kung	PIPseq	Retrospective review of prospectively recruited pts	101	16%	Whole-exome, RNA sequencing	Potentially targetable genomic alterations were identified in 38/ 101 (38%) pts	Columbia University Medical Center, USA	Pediatric solid and hematologic malignancies

Abbreviations: NGS = next-generation sequencing, PFS = progression-free survival, OS = overall survival, pts = patients.

Table 2
Functional implications of transcriptomics.

Implication	Description	Examples/References
Identification of therapeutic targets	Identify actionable molecular alterations using RNA-seq and navigate to therapy based on the result	WINTHER trial [7,32,33,34]
Detection of gene fusions	Detect a hybrid gene formed from 2 different genes as a result of chromosomal rearrangements	Targeted RNA-seq for fusion gene detection [37,38,39,40,41]
Transcript annotation	Discover novel transcripts	Annotating genomes [42,43]
Regulation by miRNA sequencing	Explore the role of miRNA in mRNA regulation	miRNA role in mechanisms of tumorigenesis and as prognostic and predictive biomarkers [44,45,46,47]
Influence of noncoding RNA sequencing	Explore the role of noncoding RNA molecules in mRNA regulation	Noncoding RNA molecules as diagnostic, prognostic, and predictive biomarkers [36,48]
Use of prognostic gene expression signatures	Identify gene expression signatures used to assess patient prognosis	Oncotype DX, [49,71] MammaPrint [50,51]
Use of predictive gene expression signatures	Identify gene expression signatures used to assess the benefit from treatments	Oncotype DX [49,71]
Identification of tissue of origin for cancer of unknown primary (CUP)	Identify the primary tumor site using gene expression profiling	Transcriptome-based prediction of primary tumor in patients with CUP [52,53,54,55,56] [120],
Understanding of tumor heterogeneity	Use gene expression profiling to identify intrinsic cancer subtypes	Tumor classification into subtypes [57,58,59]
Interrogation of biomarkers for immuno-oncology	Use immune cell profiling to explore mechanisms of immune escape and identify immune cell phenotypes	Transcriptomics in cancer immunotherapy [60,61,62] [157,158],
Silencing of the transcriptome	Understand genomic alterations that are silenced at the transcript level	Identification of discrepancies between DNA molecular alterations and RNA expression and effect on therapeutic resistance [142] Silencing may also be exploitable for therapeutic purposes
Interrogation of expression levels	Understand differences between amplified and expressed genes	Gene amplification is not always associated with increased gene expression [64]

cancer progression, aggressive tumor behavior, and deregulated cellular processes [76,77]. Given the oncogenic nature of these modifications, their regulators could be targeted for novel therapies.

3.3. Functional uses of transcriptomics

3.4. Identification of therapeutic targets

Transcriptomic data have been incorporated into many different tumor molecular profiles to increase the number of targetable molecular alterations and provide additional therapeutic options to patients with advanced cancer [7,32–34,78,79]. In one study, gene expression profiling of longitudinally collected primary breast tumors and metastatic lesions identified several highly targetable genes [78]. Administration of therapeutic agents against these alterations in patient-derived xenograft models led to a statistically significant antitumor response vs. controls. In another study of 1049 children and young adults with *de novo* acute megakaryocytic leukemia, RNA-seq revealed druggable targets[79]. Importantly, in our WINTHER precision medicine clinical trial, which prospectively assessed both genomics and transcriptomics in diverse solid malignancies, the use of transcriptomic analysis increased the number of targetable -omic changes by a third over NGS [7].

3.5. Detection of gene fusions

Conventional cytogenetic analyses, including fluorescence in situ hybridization and RT-qPCR, have been widely used for fusion gene detection. However, these methods are designed to discern the presence of specific known gene fusions, not identify novel ones. Newer techniques and algorithms have been developed for the performance of wide-scale RNA-seq to detect novel gene fusions [38–40].

