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The emerging value of circular non-coding RNA research in cancer diagnosis and treatment

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Abstract

Circular RNAs (circRNAs) are a recently described class of RNA molecules that have attracted substantial attention as new components of disease mechanisms and as potential biomarkers in multiple diseases, including cancer. CircRNAs are often highly conserved and exhibit developmental stage- and disease-specific expression. Several studies have reported circRNA expression patterns that are associated with specific cancer types and with patient prognosis. Here, we overview the active registered clinical trials that investigate the value of circRNAs as cancer biomarkers and discuss the potential of circRNAs in clinical cancer care. Taken together, circRNAs are actively being investigated as diagnostic, predictive, and prognostic biomarkers, and their potential to serve as therapeutic intervention points motivates ongoing translational and clinical research.

Introduction

Circular RNAs (circRNAs) are RNA molecules generated from canonical splice sites in a back-splicing reaction in which the 3'-end of an exon ligates to the 5'-end of its own or an upstream exon through a 3',5'-phosphodiester bond to create a circular, covalently bonded molecule, in concert with traditional linear splicing reactions (1). The Alu family of repeats is often associated with back-splicing and is responsible for the fate of RNA moiety. Inverted Alu pairs from the same intronic region cause extrusion of themselves, leading to canonical splicing, while the pairs coming from the flanking introns facilitate circularization (2). Through variations in the splicing template, circRNAs may contain exonic or intronic sequences and may interact with cis-acting sequence elements or trans-acting RNAs and RNA-binding proteins.

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Based on the nature of splicing, circRNAs classify into four subtypes: 1) Exonic circRNAs (ecircRNAs), which are derived from single or multiple exons and often located in the cytoplasm; 2) circular intronic RNAs (ciRNAs), which contain only introns and are found in nuclei; 3) exonic-intronic circRNAs (ElciRNAs) which constitute both exons and introns, mostly intronic regions located between exons and found in nuclei and 4) tRNA intronic circRNAs (tricRNAs) that are formed by splicing pre-tRNA introns. Once circularized, they are either retained in the nucleus, secreted through extracellular vesicles, or exported to the cytoplasm, where they may interact with proteins or RNAs or be translated through a cap-independent mechanism (2). Proposed mechanisms of cancer modification described to date include RNA or microRNA sponging, protein scaffolding, modulation of transcription/translation, and serving as translation templates (3).

Methods of circRNA detection vary widely, and thus reliable standards for efficient and reliable detection are being developed (4). High-throughput RNA sequencing from tumor tissue may represent the gold standard for circRNA detection and discovery (2). However, its utility for disease monitoring and routine clinical application can be financially and logistically cumbersome. In addition, much of the RNA-seq data usually contain single-end reads, such as that in cross-linking immunoprecipitation seq (CLIP-Seq) sets. However, combining the methods for RNA-seq and CLIP-seq has been shown to improve the precision of identifying circRNAs from single-end read sequence data significantly. CircRNA algorithms such as Find_circ, circRNADb, CircNet, CSCD, CircAtlas, TSCD, CircView, circBase, CIRCpedia, CIRCexplorer, CircFunBase, Mapsplice, CIRI and CircInteractome are routinely used to identify circRNAs in the datasets independently or in combination (18). Also, methods for specific circRNA detection, e.g., digital droplet PCR, RT-qPCR, isothermal exponential amplification and NanoString nCounter, have been developed as reliable laboratory methods to monitor candidate biomarkers on a larger scale (4).

A growing number of studies have identified a cell, tissue, and developmental-time-dependent expression of circRNAs (5). Importantly, a key feature of circRNAs is increased stability in serum due to the closed ring structure. In addition to this, their high conservation and abundance make them attractive candidates as biomarkers in blood as well as in other less-invasive fluid samples such as saliva. As the complete biological functions of circRNAs are a focus of active research, their levels and dysfunctions have closely been linked to various diseases (1), including cancer. This has led to studies highlighting the value of circRNAs as diagnostic, predictive and prognostic biomarkers in various cardiovascular, neurological, and immune-related diseases.

Clinical circRNA Research

Intriguing pre-clinical and basic science data supports the investigation of circRNAs in clinical decision-making. The value of circRNAs as therapeutic targets and biomarkers begins with an extensive evaluation of data linking their expression to disease. Multiple groups have shown specific circRNAs to be aberrantly expressed in certain cancer types, including both over- and under-expression (6). Most of these studies compare the levels of circRNAs in normal vs tumor tissue; however, the analysis of serum or blood circRNAs, a requirement to use them as non-invasive biomarkers, has emerged (7). Much of the

data characterizing circRNAs as tumor suppressors or oncogenes stems from cell line assays, though initial studies *in vivo* have provided evidence for the roles of circRNAs as therapeutic targets (8).

