Computers in Biology and Medicine 188 (2025) 109846

Contents lists available at ScienceDirect

Computers in Biology and Medicine

journal homepage: www.elsevier.com/locate/compbiomed

EasyCircR: Detection and reconstruction of circular RNAs post-transcriptional regulatory interaction networks

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ARTICLE INFO

Keywords:
CircRNA
MiRNA
mRNA
CircRNA-miRNA-mRNA network
Post-transcriptional regulation

ABSTRACT

Circular RNAs (circRNAs) are regulatory RNAs that play a crucial role in various biological activities and have been identified as potential biomarkers for neurological disorders and cancer. CircRNAs have emerged as significant regulators of gene expression through different mechanisms, including regulation of transcription and splicing, modulation of translation, and post-translational modifications. Additionally, some circRNAs operate as microRNA (miRNA) sponges in the cytoplasm, boosting post-transcriptional expression of target genes by inhibiting miRNA activity. Although existing pipelines can reconstruct circRNAs, identify miRNAs sponged by them, retrieve cascade-regulated mRNAs, and represent the regulatory interactions as complex circRNA-miRNA-mRNA networks, none of the state-of-the-art approaches can discriminate the biological level at which the mRNAs involved in the interactions are regulated, avoiding considering potential target mRNAs not regulated at the post-transcriptional level. EasyCircR is a novel R package that combines circRNA detection and reconstruction with post-transcriptional gene expression analysis (exon-intron split analysis) and miRNA response element prediction. The package enables estimation and visualization of circRNA-miRNA-mRNA interactions through an intuitive Shiny application, leveraging the post-transcriptional regulatory nature of circRNA-miRNA relationship and excluding unrealistic regulatory interactions at the biological level. EasyCircR source code, Docker container and user guide are available at: https://github.com/InfOmics/EasyCircR.

1. Introduction

The RNA is a fundamental molecule found in our cells that is essential for a variety of biological functions, including gene regulation, protein synthesis, and transmission of genetic information. Within our cells, RNA manifests in diverse forms, which are usually classified as protein-coding and non-coding sequences. Extensive research has revealed associations between RNA alterations and a significant fraction of diseases [1,2], encompassing both coding and non-coding RNA. Although there is an increasing focus on researching RNA interaction effects [3,4], there remains a gap in understanding the role of specific RNA molecules and how they interact with the molecular environment. Circular RNAs (circRNAs) are a type of non-coding single-stranded RNAs that play critical roles in a variety of biological processes, including cell proliferation, differentiation, and development. Additionally, they have been related, also in a recent review [5], to cancer initiation and progression, and their resistance to endonucleases makes them ideal biomarker candidates. Unlike linear RNAs, long non-coding RNAs, and microRNAs (miRNAs), circRNAs have covalently closed loop structures without free 3' poly(A) tails or 5' caps. They include several miRNA response elements (MREs) which allow them to bind miRNAs and act as competitive endogenous RNAs.

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https://doi.org/10.1016/j.compbiomed.2025.109846
Received 12 March 2024; Received in revised form 6 February 2025; Accepted 10 February 2025
Available online 22 February 2025