3.6. MicroRNA sequencing

Transcription is the process by which individual genes are copied into RNA molecules to build the transcriptome. The translation of these RNA molecules into proteins constructs the proteome. The RNA content of a cell includes coding and noncoding RNA. The coding RNA, which makes up the transcriptome, consists of messenger RNAs (mRNAs). The noncoding RNAs primarily include ribosomal RNAs (rRNAs), which are components of ribosomes (the structures on which protein synthesis takes place), and transfer RNAs (tRNAs), which are small molecules that are involved in protein synthesis by carrying amino acids to the ribosome and ensuring that they are linked together in the order specified by the nucleotide sequence of the mRNA that is being translated into a protein. In addition to rRNAs and tRNAs, short regulatory non-coding RNAs, including piwi-associated RNAs, endogenous short-interfering RNAs, and microRNAs (Fig. 1), have received significant attention due to their role as regulators of gene expression.

3.7. Regulation by microRNAs

Over the past decade, several studies have focused on revealing the microRNA (miRNA) repertoire [80–83]. These small non-protein-coding RNA molecules (18–25 nucleotides) are capable of controlling gene expression by binding to mRNA targets, thus interfering in the final protein output. MiRNAs have been recognized as major regulators of biological features including proliferation [84,85], migration [86], and apoptosis [87]. MiRNA profiling has been successfully used to molecularly classify tumors [88,89], to assess the prognosis of patients with different tumor types [90–92], and to predict the development of resistance to treatments [93–95]. MiRNAs and miRNA mimics are currently being evaluated in clinical trials as therapeutic agents (NCT02369198, NCT01829971, NCT02580552). Intriguing data have emerged from the study of circulating miRNAs. Studies show that miRNAs, encapsulated in exosomes, are released from cells and can be detected in biological fluids [96,97]. The noninvasive assessment of circulating miRNAs is an appealing approach for disease monitoring and diagnosis. Several studies are investigating the role of circulating miRNAs as cancer diagnostic and prognostic biomarkers [98].

3.8. Noncoding RNA sequencing

In addition to miRNAs, gene expression profiling can reveal other molecular elements, including other noncoding RNAs. Noncoding RNAs are transcribed from non-protein-coding regions of the genome and have a wide range of regulatory functions [99,100]. Depending on their transcript size, they are categorized as small (< 200 bp) or long noncoding RNAs (lncRNAs; > 200 bp, up to ~100 kb). Noncoding RNAs have been shown to play an important role in cancer, and noncoding RNA expression profiling has diagnostic, prognostic, and predictive value in patients with solid and hematologic malignancies [36,101].