Though the contributions of circRNAs to tumor suppression and oncogenesis are still in their infancy and in the process of being fully elucidated, ample studies have shown their potential as cancer diagnostic and prognosis tools in various cancer types, including head and neck cancer, esophageal, lung, breast, gastric, pancreatic, colorectal, bladder, endometrium, cervical, hepatocellular carcinoma, leukemia, melanoma and osteosarcoma in variety of animal models as well as in patient cohorts (9,10).

As the understanding of circRNA biology improves and detection capabilities become more readily utilized, the clinical investigation into their importance is also rising. In fact, a significant amount of pre-clinical research has impelled clinical trials targeting circRNAs as therapeutics, yet challenges for delivering or targeting circRNAs remain. This is reflected by a search of clinicaltrials.gov, which shows that of dozen or so current clinical trials, none are therapeutic.

Clinical Trials

An August 2022 review of active clinical trials on circRNAs in ClinicalTrials.gov revealed 14 registered clinical trials, including 5 investigating circRNAs in cancer, as highlighted in Table 1. Shared features of the ongoing clinical investigations include the phase of research (i.e., discovery) and the requirement for subsequent validation in independent cohorts, as well as laboratory investigation for molecular mechanisms of tumor-modifying behavior. We describe each trial below.

1. CIRcular and Non-coding RNAs as Clinically Useful Biomarkers in Pancreaticobiliary Cancers (CIRCUS). Updated March 8, 2021. Assessed Aug 15, 2022. https://clinicaltrials.gov/ct2/show/NCT04584996

   Sponsored by the Royal Surrey County Hospital NHS Foundation Trust, the CIRcular and Non-coding RNAs as Clinically USeful Biomarkers in Pancreaticobiliary Cancers (CIRCUS) is a discovery trial aimed to uncover noncoding RNA links to pancreatic ductal adenocarcinoma (PDAC). The initial phase of the trial will examine eight paired samples of PDAC and normal pancreas tissue during standard-of-care surgery. A panel of dysregulated circRNAs will be established and subsequently investigated prospectively in the trial. These studies will further examine the levels of circRNAs in blood samples to prospectively monitor the disease course after surgery. They will be compared to tumor marker CA19–9, the only widely accepted biomarker for PDAC surveillance after primary therapy or to monitor response to systemic therapy. For patients with available specimens, the authors will make measurements in bile and tumor tissue, while it is expected that control patients will have blood samples only (or bile sample in cases of benign cholecystectomy).

   The unbiased approach taken in these studies is expected to yield circRNA biomarkers correlating to PDAC burden of disease. From a translational

medicine perspective, it is possible that targets for disease-modifying therapy will be uncovered through further laboratory characterization, which will be required for hypothesis-generating discoveries from this dataset. Furthermore, comparing serum levels of circRNAs to a reliable serum marker for disease burden (namely CA19–9) will strengthen the utility of specific circRNAs as biomarkers. Potential limitations include the size of the discovery panel to generate candidate circRNAs, the multiple types of concurrent sample collection (e.g., tumor tissue, blood, bile) and the lack of a clear standard treatment plan for the PDAC patients.

2. Rediscovering Biomarkers for the Diagnosis and Early Treatment Response in NEN (REBORN). Updated Dec 4, 2020. Assessed Aug 15, 2022. https://clinicaltrials.gov/ct2/show/NCT04464122

   The study sponsored by the University of Roma La Sapienza, Rediscovering Biomarkers for the Diagnosis and Early Treatment Response in NEN (REBORN), is a multicentered, controlled, and observational study that aims to collect data from patients of pulmonary and gastro-entero-pancreatic Neuroendocrine Neoplasm (NEN) and study immune profiling, angiogenetic markers and circRNAs.

   The study consists of 3 groups:

   a. Control group- patients affected by other non-malignant endocrine diseases

   b. Neuroendocrine tumor group: patients affected by histologically proven NENs, locally advanced or metastatic, originating from pulmonary or gastro-entero-pancreatic (GEP) tract, candidate to medical therapy

   c. Patients treated with somatostatin analogs or chemotherapy

   In order to avoid the limitation of tissue acquisition, the NEM patients will be evaluated using liquid biopsy samples after 1 and 3 months of first-line treatment. The liquid biopsy samples will be used for cytometric analysis, which would provide insights into the immunological alterations that could represent the signal for the neoplastic spread of the tumor. In addition, the samples would also be evaluated for circRNA sequencing and angiogenesis mediators, especially the modification of the angiogenic mediator sTie2 after treatment.