By sponging miRNAs, circRNAs can lower the inhibition of miRNA-targeted genes, consequently modulating their expression level [6]. Many disorders, including cancer, have been linked to dysregulation of circRNA-miRNA-mRNA interaction network. Therefore, after circRNA detection [7], it is biologically important to infer interactions between RNA molecules in the form of circRNA-miRNA-mRNA network by predicting the sponged miRNAs and the putatively regulated target genes [8,9]. Several pipelines and tools for RNA-RNA interaction prediction [10–12] and specifically for circRNA-miRNA-mRNA interaction estimation already exist [13,14], and many of them were recently published confirming the importance of studying circRNAs regulatory mechanisms in greater depth. Nguyen et al. [15] recently developed CircNetVis an interactive application that allows the exploration of circRNA-miRNA-mRNA interactions taking as input a circRNA ID, its coordinates or the circRNA sequences in the FASTA format. Moreover, even more recently Fraboulet et al. [16] developed Cirscan a tool that starting from normalized circRNA, miRNA and gene expression matrices, automatically infers circRNA-miRNA-mRNA interaction networks by estimating sponging scores. Despite several tools released, only a few of them allow for the inference of different circRNA regulatory effects between two sample conditions, and none of them takes advantage of the post-transcriptional regulatory nature of the circRNA-miRNA interactions. CircRNAs regulatory analysis typically involves identifying potential target genes by querying databases or exploring if differentially expressed genes between sample conditions are up or down-regulated in response to the presence of specific circRNAs [17,18]. Identifying prospective target genes while neglecting the level at which regulation occurs may be misleading, resulting in a erroneous estimation of regulatory outcomes. In this context, we developed EasyCircR, an R package that allows users to easily perform the entire circRNA regulation analysis and visualize interaction results through an intuitive graphical interface. Unlike Circscan and CircNetVis, the tool reconstructs full-length circRNAs from FASTQ files collected from samples of multiple groups (e.g., Control vs Tumor, Treated vs Untreated), retrieves the genes that are modulated between the conditions of interest, and predicts their binding miRNAs. Differently from any other circRNA analysis tool, to filter out potential unrealistic regulatory interactions, only the post-transcriptionally regulated genes are kept in the analysis. To search for them, EasyCircR applies the exon-intron split analysis [19] to identify variations in transcriptional and post-transcriptional regulation by estimating differences in exonic and intronic changes across different conditions. Finally, EasyCircR provides an interactive R Shiny application where regulatory interactions can be examined individually, filtered based on specific criteria, and exported in textual format for additional research. EasyCircR was tested on two distinct RNA-seq datasets. The first one was collected from diffuse large B cell lymphoma (DLBCL) cell line [20] exposed to Bimiralisib, an inhibitor with preclinical and early clinical anti-lymphoma activity [21] while the second dataset was collected from triple-negative breast cancer (TNBC) slow-cycling and rapid-cycling cells [22]. Interestingly, the DLBCL case study results revealed, in the treated samples, the down-regulation of a tumor promoter circRNA and the up-regulation of circRNAs (Table 1) which induce the positive post-transcriptional regulation of tumor suppressor genes capable of inhibiting the growth and diffusion of tumor cells. Moreover, consistently with what was described in Dong et al. [22], the analysis of slow-cycling and rapid-cycling TNBC revealed the down-regulation of a circRNA of chromosome 10 (Table 2), regulating at the post-transcriptional level several genes involved into the functionality and structure of the extracellular matrix, which has been shown to be altered in slow-cycling tumor cells.

2. Methods

EasyCircR is a powerful tool for identifying circRNA-miRNA-mRNA interactions from RNA-seq data. It offers various options for exploring reconstructed circRNA structures and exporting filtered results for further analysis. EasyCircR performs four key steps: identifying differentially expressed (DE) circRNAs, predicting circRNA-miRNA interactions, studying post-transcriptional regulation, integrating and visualizing data (see Fig. 1).

Starting from RNA-seq FASTQ files, the first step begins with the reconstruction of full-length circRNAs using CIRI-full [23]. CIRI-full uses the reverse overlap (RO) and back-splice junction (BSJ) features to identify low-abundance circRNAs. CIRI-full relies on CIRI2 [24] for circRNA detection [25] and CIRI-AS [26] to detect cirexon (circRNA's exon) and alternative splicing events in circRNAs. Since CIRI-AS cannot process sequencing reads with different lengths, EasyCircR accepts reads of the same length or trims reads using the Trimmomatic tool [27] according to the user preferred length supplied as a parameter to the package. To preserve the highest number of reads, EasyCircR includes a visualization option that allows exploring the distribution of read lengths before trimming. After circRNA detection, EasyCircR computes circRNA differential expression analysis (DE) by using limma+voom [28] R packages. Given CIRI-full ability to detect circRNAs at the isoform level, DE is performed considering alternative splicing of transcripts. Additionally, EasyCircR provides the functionality to map significant differentially expressed circRNAs to their host gene annotation [29] supplied by the circBank database [30], which contains over 140,000 human-annotated circRNAs. In the second step, EasyCircR computes the circRNA-miRNA interactions by using TargetScan [31], which predicts miRNA response elements (MREs) from the entire circRNA sequences and it is considered one of the most robust sequence-based tools for miRNA targets prediction [32]. As the third step, EasyCircR identifies, through the EISA algorithm from the eisaR package [19], the post-transcriptionally regulated genes. Gaidatzis et al. established an association between the number of reads that map the intronic and exonic regions of a gene and the type of regulation in progress. In particular they observed that, considering two sample conditions, if we have a significant difference in the number of reads that map the exonic regions of a gene we are dealing with regulation at the post-transcriptional level. On the other hand, if the number of both reads mapping a gene's intronic and exonic regions differs significantly, we are dealing with an alteration at the transcriptional level. As a result, eisaR quantifies genes starting from FASTQ input files and sorts them based on differences in exonic and intronic read counts between conditions. Only statistically significant genes (FDR < 0.05) are kept as potential circRNA target genes. Finally, circRNA-miRNA-mRNA interaction networks are constructed by retrieving the miRNAs targeting the genes returned in the third step with multiMiR [33]. Interactions are visualized through a Shiny app including multiple types of feature filters as well as the option of investigating reconstructed circRNA structures and exporting filtered results in textual format for further analysis. All the EasyCircR features and tools comprising the package workflow, along with their respective inputs and outputs, are summarized in Fig. 2.

