The Potential CircRNAs in Imatinib Resistance of GIST

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Research

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Abstract

Background: Recent studies have found that circular RNA is an abundant RNA species, belongs to part of the competing endogenous RNA network(ceRNA), which was proved to play an important role in the development, diagnosis and progress of diseases.

Methods: We determined the expression of circular RNAs in paired normal gastric tissues(N), primary GIST (gastrointestinal stromal tumor) tissues (Y or YC) and imatinib mesylate secondary resistance GIST tissues(C) with microarray and predicted 8677 dysregulated circular RNAs.

Results: We identified 15 circRNAs was up-regulated and 8 circRNAs were down-regulated in C group. Gene ontology (GO)and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis indicated that these host linear transcripts that differentially express circular RNAs are involved in many key biological pathways., predicting the potential tumor-genesis and drug resistance mechanism was related with HIF-1 pathway, later we draw the cirRNA-miRNA-mRNA network involved in the HIF-1 pathway, found that several dysregulated circRNAs and the relationship between circRNA-miRNAs-mRNA, such as circRNA_06551, circRNA_14668, circRNA_04497, circRNA_08683, circRNA_09923(Green, down-regulation) and circRNA_23636, circRNA_15734(Red, up-regulation).

Conclusions: Taken together, we identified a panel of dysregulated circRNAs that may be potential biomarkers even therapy relevant to the GIST, especially imatinib secondary resistance GIST.

Background

Gastrointestinal stromal tumor (GIST) is the most common gastrointestinal mesenchymal tumors[1].The pathogenesis of GIST is mainly due to the protooncogene tyrosine kinase receptor KIT or PDGFRA- α gene activation mutation, as a result, abnormal activation of downstream signaling pathways, cell proliferation, apoptosis is inhibited and transformed into tumor cells[2, 3]. According to the type of gene mutation, GISTs were divided into KIT mutant (80%~85%), PDGFRA mutant (5%~10%) and wild type (10%)[4]. This provides a theoretical basis for the molecular targeted drug imatinib (IM) mesylate. IM is a drug that inhibits the activity of tyrosine kinase of KIT and PDGFRA gene, which is effective in the treatment of advanced GIST, and achieves satisfactory results[5]. However, more and more studies have found that IM occurs primary and secondary resistance in the treatment process of GIST, and the mechanism of drug resistance is complicated. Thus, targeting of KIT inhibitors alone does not benefit all GIST patients, especially in patients with wild-type GIST.

CircRNAs is a class of non coding RNA (ncRNAs) molecules usually composed of more than one exon, formed mainly by back-splicing and covalent binding, which misinterpreted as a rare event (splicing error)[6]. CircRNA functions as competitive endogenous RNA (ceRNA) efficiently targeting miRNA and inhibition miRNA transcription like a molecular sponge, indirectly regulate mRNA expression[7]. CircRNA could regulate downstream gene expression through targeting miRNA and it may play an important effect in disease mechanisms. Inhibition of circ_0067934 could block metastasis, proliferation, and EMT in NSCLC cells via miR-1182/KLF8 axis [8]. Regulation of Circ_0014130 could inhibit cell apoptosis in NSCLC cells by sponging miR-136-5p[9]. Over-expression of circ_0004015 could enhance resistance to gefitinib in NSCLC cells through miR1183- PDK1 axis[10].

In this present study, we investigated the differentially expressed circRNAs using human circRNAs array in GIST tissues. We firstly demonstrated circRNAs of imatinib mesylate secondary resistance GIST and primary GIST, and found a correlation of circular RNA abundance and imatinib mesylate relapse resistance and make a function prediction.

Methods

Patients and clinical specimen

The tumor tissues (all the tumor are greater than 5 cm) and matched normal gastric tissues were collected from 9 patients (three normal gastric tissue samples (N), three primary GIST samples (Y or YC) and three GIST samples secondarily resistant to IM (C)) who undergoing surgical resection at the First Affiliated Hospital of Wenzhou Medical University. All tissues samples were determined to be malignant GIST by pathology and immunohistochemistry (CD117(+), CD34(+), mitotic phase greater than 5/50HPF) and then stored in liquid nitrogen for further use. This study was approved by the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University and written informed consent was given before operation.