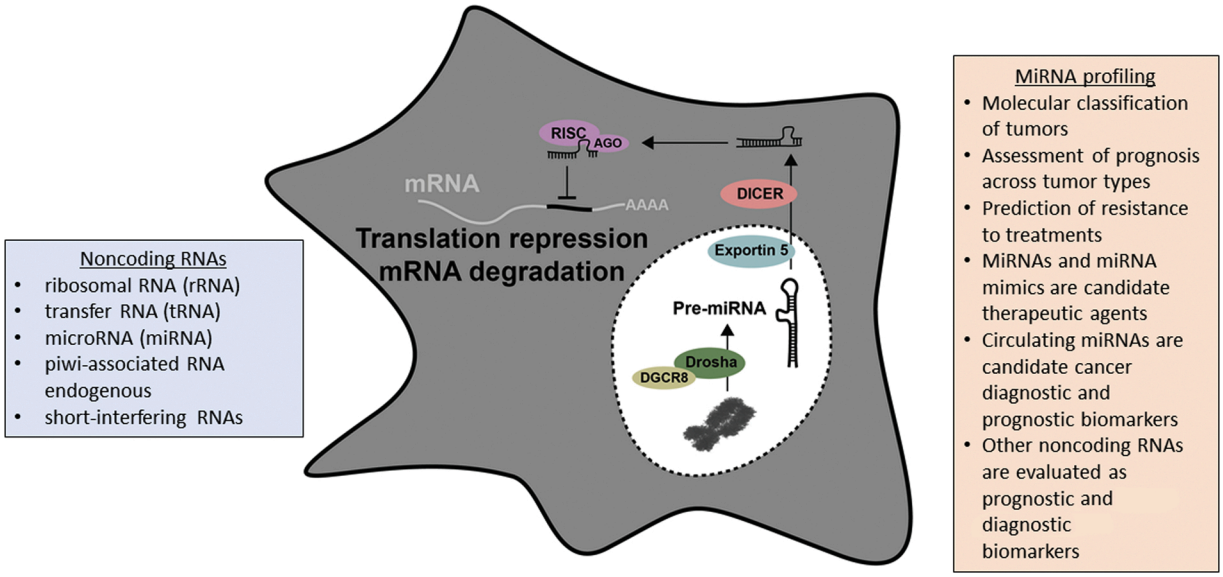

Fig. 1. Noncoding RNAs. The role of noncoding RNAs in cancer diagnosis, prognosis, prediction of response to cancer therapy and disease monitoring is currently under investigation. MicroRNAs (miRNAs) are noncoding RNAs that play an important role as regulators of gene expression. A primary miRNA transcript is cleaved by the microprocessor complex Drosha-DGCR8 in the nucleus. The resulting pre-miRNA is exported from the nucleus. In the cytoplasm, Dicer cleaves the pre-miRNA hairpin to its mature length. The mature miRNA is loaded into the RNA-induced silencing complex (RISC), where it guides RISC to silence target mRNA through mRNA degradation or translational repression.

Recent studies show that circulating noncoding RNA levels in body fluids (eg, serum, urine) differ between individuals with and without cancer [101–104]. These data suggest that noncoding RNAs could be used as diagnostic biomarkers in cancer screening. Other investigators have shown that profiling of noncoding RNAs has prognostic utility in patients with cancer [36,101]. Finally, studies are currently evaluating the role of circulating noncoding RNAs in predicting response or resistance to various treatments. Preliminary data suggest that this approach might provide useful insights with which to identify patients who are likely to have a response to therapy [105–108]. The clinical appeal of the use of noncoding RNAs and miRNAs as biomarkers is that they can be obtained noninvasively via liquid biopsies. However, the clinical utility of these approaches has yet to be prospectively defined and validated.

3.9. Prognostic gene expression signatures

Several studies have evaluated the presence of prognostic biomarkers through transcriptomic analysis. Gene expression signatures are alterations in the expression of single genes or sets of genes with a validated association with disease prognosis, therapeutic benefit, or cancer diagnosis. Despite recent technological advances, robust molecular prognostic biomarkers are still lacking in clinical practice. As a result, there is a great need to improve risk assessment in patients with cancer in order to identify patients at high risk of recurrence or death. When identified, these patients might be treated more aggressively or with different therapeutic strategies.

To improve risk stratification, prognostic multigene assays have been developed and validated in lung [109], breast [51,57,110], colon [111, 112], and other tumor types [113–117]. These gene expression signatures provide prognostic information independently of clinicopathologic features and have been shown to improve the stratification of patients based on risk of recurrence. However, only a few, such as Oncotype DX in breast cancer, are recommended in international patient management guidelines for use in clinical practice to predict whether a patient is likely to have a recurrence of the disease [71]. Future studies might reveal the prognostic value of additional gene expression signatures in different tumor types.

3.10. Predictive gene expression signatures

Other studies have employed transcriptome analysis to identify gene expression signatures that can predict response to specific cancer therapies [118,119]. However, only a small number of predictive signatures have been validated in prospective randomized clinical trials. Therefore, only a few gene expression signatures with verified clinical validity are used in daily practice. For instance, the validated predictive model Oncotype DX is often used in patients with hormone-receptor–positive, human epidermal growth factor receptor type 2–negative, early-stage breast cancer to predict benefit from adjuvant chemotherapy [71]. It is critical that these signatures are robust, highly reproducible, and validated in diverse populations beyond the tightly controlled environment of clinical trials.

3.11. Classification of cancer of unknown primary

Transcriptomics holds promise as an additional tool for the accurate classification of cancer of unknown primary (CUP). Several investigators have explored gene expression profiles and revealed biomarkers indicative of the origin of the tumor in patients with CUP [52–56,120]. In one study, transcriptome analysis of 16 674 tumors corresponding to 22 tumor types revealed a 154-gene expression signature that aided the identification of tumor origin [120]. Independent validation of the signature was successfully performed using 9626 primary tumors. In another study, a cancer type classifier was developed using gene expression data from more than 10 000 tissue samples from 30 tumor types [56]. The accuracy of the classifier was high (77 %–88 %) and varied according to the primary tumor type, the purity of the tumor sample, and the site of tumor tissue (primary or metastatic). Finally, computational algorithms have been employed to mine RNA expression datasets and identify diagnostic classifiers [55]. Gene expression profiling can be incorporated into diagnostic algorithms for patients with CUP to increase rates of accurate classification and improve understanding of patients' prognoses. However, in randomized trials, treating CUP according to tissue-of-origin signatures did not effectively improve outcomes [121,122].