3. Using EasyCircR Shiny app

As outlined in the methodology section, after completing computational analysis with EasyCircR, the package provides a Shiny web application for visualizing the results. Fig. 3 provides an overview of the web-app main visualization functions. The application comprises three tables containing comprehensive listings of identified circRNAs, miRNAs, and mRNAs derived from the analysis (Fig. 3a).

When a specific entry is selected from any of these tables (such as a gene, miRNA, or circRNA), only the relationships associated with that entry are displayed. Activating the "View" button displays the structure of the selected circRNA from the CIRI tool [23], as well as the regions where miRNAs are bound (Fig. 3b,c). As one goes the web application interface, a filter section becomes available. This filter technique allows interactions to be refined using designated criteria such as the source database, the circRNA chromosome, the start or termination positions

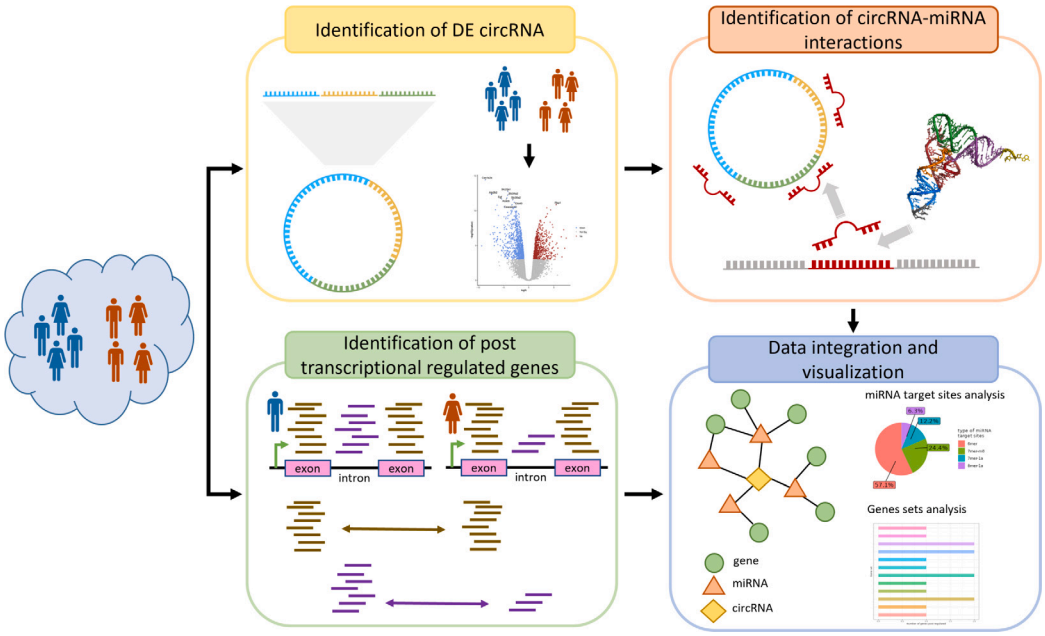

Fig. 1. A graphical overview of EasyCircR pipeline: starting from RNA-seq data collected from two sample groups, the package quantifies the circularRNAs, identifies those differentially expressed between the two sample conditions and, for each of them, detects the binding microRNAs. To conclude, EasyCircR infers, starting from the sequencing data, the post-transcriptional regulated genes and constructs putative circularRNA-microRNA-gene regulative interactions that could be explored and visualized through an intuitive Shiny app.

of the circRNAs, and many more. In addition to filter options, the application includes graphical representations depicting the distributions of miRNA target site types and gene sets (Fig. 3d). A navigable table at the bottom of the web application page contains an exhaustive list of potential associations. This table is dynamic, updating in real-time based on the applied filters. Users can also export the table in a variety of formats (csv, xlsx), facilitating further in-depth analyses. Finally, EasyCircR allows to visualize as network plot all the molecules composing a circRNA-miRNA-mRNA regulatory network (Fig. 3e).