RNA extraction, library construction and sequencing

Total RNA was extracted using the mirVana miRNA Isolation Kit (Thermo) following the manufacturer's protocol. RNA purity and quantification were evaluated using the NanoDrop2000 spectrophotometer (Thermo). RNA integrity was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies). Samples with RNA Integrity Number (RIN) ≥ 7 were used for subsequent library construction. After rRNA depleted and linear RNA digested by Ribonuclease R (Epicentr), library construction using TruSeq total RNA and Ribo-Zero Gold (Illumina). Then we sequenced these libraries on the Illumina sequencing platform (HiSeq X Ten) and 150 bp paired-end reads were generated. circular RNA sequencing analysis and conducted by OE Biotech Co., Ltd. (Shanghai).

Bioinformatic analysis

Raw data (raw reads) of fastq format were firstly processed using the Trimmomatic software[11]. Clean data (clean reads) were obtained by removing reads containing adapter, reads containing ploy-N and lower quality reads from raw data. Clean reads were aligned to the reference genome GRCh38 utilizing the MEM algorithm of Burrows-Wheeler aligner (BWA, version 0.7.5a)[12]. Based on the junction reads and GT-AG splicing signals, circRNAs were verified using CIRI2 software[13]. Combined with annotation information in protein database, circRNAs were [annotated](#) for further analysis. RPM determined circRNAs level. To identify differentially expressed circRNAs, statistical comparison between two different groups was determined by the DESeq (2012) R package[14], with setting the threshold of adjusted p-value < 0.05 and foldchange > 2 or foldchange < 0.5 . FDR (false discovery rate) is used as the p-value threshold for multiple tests to judge the significance of gene expression differences. The interaction of circRNA-miRNA was predicted by miRanda software with the threshold of score > 150 , energy < -30 and strict paired in the seed region[15]. The target genes of miRNA were predicted based on intersection of the results on miRWalk and miRDB database. Differentially expressed genes data of the group C vs YC were obtained from another research[16].

Gene function analysis

(GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were used to predicate the functions of circRNAs [17, 18]. KEGG analysis was performed to determine the involvement of target genes in different biological pathways using KOBAS software [19].

Construction of circRNA-miRNA-mRNA interaction Networks

miRanda software was used to predict the interaction of circRNA-miRNA with the threshold of score > 150, energy < -30 and strict paired in the seed region. The target genes of miRNA were predicted based on intersection of the results on miRWalk and miRDB database. CircRNA-miRNA-mRNA network was visualized by the Cytoscape software.

Results

C-KIT two mutation sites in patients with imatinib mesylate secondary resistance

All specimens were analyzed by RT-PCR amplification, DNA sequencing and analysis and then comparison with wild type c-KIT gene, C-KIT/PDGFR- α mutation were detection, secondary mutation were found in 3 GIST specimens with drug resistance. one case locus in the v654a of exon 13, one case locus in the T760I of exon 14, the other locus in the v654a of exon 17(Figure 1), which confirm the resistance of imatinib mesylate and offer a potential theoretical basic of the application probability of circRNAs.

Identification And Characteristics Of Circrnas

To explore circRNA expression profiles in N, Y or YC, and C group, we performed ribosomal RNA-depleted RNA sequencing and the number of circRNAs identified in each sample is shown in Figure 2A. Venn analysis showed that 11935 circRNAs were found between predicted circRNAs and circBase (Figure 2B). According to the circular RNAs array, a total of 30,550 were detected in 9 samples, and the length mostly distribute in 201-400bp and >2000bp (Figure 2C). Chr1, Chr2 and Chr3 are the three most located chromosomes (Figure 2D). Most circular RNAs have less than 6 exons (Figure 2E). Similarly, most of the identified circular RNAs (27050, 88.54%) came from the overlapping regions of meaning, indicating that the formation of circular RNAs is closely related to the pre-mRNA splicing mechanism (Figure 1F). Approximately 3.82% (1167) and 5.41% (1652) circular RNAs were derived from exons and intergenic regions. A small part of circular RNA is antisense circular RNA (383, 1.25%) and intronic circRNAs (298, 0.98%). General features of the circRNA sequencing data were list in Table 1.