   The study proposes to utilize a novel approach, i.e., to study circRNA that are taken up by tumor-educated platelets in the form of membrane vesicles as biomarkers for pulmonary and gastro-entero-pancreatic NEN. The study protocols aim to identify the immunological component of the disease, which would be valuable in terms of discerning its clinical presentation.

3. Thyroid Nodule Gene Sequencing in a Danish Population. Updated May 17, 2022. Assessed Aug 15, 2022. https://clinicaltrials.gov/ct2/show/NCT05377736

   Aarhus University Hospital, along with the University of Aarhus and Odense University hospital, are leading the study ‘Thyroid Nodule Gene Sequencing in

a Danish Population' and enrolling its participants by invitation. The study aims to study the thyroid nodules of benign and malignant origin in 30 patients from the Danish population and describe their molecular alterations at the DNA and RNA level, including circRNAs. In addition, the study also includes exploring the DNA methylation and presence of circulating tumor DNA in thyroid tissue and blood samples, respectively.

4. A Study of Blood Based Biomarkers for Pancreas Adenocarcinoma. Updated Nov 11, 2021. Assessed Aug 15, 2022. https://clinicaltrials.gov/ct2/show/NCT03334708

   Memorial Sloan Kettering Cancer Center is leading a prospective discovery study using a panel of patients with pathologic pancreatic processes to uncover novel biomarkers of early pancreatic cancer. In collaboration with Sheba Medical Center, Weill Medical College of Cornell University, Weizmann Institute of Science, 700 patients are planned for accrual into three broad categories and compared to a group of healthy controls:

   a. Locally Advanced or Metastatic Pancreatic Cancer Cohort
   b. Acute Benign Pancreatic Pathology Control Cohort (e.g., acute pancreatitis)
   c. Chronic Benign Pancreatic Path, IPMC & Pancreatic Cyst Ctrl

   While this study does not focus on circRNA exclusively, they are included in a robust group of novel biomarkers to be investigated, including proteins, proteases, functional DNA repair assays, exosomes, stromal elements, circular RNAs (cRNAs) and circulating tumor DNA (ctDNA).

   The strengths of this study are in its patient recruitment goals, range of pathology and standardized sample collection. Presuming an equal distribution of patients recruited to each cohort, the probability of identifying disease-associated circRNAs is increased for PDAC. Furthermore, by using blood samples across all patients, the feasibility of circRNAs as clinical markers will be validated. Finally, through comparative studies, including DNA repair and ctDNA, hypotheses of mechanistic links between circRNAs and tumorigenesis or treatment response will be generated. Specifically linked to the PDAC cohort is the coordination of blood collection with diagnostic surveillance imaging and comparisons to baseline tumor pathology. While circRNAs are not the primary focus of this study, it would be expected that the study would create a robust database in which circRNAs may be analyzed. It is not clear from the published trial design whether specific candidate circRNAs will be assessed or whether these samples will be subjected to high throughput sequencing for all RNAs present in blood samples, and therefore the scale of discovery is unknown.

5. Research on Precise Immune Prevention and Treatment of Glioma Based on Multi-omics Sequencing Data. Updated Mar 24, 2021. Assessed Aug 15, 2022. https://clinicaltrials.gov/ct2/show/NCT04792437

A discovery study based in Huashun Hospital at Fudan University in Shanghai; China aims to uncover novel prognostic markers in glioma patients. In this study, multi-omic technology, including RNA and protein expression analyses, will be performed on the samples collected from 120 patients to identify neoantigens on a per-patient basis. CircRNAs are not the focus of this study; however, the utility of circRNAs in predicting response rates for immunotherapy in glioma patients will be evaluated.

This ambitious study will attempt to utilize an intelligent evolution mode of glioma immunotyping to predict treatment response. While it is not known to what degree circRNA and other ncRNAs will contribute to the model, this study has the potential to identify circRNAs as predictive biomarkers for therapy.

Current and Future Value of CircRNAs in Cancer Care

While we are still in the “early adopter” phase of the clinical research, these highlighted studies represent the burgeoning interest in circRNAs in cancer care and illustrate the initial applications of circRNAs, namely as diagnostic, prognostic and potentially predictive biomarkers. These studies also underscore the need for high-quality prospective registration studies examining circRNA expression patterns in cancer. Only through a detailed examination of circRNA expression in a large number of cancer samples associated with matched normal tissues, somatic mutation patterns, and high throughput RNA and protein expression profiling will valuable associations be made.

To the goal of identifying predictive biomarkers, circRNA expression patterns must be studied in patient populations undergoing standard or controlled trial therapy. This way, specific circRNAs that associate with treatment response will guide investigational trials in which therapy is tailored to circRNA expression profiles or mutation status. We would hypothesize that available biospecimens from large, randomized trials with standard and investigational therapies may be examined for circRNA expression changes that will drive innovation in this field. Finally, ongoing laboratory investigation of the molecular mechanisms of circRNA impacts on tumor behavior will uncover novel therapeutic intervention points and fuel translational discovery.