3.12. Assessment of tumor heterogeneity

Investigators exploring the intratumor heterogeneity of renal tumors (primary and corresponding metastatic sites) demonstrated that tumors are not only genomically but also transcriptomically heterogeneous [123]. Specifically, they showed that gene expression signatures suggestive of good and poor prognoses can be identified within the same tumor. Others explored tumor heterogeneity between foci of multifocal or multicentric invasive lobular breast carcinoma and observed heterogenous transcriptional profiles in expression analysis of 730 genes [124]. More recently, studies have incorporated single-cell RNA-seq methods to explore tumor heterogeneity in detail [59,125–127].

3.13. Prediction of response to immuno-oncology

Despite unprecedented improvement in patient outcomes by the use of immune checkpoint inhibitors, mechanisms of resistance significantly limit the benefit from these treatments. Several genomic alterations are being evaluated as predictive biomarkers for immunotherapy [128–132].

In recent studies, RNA-seq and gene expression analysis have been incorporated to predict responsiveness to immunotherapy. In 1 study, analysis of the genome and transcriptome of melanoma tissue samples identified biomarkers that predicted response to anti-PD-1 therapy [133]. In addition, transcriptomic profiles suggested that innate tumor resistance to anti-PD-1 immunotherapy was associated with mesenchymal and inflammatory tumor phenotypes [133]. In a study of metastatic melanoma tumors, gene expression profiling showed that tumors with *PTEN* loss had lower expression of inflammation-related genes, suggesting that *PTEN* loss could be associated with resistance to immunotherapy [134]. Other studies identified gene expression profiles associated with response or resistance to immunotherapeutic agents [135,136]. Transcriptome analysis has also been used to study how the tumor microenvironment evolves after treatment with immunotherapy [137], as well as tumor immune heterogeneity136] and tumor immune classification [138].

3.14. Transcriptomic silencing

Gene expression silencing is a mechanism of transcriptional regulation in mammalian cells. It is mediated by RNA interference (RNAi), in which a small noncoding RNA associates with RNA-induced silencing complex and degrades target mRNAs [139]. Gene silencing is seen with exogenous (e.g., viral, bacterial) and endogenous (e.g., transgene, transposon) sequences. In addition, RNAi is involved in the regulation of gene expression and other biologic processes. Transcriptomic silencing is used in combination with gene expression profiling to identify novel biomarkers and therapeutic targets [140]. For instance, in 1 study of 9873 prostate tissue samples, gene expression profiling revealed 295 genes that had high mRNA expression in prostate cancer samples. An RNAi-based cell viability assay was incorporated to demonstrate the role of gene silencing in prostate cancer cell lines, and silencing of a candidate gene (*ERGIC1*) led to inhibition of ERG mRNA expression and decreased proliferation of ERG-positive prostate cancer cells [140]. These data suggested that ERG could be tested as a candidate drug target.

RNAi-mediated gene silencing is an appealing approach for use in cancer treatment because it can silence oncogenes and other driver genes involved in cancer cell proliferation, the cell cycle, and tumor progression. In this vein, clinical trials are currently evaluating the clinical benefit from the use of short interfering RNA (siRNA)-based treatments in patients with cancer. PROTACT is a phase II trial assessing the clinical utility of siG12D LODER in combination with chemotherapy in patients with locally advanced pancreatic cancer (NCT01676259). In this study, siG12D LODER, a miniature biodegradable polymeric matrix that encloses siRNAs targeting G12D-mutated *KRAS,* is implanted in the tumor via endoscopic ultrasound. Published data from another phase I/IIa trial evaluating the administration of siG12D LODER as first-line treatment (in combination with chemotherapy) in 15 patients with locally advanced pancreatic cancer showed that this therapeutic approach was safe and well tolerated [141]. Another phase I trial is evaluating the administration of a liposomal agent consisting of siRNAs against EphA2 that are encapsulated into 1,2-dioleoyl-sn-glycero-3--phosphatidylcholine liposomes in patients with advanced cancer (NCT01591356). Both trials are currently recruiting patients.