Table 1
General features of the circRNA sequencing data

Sample	raw reads	raw bases	clean reads	clean bases	valid bases	Q30	GC
Sample_N1	84587074	10573384250	81651670	10201851969	96.48%	93.56%	55.50%
Sample_N2	91507150	11438393750	88469786	11053620694	96.63%	93.73%	56.00%
Sample_N3	91320298	11415037250	88647372	11076539842	97.03%	94.29%	57.00%
Sample_C1	84193988	10524248500	81676720	10204694315	96.96%	94.20%	58.00%
Sample_C2	82799646	10349955750	80243548	10025345565	96.86%	93.93%	58.00%
Sample_C3	85145886	10643235750	82621864	10322885971	96.99%	94.11%	55.50%
Sample_YC_1	84743526	10592940750	81602896	10186655571	96.16%	93.57%	57.00%
Sample_YC_2	83362440	10420305000	80906514	10108276460	97.00%	94.37%	50.50%
Sample_YC_3	82891684	10361460500	80622518	10073190258	97.21%	94.35%	55.50%

The Potential Functions Identification

Recent study has reported that circRNAs possess has tissue-specific expression characteristics. we used DEseq software to analyze circRNA expression profiles RPM to screen dysregulated circRNAs in three different GIST samples, found that that no abnormal expression was observed in three different GIST samples (Figure 3A). PCA (Principal Component Analysis) was performed to analyze the circRNA expression profiles of the three groups samples. The distance between points represented the similarity between the two samples, and the repeatability of the three groups of samples was ideal in Figure 3B. Differential analysis was conducted among the three comparison groups by Volcano plots. The circRNA differentially expressed was screened using the criteria of "adjusted pvalue < 0.05 and absolute value of log2Foldchange >1". The red dot on the volcano map was significantly increased circRNA, the green dot was significantly reduced circRNA, and the gray dot showed no obvious difference (Figure 3C). These were 159, 98, and 37 circRNAs up-regulated, 277, 284, and 23 circRNAs down-regulated in comparison C-vs-N, YC-vs-N and C-vs-YC, respectively(Figure 3D). Venn analysis of the three comparison groups was shown in Figure 3E. In general, the same kind of samples can be clustered in the same cluster, and the genes in the same cluster may have similar biological functions, our results show that all samples in paired groups have the co-regulated (up or down) genes (Figure 3F). The top ten different expression circRNA in the three comparison groups was shown in Table 2.