4. Results

To show the utility of EasyCircR, we tested the package in two different scenarios. In a first case study, we investigated RNA-seq data from DLBCL cell lines [20] treated with Bimiralisib. Our findings confirmed the post-transcriptional and anti-lymphoma regulatory effect of the drug via circRNA-miRNA-mRNA interactions. By comparing treated with untreated samples of the TMD8 cell line, EasyCircR identified 10 significantly differentially expressed circRNAs that potentially sponge 1214 miRNAs and regulate 135 genes at the post-transcriptional level. The analysis revealed circRNA-miRNA-mRNA interactions that support the biological findings discussed in [20]. In the treated samples, EasyCircR highlighted the down-regulation of hsa_circXPO1_001 circRNA, a known tumor growth promoter [34], and the up-regulation of hsa_circUBXN7_003 and four circRNAs on chromosome 7 with binding sites for miR-429 and miR-148b-3p (Table 1). These two miRNAs have been identified as cancer biomarkers [35], promoting cancer cell proliferation and migration. Moreover, miR-148b-3p is also known for its ability to target tumor-suppressor genes, negatively affecting the PI3K/Akt pathway [36] and promoting tumor growth. Finally, we investigated the post-transcriptionally regulated genes that compose the circRNA-miRNA-mRNA interaction network, discovering that some of them are involved in the Reactome pathway "Cellular response to starvation" [37], as well as in the regulation of translation processes, such as RPS23 and RPL41. To further confirm the efficacy of EasyCircR and the biological significance of investigating the regulatory functions of circRNAs, we evaluated RNA-seq data collected from three slow-cycling and three rapid-cycling triple-negative breast cancer (TNBC) samples [22] (GSE267759). Slow-cycling tumoral cells are resistant to chemotherapy treatments due to their dormant nature, which has been associated to modifications in the extracellular matrix [38,39]. Our analysis revealed a potential regulatory cascade that supports the findings discussed in the article [22]. In slow-cycling cells, we found the down-regulation of a circRNA located on chromosome 10 (Table 2), which regulates 55 genes at the post-transcriptional level. These genes are associated with Gene Ontology cellular components such as Extracellular Vesicles and Exosomes, as well as with the molecular function of cell adhesion molecule binding, further confirming the alterations at the extracellular matrix level in slow-cycling tumor cells. Furthermore, the modulated circRNA can harbour two miRNAs miR-15a-3p and miR-224-5p known for their breast cancer cells proliferative functions [40,41]. In conclusion, both case studies emphasized the need of investigating the post-transcriptional regulatory role of circRNAs in order to acquire a better understanding of the potential sources of individual gene expression changes.

5. Conclusion

We have developed a new R package named EasyCircR, specifically designed for comprehensive circRNA regulatory analysis. EasyCircR leverages the post-transcriptional regulatory properties of circRNAs to expand our understanding of their regulatory roles, providing reliable and consistent relationships between circRNAs, miRNAs, and mRNAs. Starting from RNA-seq FASTQ files of two independent sample groups, our tool detects differentially expressed circRNAs between these conditions. It then identifies the miRNAs sponged by these circRNAs and determines their cascade target genes subjected to post-transcriptional regulation. The analysis outcomes can be displayed, explored and downloaded through a Shiny app included in the package. We applied EasyCircR in two different scenarios. First, we investigated diffuse large B cell lymphoma cell lines treated with Bimiralisib, successfully validating the drug's post-transcriptional regulatory impact. Second, we used the package to analyze slow-cycling and rapid-cycling triple-negative breast cancer cells (TNBC) suggesting how extracellular matrix alterations in slow-cycling cancer cells could be due to the post-transcriptional regulative function of circRNAs. Although the findings