In addition, RNA-seq, along with NGS, has revealed transcriptomic silencing of DNA mutations in advanced cancers, which has important implications for resistance to targeted therapeutics. In a study of 45 patients with cancer, 86 pathogenic DNA alterations were identified, including 17 (19.8 %) alterations that were not observed at the RNA level [142]. Among these patients, 31 % (14/45) had 1 or more DNA alterations that were not expressed at the RNA level. Examples of genes that had pathogenic DNA alterations not seen at the RNA level included *ALK*, *KDR*, and *GNAS*. On the other hand, alterations involving other genes, including *TP53*, *PIK3CA*, and *FGFR3*, showed 100 % concordance between DNA and RNA. This study concluded that a significant number of patients had DNA alterations that are silenced at the RNA level and that transcriptomic silencing merits additional investigation as a mechanism of therapeutic resistance.

3.15. Interrogation of gene expression levels

Gene amplification has frequently been described as a mechanism leading to carcinogenesis. Even though gene amplification would be expected to correlate with overexpression, there are cases where these phenomena are not associated [64]. In order to characterize a gene amplification as a “driver” alteration, overexpression is required. Gene expression profiling alone or in combination with copy-number variation (CNV) analysis is used to identify candidate driver genes for molecular therapeutic targeting [34,143,144]. In 1 study, investigators analyzed gene CNV and mRNA expression data from The Cancer Genome Atlas project. They identified 42 candidate cancer driver genes and validated their oncogenic activity via siRNA knockdown [143]. In another study, transcriptome and CNV analysis enabled the identification of targetable molecular alterations as well as prognostic and diagnostic biomarkers [34]. Importantly, some studies suggest that the variability in normal transcript levels between tissues and between individuals means that cancer transcript expression levels must be compared to their normal-tissue counterparts for accuracy [7].

Novel bioinformatic approaches in large datasets are essential for exploring and describing the association of gene amplification and expression with mechanisms of carcinogenesis. Launched by the National Institutes of Health in 2010, the Genotype-Tissue Expression (GTEx) Program aims to explore the association between genetic variants and gene expression [145] and consists of a tissue bank with multiple tissue samples from each donor along with whole-genome and RNA-seq data from approximately 960 deceased adult donors. This program provides publicly available data to the research community, which investigators can use in studies of mechanisms of gene regulation, genetic variation and its association with gene expression and disease risk, and novel methods of gene expression analysis [145,146].

3.16. Machine learning

Machine learning, a subfield of artificial intelligence, has been playing an increasingly central role in biomedical and pharmaceutical sciences [147], primarily owing to the need to develop new tools to analyze the influx of complex, heterogeneous, and multidimensional biological datasets. Machine learning approaches can be used to detect key genomic and epigenetic features that can help classify patients who may have different responses to a drug. For this purpose, multiple feature-selection algorithms have been proposed, including filters (e.g.,

Markov blanket filtering), wrappers (e.g., gradient-based-leave-one-out gene selection), and embedded techniques (e.g., block diagonal linear discriminant analysis). Next, a variety of established machine learning algorithms, including artificial neural networks, support vector machines, and decision trees can build predictive drug response models based on the features. As an example, a multilayer perceptron neural network can be trained to generate an output (e.g., whether the patient responds to targeted treatments) in response to a set of input variables (e.g., genetic variations). Most recent developments focus on deep learning methods, a subset of machine learning based on artificial neural networks. Currently, deep learning approaches hold significant promise as they are capable of unsupervised learning using data that are unstructured or unlabeled [148], potentially addressing some of the inherent limitations of machine learning [149].