Table 2
Top 10 differentially expressed circRNAs in the three comparison groups

circRNA_id	circBase_id	log2FoldChange	P-value	Adjusted P-value	Regulation	Host genes
C-vs-N						
circRNA_09533	hsa_circ_0006867	-8.62	9.25E-90	2.61E-85	Down	LRBA
circRNA_17427	hsa_circ_0018064	-inf	3.05E-64	4.30E-60	Down	SVIL
circRNA_20776	hsa_circ_0026782	-9.86	7.21E-53	6.78E-49	Down	ITGA7
circRNA_09530	-	-inf	2.72E-46	1.92E-42	Down	LRBA
circRNA_13055	hsa_circ_0079284	-5.41	2.84E-43	1.60E-39	Down	RNF216
circRNA_11884	hsa_circ_0004119	-5.40	1.63E-41	7.69E-38	Down	RAB23
circRNA_01991	hsa_circ_0005230	5.26	1.57E-35	6.32E-32	Up	DNM3
circRNA_13811	hsa_circ_0004365	-inf	7.44E-35	2.62E-31	Down	SEMA3C
circRNA_03576	hsa_circ_0000994	-4.03	2.99E-30	8.43E-27	Down	SLC8A1
circRNA_26953	hsa_circ_0000825	-5.70	2.70E-30	8.43E-27	Down	MTCL1
C-vs-YC						
circRNA_01991	hsa_circ_0005230	3.92	1.33E-19	2.49E-15	Up	DNM3
circRNA_03862	hsa_circ_0004435	-2.74	1.07E-12	6.67E-09	Down	FANCL
circRNA_15734	-	Inf	9.93E-13	6.67E-09	Up	-
circRNA_11269	hsa_circ_0003718	-4.00	6.83E-12	3.20E-08	Down	RANBP17
circRNA_02957	hsa_circ_0002922	-2.17	2.05E-11	7.68E-08	Down	ZNF124
circRNA_30179	-	-inf	3.18E-11	9.95E-08	Down	DIAPH2
circRNA_30540	hsa_circ_0009024	-inf	7.27E-11	1.95E-07	Down	-

circRNA_id	circBase_id	log2FoldChange	P-value	Adjusted P-value	Regulation	Host genes
circRNA_03489	hsa_circ_0000992	3.91	4.07E-10	9.55E-07	Up	PRKD3
circRNA_04497	-	-2.60	5.75E-10	1.20E-06	Down	DPP10
circRNA_25651	-	Inf	9.34E-10	1.75E-06	Up	ZC3H18
YC-vs-N						
circRNA_09533	hsa_circ_0006867	-8.15	1.75E-86	4.41E-82	Down	LRBA
circRNA_17427	hsa_circ_0018064	-inf	1.81E-67	2.28E-63	Down	SVIL
circRNA_13055	hsa_circ_0079284	-7.80	8.02E-60	6.75E-56	Down	RNF216
circRNA_04497	-	Inf	5.19E-58	3.27E-54	Up	DPP10
circRNA_09530	-	-inf	4.50E-47	2.27E-43	Down	LRBA
circRNA_11884	hsa_circ_0004119	-4.77	1.01E-39	4.26E-36	Down	RAB23
circRNA_20776	hsa_circ_0026782	-6.94	1.03E-33	3.70E-30	Down	ITGA7
circRNA_26999	hsa_circ_0008821	-inf	5.42E-32	1.71E-28	Down	RAB31
circRNA_13811	hsa_circ_0004365	-6.68	1.69E-29	4.74E-26	Down	SEMA3C
circRNA_18913	hsa_circ_0000277	6.50	1.12E-27	2.82E-24	Up	PDE3B

GO enrichment analysis for the host genes of differentially expressed circRNAs

After get the differentially expressed genes, we selected the top10 functional enrichment analysis. The enriched functional terms were used as the predicted functional term of given circRNAs. Analysis the difference gene expression with GO analysis, to describe its function (with GO annotation). GO analyses covered three subgroups: biological process (BP), cellular component (CC), and Molecular function (MF). The GO analysis with the most significant enrichment in the BP, CC, and MF subgroups by C-vs-N comparison groups is regulation of transcription, DNA-templated, cytosol and metal ion binding, respectively. In C-vs-YC group, the GO analysis with the most significant enrichment in the BP, CC, and MF subgroups is regulation of transcription, DNA-templated, nucleoplasm and double-stranded DNA binding. In YC-vs-N group, the GO analysis with the

most significant enrichment in the BP, CC, and MF subgroups is regulation of transcription from RNA polymerase II promoter, cytosol and metal ion binding (Figure 4A-C).