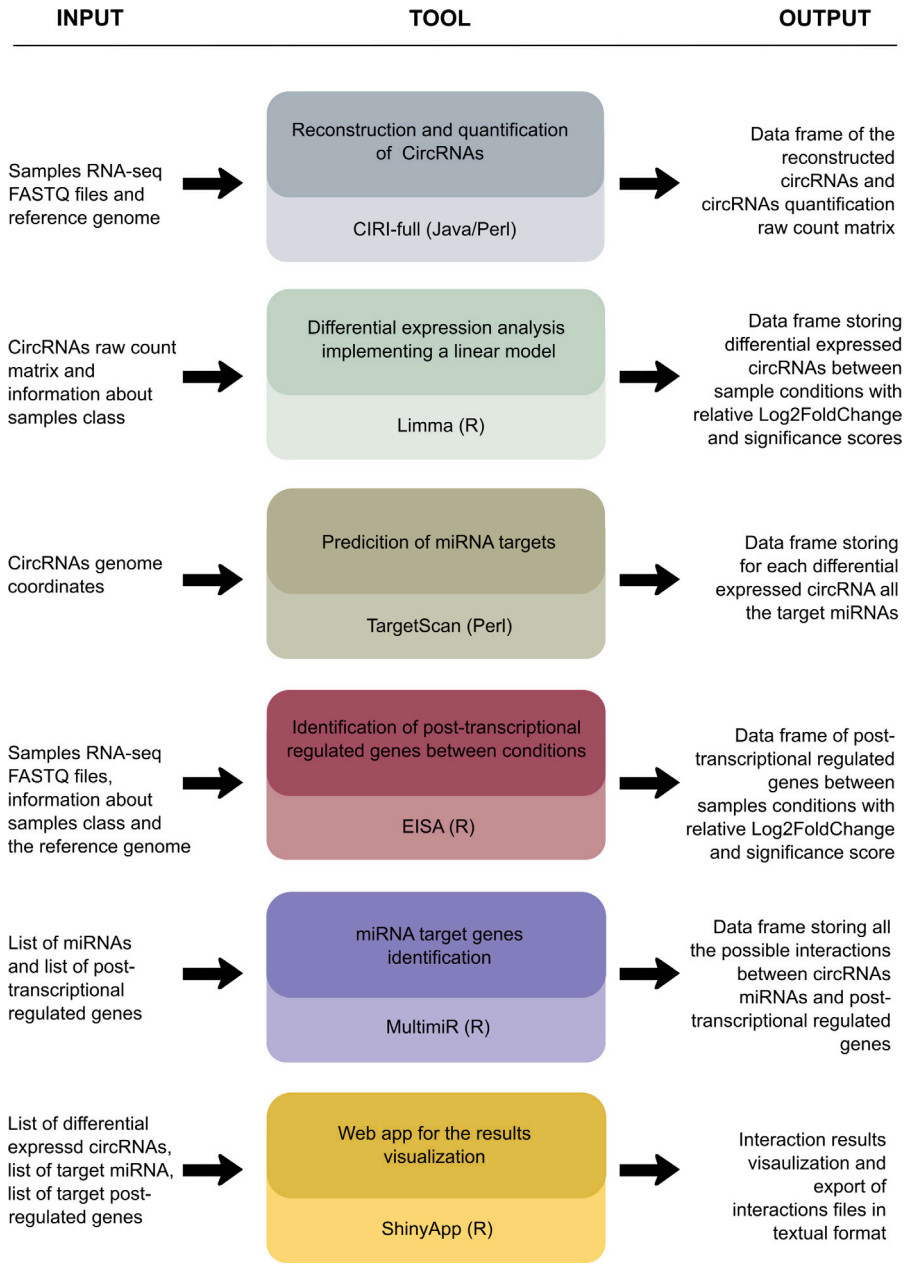

Fig. 2. Schema of EasyCircR tasks, including the tools implemented to perform each task and relative inputs and outputs.

Table 1
Table showing the significant differential expressed circRNAs of DLBCL case study. For each circRNA are shown the Ensembl ID and symbol of the gene where the circRNA is located, the statistics results of circRNAs differential expression analysis including log fold change, average expression, p-value and adjusted p-value, the validated name of the circRNA retrieved from circBank and the number of harboured miRNAs.