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Table 1:

Ongoing clinical trials utilizing circRNA as biomarkers

NCT ID	Other ID	Tudy title	Study design	Condition	Study/ start-Completion	Type of intervention	Intervention	Outcome-measures	Status
NCT04584996	IRAS 277406	CIRcular and Non-coding RNAs as Clinically USeful Biomarkers in Pancreaticobiliary Cancer	Observational Model: Case-Control, Time Perspective: Prospective (N=186)	Pancreatic Cancer, Biliary Tract Cancer	Oct 4, 2020-Nov5, 2023			circRNAs for diagnosis Describe circRNAs expression profile Diagnostic features of blood circRNAs circRNAs in other biomaterials Bioinformatics	Recruiting
NCT04464122	Reborn Study	Rediscovering Biomarkers for the Diagnosis and Early Treatment Response in NEN (REBORN)	Observational Model: Case-Control, Time Perspective: Prospective (N=60)	Neuroendocrine Tumors, Neuroendocrine Neoplasm, Neuroendocrine Tumor Grade 1, Neuroendocrine Tumor Grade 2, Neuroendocrine Carcinoma	Sept 14, 2020-Dec 31, 2022	Drug	Somatostatin analog; chemotherapy	To evaluate the modification of the angiogenetic mediator sTie2 after treatment, To evaluate the difference in the angiogenetic mediator sTie2 between patients and controls, To evaluate the difference in the angiogenetic mediator sTie2 between patients and controls, To validate the use of circular RNAs from TEPs in NEN diagnosis, To evaluate the changing in circular RNAs from TEPs in NEN patients after somatostatin analogs treatment, To compare circular and cellular angiogenesis mediators between patients and controls, To evaluate the changing in circular and cellular angiogenesis mediators after treatment, To quantify	Recruiting

NCT ID	Other ID	Tudy title	Study design	Condition	Study/ start-Completion	Type of intervention	Intervention	Outcome-measures	Status
								PBMC subpopulation in patients and controls, To evaluate the modification of PBMC subpopulation in patients after treatment, To evaluate the modification of PBMC subpopulation in patients after treatment, To compare classical neuroendocrine markers serum levels between patients and controls, To evaluate the modification of classical neuroendocrine markers in patients after treatment, To evaluate infectious diseases frequency and severity between patients and controls, To evaluate the difference in quality of life questionnaire in patients and controls, To evaluate the modification in quality of life questionnaire in patients after treatment	
NCT05377736	Thy-sec-da 032022	Thyroid Nodule Gene Sequencing in a Danish Population	Allocation: N/A, Intervention Model: Single Group Assignment, Masking: None(Open Label), Primary Purpose: Diagnostic (N=30)	Thyroid Nodule (Diagnosis), Thyroid Cancer	Mar 1, 2022-Jan 18, 2027	Genetic	Molecular analyses	DNA alterations RNA alterations DNA methylation Circulating tumor DNA	Enrolling by invitation
NCT03334708	17–527	A Study of Blood Based Biomarkers for Pancreas Adenocarcinoma	Observational Model: Case-Control, Time Perspective: Prospective (N=700)	Pancreatic Cancer, Pancreatic Diseases, Pancreatitis, Pancreatic Cyst	Oct 30, 2017-Oct 30, 2022	Diagnostic Test	Blood Draw, Tumor Tissue Collection, Cyst Fluid	Change in biomarkers to determine sensitivity and specificity of the assay to diagnose early	Recruiting

NCT ID	Other ID	Tudy title	Study design	Condition	Study/ start-Completion	Type of intervention	Intervention	Outcome-measures	Status
								stage pancreatic cancer	
NCT04792437	KY2021-059	Research on Precise Immune Prevention and Treatment of Glioma Based on Multi-omics Sequencing Data	Observational Model: Other, Time Perspective: Other (N=120)	Transcriptomics, Radiomics, Glioma	Mar 10, 2021-Sept 30, 2022	Procedure	surgery	Overall Survival (OS), month Progress Free Survival (PFS), month Tumor Mutation Burden (TMB), mutations/mb, derived from Genomic sequencing Reads Per Kilobase Million (RPKM), KB^(−1), derived from Transcriptome sequencing expression level of protein, derived from Protein sequencing expression level of low molecular weight protein, derived from Metabonomics expression level of DNA, derived from Metagenomics frequency of TCR/BCR clone, derived from Immunomics expression level of protein, drived from paraffin-embedded specimens, retrospectively collected.	Recruiting