Construction Of The Circrna-mirna Interaction Network In Drug Resistance/hif-1

We combined the chip data (OE2016Q1031Y) from another of our published articles to draw the circRNA-miRNA-mRNA network in the group C vs YC, and found that, 15 circRNA were up-regulated (Red), 8 circRNA was down-regulated (Green) (Figure 5A). GO enrichment analysis of the circRNA-miRNA-mRNA network, the bubble diagram shows the top 20 enriched GO terms ($P < 0.05$) (Figure 5B). KEGG pathway enrichment analysis of the circRNA-miRNA-mRNA network, and found out potential relationship between differential expression genes with changes of cell pathways, such as HIF-1 pathway, Central carbon metabolism in cancer, AMPK signaling pathway, Autophagy-animal and so on (Figure 5C). Later, we analyzed the circRNA-miRNA-mRNA network involved in the HIF-1 pathway, found that the correlation between each dysregulated circRNA-miRNAs-mRNA, circRNA_06551, circRNA_14668, circRNA_04497, circRNA_08683, circRNA_09923 (Green, down-regulation) and circRNA_23636, circRNA_15734 (Red, up-regulation) (Figure 5D).

Discussion

CircRNAs, has a covalently closed loop structure without 5' and 3' termini, mainly caused by back-splicing and covalently binding, which was detected 20 years ago but considered as a rare event for so long [20, 21]. Recent studies have proved that circRNAs plays critical roles in the development and prognosis of many diseases, such as Alzheimer, cardiac hypertrophy and heart failure, cancers [22]. Now a greater appreciation rose that circRNAs which thought to be a promising direction in diverse diseases. More and more studies have shown that circRNA is deregulated in different types of human cancers [6, 23–25]. CircRNA plays a vital effect in the biological processes involved in tumor progression and drug resistance [26]. It also acts as a microRNA (miRNA) sponge and RNA binding protein sponge, gene transcription. For instance, over-expression of circRNA_0025202 could regulate tamoxifen sensitivity through regulation of the miR-182-5p/FOXO3a axis in breast cancer [27]. Circular RNA AKT3 could regulate drug resistance in gastric cancer (GC) cells via inhibition of miR-198 and upregulation PIK3R1 [28]. Up-regulation of Circular RNA MCTP2 could inhibit resistance to cisplatin in GC by regulation of miR-99a-5p/MTMR3 axis [29]. Inhibition of circCELSR1 could enhance sensitivity to paclitaxel in ovarian cancer cells via FOXR2 /miR-1252 axis [30]. However, the expression profiles and functions of circRNA in GIST IM resistance are still unclear.

Supporting the theory that circRNAs may be a new and stable biomarker and breakthrough therapeutic direction for now known intractable diseases. In the present study, we explored the circRNA expression patterns in 9 patients N, Y or YC and C group using high-throughput RNA sequencing. Obtain microarray specimen results as shown that most of the circRNA is about 201-400bp in length, which is consistent with the previous report that the median length of circRNA is about 500 nt [31]. Circular RNA is mainly produced by the exons or introns of its host linear transcript and participates in the regulation of host gene expression [32, 33]. Therefore, following screened out the differentially expressed circRNAs between Y and C tissue samples, we used GO and KEGG pathway analyses to predict the biological functions of their host linear transcripts, indicating that it was involved in HIF-1 signaling pathway.