4. Transcriptomics in clinical trials

The WINTHER trial was one of the first studies to incorporate transcriptional analysis, in addition to genomics, in order to match patients with solid tumors to therapeutic agents (Table 1) [7]. WINTHER was an international clinical trial directed by the Worldwide Innovative Network for Personalized Cancer Medicine (WIN Consortium) and involved centers in 5 countries. The rationale for the trial was the need to expand the identification of predictive biomarkers beyond genomic aberrations. Therefore, a double biopsy of tumor (primary or metastatic) and normal tissue from each patient was performed after study enrollment. Tissue was used for DNA analysis by NGS of 236 cancer-related genes. In addition, the study included gene expression analysis of matched tumor and normal tissue pairs. Transcriptional data were evaluated for treatment selection if genomic data generated no recommended treatment option. The selection of targeted therapy using transcriptomic analysis occurred in a stepwise process as follows: gene expression analysis was performed on tumor tissue and paired normal samples from individual patients. Bioinformatic analysis was performed, and the differential expression of these genes in tumor versus analogous normal tissue was used to select treatment. Details for each overexpressed or under-expressed mRNA were provided, including gene fold changes, expression intensity, and type of molecular abnormality. Each alteration was evaluated as a potential match with a targeted agent on the basis of a knowledge database. The WINTHER database included information on the gene expression "targeting" and efficacy of both registered drugs and drugs evaluated in clinical trials. Treatment selection was based on the recommendations of a clinical management committee. Finally, the trial evaluated the use of an exploratory matching score. The score, which was calculated in a *post hoc*, blinded fashion, was derived by dividing the number of molecular alterations that could be associated with a therapeutic agent by the total number of alterations (for DNA analysis) or by adding the reciprocal of the ranks of each matched drug received by the patient according to the WINTHER algorithm (for RNA analysis).

Of 303 patients who consented, 107 (35 %) patients received 1 or more agents and were evaluable for analysis. Patients were heavily pretreated with a median of 3 prior therapies. Among the 107 evaluable patients, 15 % had stable disease for 6 months or longer, and 11 % had partial or complete responses. Having 2 or fewer previous therapies, an Eastern Cooperative Oncology Group (ECOG) performance status of 0, and a higher matching score were independently associated with longer progression-free survival. ECOG performance status of 0 and a higher matching score were also associated with longer overall survival on multivariate analysis. The WINTHER trial demonstrated that transcriptomic analysis can be an indispensable tool for the navigation of treatment in selected patients. However, the analysis was complex and required bioinformatic expertise.

Other clinical trials focusing on pediatric patients have matched therapies to molecular alterations identified by transcriptomic analysis [32–34]. In 1 study, gene expression profiling was performed in 20 patients with refractory pediatric sarcoma to identify overexpressed genes and deregulated pathways that could be therapeutically targeted [32]. The actionable targets most commonly identified were TOP2A and FGFR1 upregulation. Nine of the 20 patients received a targeted therapy. Patients who received targeted treatments had higher overall ($P = 0.0014$) and progression-free ($P = 0.0011$) survival rates compared to patients who did not.

The Individualized Therapy for Relapsed Malignancies in Childhood (INFORM) study used RNA sequencing in addition to whole-exome and low-coverage whole-genome sequencing in prospectively recruited children with high-risk relapsed or refractory malignancies [33]. An expert multidisciplinary panel prioritized the identified molecular alterations using a customized prioritization algorithm. The algorithm used a 7-step priority scale, ranging from "very low" to "very high," based on the biological relevance, type of alteration, and potential druggability. Investigators created an internal database comprising genes considered to be potential therapeutic targets. Treatment was selected on the basis of physician choice. In the initial report of 52 patients, candidate targetable molecular alterations were identified in 26 (50 %) of the patients, 10 of whom received the respective treatment. Even though patients were heavily pretreated, clinical responses were noted.

The investigators recently reported updated results of the INFORM study. Of 1300 patients who were enrolled at 72 centers, 525 were included in the analysis [150]. A "very high-" or "high-" priority actionable target was identified in 8 % and 14.8 % of patients, respectively. Patients who received targeted treatment had longer progression-free survival compared to patients who received non-targeted therapy (204.5 vs. 114 days, $P = 0.0095$), although no difference was noted in overall survival between the 2 groups. Patients who received treatment matched to a "very high-" priority target had higher time-to-progression ratios (time to progression before/time to progression after enrollment in INFORM) compared to the remaining patients.