circRNA (BSJ ID)	Ensembl ID	Gene name	logFC	AveExpr	p-value	adj.p-value	Validated	#miRNA
1:247155566|247159813:-:1	ENSG00000196418	ZNF124	−5.121	11.153	2.459347e−12	1.337e−09	hsa_circZNF124_005	201
2:61522611|61533903:-	ENSG00000082898	XPO1	−4.050	11.013	6.593850e−07	1.793e−04	hsa_circXPO1_001	170
3:196391813|196403019:-	ENSG00000163960	UBXN7	3.052	12.879	1.807034e−06	2.579e−04	hsa_circUBXN7_003	129
7:116092137|116112038:-:3	ENSG00000105967	TFEC	3.379	11.069	3.429041e−06	2.579e−04	–	220
7:116092137|116112038:-:7	ENSG00000105967	TFEC	3.379	11.069	3.429041e−06	2.579e−04	–	235
7:116092137|116112038:-:11	ENSG00000105967	TFEC	3.379	11.069	3.429041e−06	2.579e−04	–	296
7:116092137|116112038:-:15	ENSG00000105967	TFEC	3.379	11.069	3.429041e−06	2.579e−04	–	311
18:9182382|9221999:+	ENSG00000265257	–	4.566	11.429	3.793828e−06	2.579e−04	hsa_circANKRD12_009	347
12:116230533|116237705:-:13	ENSG00000265257	MED13L	3.251	10.809	3.062081e−05	1.388e−03	hsa_circMED13L_005	229
12:116230533|116237705:-:16	ENSG00000265257	MED13L	3.251	10.809	3.062081e−05	1.388e−03	hsa_circMED13L_005	269

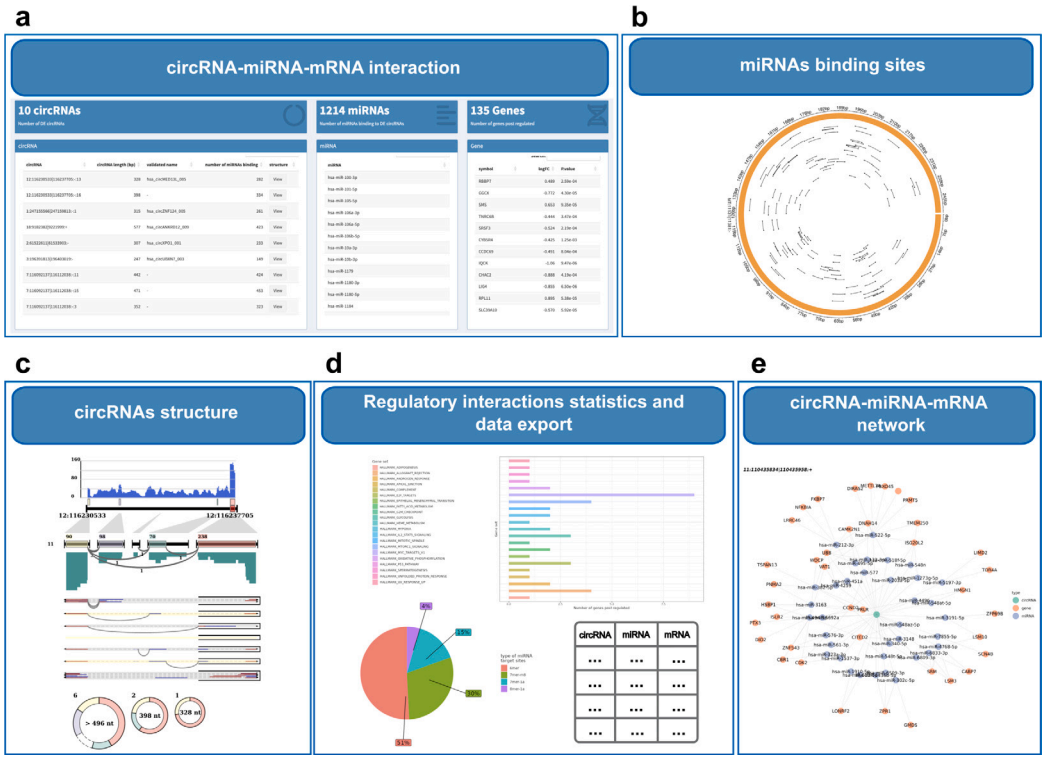

Fig. 3. Overview of the main visualization features provided by the EasyCircR Shiny app. (a) The app allows to visualize the list of all the molecules identified from the analysis including differential expressed circRNA, binding miRNAs and genes regulated at the post transcriptional level. (b) User can visualize in which region of the circRNA are the miRNAs binding site. (c) CircRNA structure provided by Ciri-Full can be explored. (d) The web app provides the full table of interactions and several plots to explore the properties of the molecules involved into the regulatory interactions including the distributions of the different types of miRNAs response elements and the list of biological pathways in which the post-transcriptional regulated genes are involved. (e) The tool also allows to visualize the complete circRNA-miRNA-mRNA regulatory network.