HIF is often up-regulated in different cancer cells and is related with the progression and poor clinical outcomes of many tumor entities[34, 35]. HIF genes regulate the expression of many genes related to angiogenesis, tumor growth, metastasis, and therapeutic resistance[36]. Hypoxia inducible transcription factor 1 α (HIF-1 α), was identified the main regulator of hypoxia-induced drug resistance, is considered to be an attractive target for tumor therapy[37]. The present study constructed the circRNA-miRNA-mRNA network involved in the HIF-1 pathway through bioinformatic prediction and clarified circRNA-miRNA-mRNA axis participation in this regulatory network. We found several circRNA were up-regulated or down-regulated, such as circRNA_23636, circRNA_15734(up-regulation); circRNA_06551, circRNA_14668, circRNA_04497, circRNA_08683, circRNA_09923(down-regulation); and there are many circRNA-miRNA-mRNA axis, such as, circRNA_23636-has-miR-6077-SLC2A1, circRNA_15734-hsa-miR-6893-5p-PFKFB3, circRNA_15734-hsa-miR-8485-VEGFA); circRNA_06551-hsa-miR-1915-3p- PFKFB3), circRNA_14668-hsa-miR-8485- VEGFA), circRNA_08683-hsa-miR-6728-5p-ENO2), circRNA_04497-hsa-miR-6808-5p-PFKFB3), circRNA_04497-hsa-miR-8485- VEGFA), circRNA_09923-hsa-miR-4755-3p- PFKFB3), circRNA_09923-hsa-miR-3155p-HK2), circRNA_09923-hsa-miR-3155a- HK2). Although the interaction between circRNA-miRNA-mRNA axis has not been completely researched, we speculate that differentially expressed circRNAs may play their biological functions in the process of IM resistance through interaction with miRNA-mRNA.

Conclusion

This study serves as the first, to our knowledge, circRNAs sequencing and functional analysis in primary GIST and imatinib mesylate secondary resistance GIST. After IM failure, few therapeutic options remain, so it is urgent to identify the mechanism of drug resistance, HIF-1 seems to be crucial in the future research.

Declarations

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University and written informed consent was given before operation.

Consent for publication

Not applicable

Availability of data and materials

We declare that all data supporting the conclusions of the study.

Competing interests

The authors declare that they have no competing f interests.

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Authors' contributions

XS and JY conceived the idea; XC, QD and JL analyzed the data; XS wrote the manuscript. All authors have read and approved the final version of the manuscript.

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Not applications.

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Figures

Figure 1

Figure 1

C-KIT two mutation sites in patients with imatinib mesylate secondary resistance C-KIT secondary mutation sites in patients with imatinib mesylate secondary resistance GIST (one case locus in the v654a of exon 13, one case locus in the T760I of exon 14, another one locus in the v654a of exon 17).

Figure 2

Identification and characteristics of circRNAs A. Identification of circRNAs in different samples. B. Venn analysis for comparison of predicted circRNAs with the data published in the circBase. C. The length distribution of circRNAs. D. Chromosome distribution of circRNAs. E. Distribution of the exon numbers of circRNAs. F. Category of circRNAs based on genomic origin.

Figure 3

Figure 3

Differentially expressed circRNAs in the N, C and YC groups A. Box plots of reads per million (RPM) values of circRNAs in each sample. B. PCA plot of all the samples in N, C and YC groups. C. Volcano plots indicated the variation of circRNA expression in different comparison groups C vs N, YC vs N, C-vs-YC. D. Column chart of differentially expressed circRNAs in each comparison. The numbers on column show the numbers of up-regulated (red) and down-regulated (green) circRNAs. E. Venn analysis of the three comparison groups. F. Heatmap of differentially expressed circRNAs between YC and C groups.

Figure 4

Figure 4

GO enrichment analysis for the host genes of differentially expressed circRNAs A-C. GO enrichment analysis was conducted based on the host genes of differentially expressed circRNAs. The bar diagrams show the top10 enriched GO terms ($P < 0.05$) in the group C vs N(A), YC vs N(B), C-vs-YC(C), respectively

Figure 5

Figure 5

the ceRNA regulatory network in the group C vs YC A. the cirRNA-miRNA-mRNA network in the group C vs YC. Red, up-regulated. Green, down-regulated. Hexagon, circRNAs. Circle, mRNA. Arrowhead, miRNA. B. GO enrichment analysis of the cirRNA-miRNA-mRNA network. The bubble diagram shows the top 20 enriched GO terms ($P < 0.05$). C. KEGG pathway enrichment analysis of the cirRNA-miRNA-mRNA network. The bubble diagram shows the enriched pathways ($P < 0.05$). D. The cirRNA-miRNA-mRNA network involved in the HIF-1 pathway.