The clinical utility of molecular analysis in pediatric tumors was also demonstrated in the Precision in Pediatric Sequencing (PIPseq) Program study [34]. In this study, whole-exome and RNA sequencing were performed in 101 patients with solid and hematologic malignancies. Molecular alterations (variant calls, copy number variations [CNV], fusions, and overexpressed genes) were initially reviewed by a multidisciplinary molecular tumor board comprising molecular pathologists, pediatric oncologists, medical geneticists, bioinformaticians, and cancer biologists. The final report included the following clinically relevant alterations: driver mutations, gain- or loss-of-function molecular alterations in oncogenes and tumor suppressor genes, respectively, and fusions known or expected to be oncogenic drivers. Investigators included "clinically meaningful" molecular alterations, used for diagnosis, prognosis, treatment (as therapeutic targets), refinement of a therapeutic plan, and/or health maintenance interventions. On the basis of the level of evidence for clinical actionability [151], a tiered report of all clinically relevant alterations was provided to the referring physician for treatment selection. Transcriptomic analysis identified diagnostic, prognostic, and predictive molecular alterations in 37 (57 %) of 65 patients. Overall, 15 (23 %) patients received targetable therapeutic agents on the basis of sequencing findings. These studies serve as proof of concept showing that the use of comprehensive gene expression profiling in daily practice is feasible and provides useful diagnostic, prognostic, and predictive data. Targeted therapeutic agents can be matched to molecular alterations, providing clinical responses even in heavily pretreated patients. Finally, future prospective trials validating the clinical utility of this approach are warranted and should also address the cost, time burden, and inconsistent analysis of transcriptomic data. Ongoing clinical trials are exploring the importance of transcriptomics in cancer therapy.

5. Challenges and limitations

The main challenges associated with transcriptomic analyses are related to the handling of tissue samples and the application of advanced computational methodologies. For example, RNA-seq protocols include sample preparation, RNA isolation and selection, and cDNA synthesis and sequencing followed by bioinformatic analysis. Formalin-fixed, paraffin-embedded tissue samples can include RNA that is degraded, fragmented [152], or contaminated [153]. Contamination caused by errors during sample preparation or by inadequate equipment sterilization can lead to the presence of sequence data from a different sample. Additionally, tumor samples can be contaminated by normal cells that infiltrate or surround the tumor. Experimental methods (cell sorting or laser capture micro-dissection) [154] and bioinformatics algorithms aim to eliminate contamination effects [155]. Novel methods have been developed to address the issue of low levels of RNA in archival tissue. Additionally, methodologic artifacts are often encountered in transcriptome analysis and require careful assessment.

Another challenge involves the application of advanced computational methodologies. High-level bioinformatic infrastructure is required to conduct complex analyses of profiling data. For instance, in the WINTHER trial, RNA analysis required the systematic development of an algorithm by bioinformaticians [7]. Therefore, the implementation of transcriptomic analysis in clinical workflows may be more complicated than that of genomic analysis. In addition, reproducibility issues need to be addressed. RNA profiling can be used to compare tumor tissue with normal tissue from the same organ, such as in the WINTHER trial; however, some investigators believe that peripheral blood or buccal swab samples could also be used for comparison. This difference may introduce variability in the interpretation of the results.

Overall, investigators who used transcriptomics in clinical trials developed diverse and complex algorithms for characterizing the actionability of molecular alterations [7,33,34]. Consequently, the use of transcriptomics in clinical practice is arduous and expensive. For instance, in the INFORM study, the average cost per patient for the molecular analysis, including tissue sample shipment, data processing and storage, labor, and general costs, was approximately €7,000 [33]. The time from tissue processing to start of analysis ranged from 0 to 112 days [33]. Therefore, transcriptomic analysis requires significant optimization, validation, and cost decrease in order to be optimally implemented in clinical practice. Standardization of bioinformatic analysis through expert consensus would make the use of transcriptomic analysis in routine clinical practice more consistent.

6. Conclusions and future perspectives

Tumor genomic profiling approaches provide average signatures and a snapshot of the tumor state at the time of biopsy but often do not reflect the complete tumor biology, all tumor components, or the intrinsic heterogeneity of individual cell populations. Innovative emerging techniques, including single-cell transcriptome profiling technologies, will improve our understanding of tumor biology in individual patients and will provide a plethora of translational discovery opportunities [59,125,156]. However, understanding tumor complexity and heterogeneity, as well as the dynamic expression of the genome, requires the incorporation of several newer methodologies. The future of precision medicine lies in the integration of genomics, transcriptomics, proteomics, and epigenetics in order to fully elucidate tumor immune and -omic profiles, optimize comprehensive tumor molecular profiles, and inform treatment decisions. By focusing on the role of transcriptomics in identifying appropriate targeted therapies, clinical trials can validate and enrich the available data demonstrating that the use of transcriptomics can increase the number of patients treated with matched targeted therapy and lead to favorable outcomes, hence providing the next frontier for precision medicine.

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Declaration of Competing Interest

The authors report no declarations of interest.

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