Table 2
Table showing the significant differential expressed circRNA identified in the TNBC case study. The table includes the Ensembl ID and symbol of the gene where the circRNA is located, the statistical results of the circRNAs differential expression analysis (log fold change, average expression, p-value, and adjusted p-value), the validated circRNA name from circBank, and the number of harbored miRNAs.

circRNA (BSJ ID)	Ensembl ID	Gene name	logFC	AveExpr	p-value	adj.p-value	Validated	#miRNA
10:78037218|78037304:+	ENSG00000138326	RPS24	−2.891	14.155	0.00248	0.0499	–	45

found require further biological validation, we uncovered promising regulatory associations that emphasize the necessity of investigating the circRNA landscape for future research. EasyCircR performance in terms of running time is closely tied to the usage of the CIRI-full tool for identifying and quantifying circRNAs. Moreover, in terms of detection power, the variability in sensitivity among circRNA detection tools is well-acknowledged [7]. To address this, EasyCircR will adopt a multi-tool approach within its pipeline to improve the reliability of results through a consensus analysis. Additionally, our future goals include providing the option to integrate miRNA sequencing datasets into the EasyCircR analysis, enabling the investigation of interactions involving miRNAs that are effectively expressed within the tissue. This will offer more realistic insights into regulatory interactions. Finally, future work could extend our current version of the software to explore the recently revealed capability of circRNAs to code for proteins [42] and to accept as input long sequencing reads for which the identification of circRNA could be challenging [43]. As previously stated, EasyCircR user-friendly design makes it ideal for exploring the non-coding part of the genome, specifically the regulatory roles of circRNAs. The potential of circRNAs as biomarkers is well-documented in the literature with a growing volume of research [44] highlighting their involvement in the regulation of biological processes associated with numerous diseases. Given the biological relevance of circRNAs, EasyCircR regulatory analysis has the potential to reveal novel disease biomarkers and contribute to the identification of new therapeutic targets.

CRediT authorship contribution statement

Antonino Aparo: Writing – original draft, Visualization, Validation, Software, Methodology. **Simone Avesani:** Writing – original draft, Visualization, Validation, Software, Methodology. **Luca Parmigiani:** Visualization, Validation, Software, Methodology, Investigation, Conceptualization. **Sara Napoli:** Conceptualization. **Francesco Bertoni:** Conceptualization. **Vincenzo Bonnici:** Writing – review & editing, Methodology, Conceptualization. **Luciano Cascione:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Investigation, Conceptualization. **Rosalba Giugno:** Writing – review & editing, Supervision, Project administration, Conceptualization.

Ethical Statement

Hereby, we assure that for the manuscript "*EasyCircR: Detection and reconstruction of circular RNAs post-transcriptional regulatory interaction networks*" the following is fulfilled:
(1) This material is the authors' own original work, which has not been previously published elsewhere.
(2) The paper is not currently being considered for publication elsewhere.
(3) The paper properly credits the meaningful contributions of co-authors and co-researchers.
(4) The results are appropriately placed in the context of prior and existing research.

(5) All sources used are properly disclosed (correct citation). Literally copying of text are indicated as such by using quotation marks and giving proper reference.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors would like to thank the Funder: Project funded under the National Recovery and Resilience Plan (NRRP), Mission 4 Component 2 Investment 1.4 - Call for tender No. 3138 of 16 December 2021, rectified by Decree No. 3175 of 18 December 2021 of the Italian Ministry of University and Research, funded by the European Union – NextGenerationEU; Award Number: Project code CN_00000033, Concession Decree No. 1034 of 17 June 2022 adopted by the Italian Ministry of University and Research, CUP B33C22000660001, Project title "National Biodiversity Future Center - NBFC"; the #NEXTGENERATIONEU and the Italian Ministry of University and Research, National Recovery and Resilience Plan (PNRR), projects MNESYS (PE0000006), HEAL ITALIA (PE0000019), and the "PREPARE" project (n. F/310130/05/X56–CUP: B39J23001730005)–D.M. MiSE 31/12/2021.

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