RESEARCH ARTICLE

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# Identification of Extrachromosomal Circular DNA Isolated from Cell Culture Supernatant and Its Potential Applications for Hepatocellular Carcinoma Diagnosis

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## Abstract

**Background:** Hepatocellular carcinoma (HCC) is the most common primary liver malignancy worldwide and is associated with a high mortality rate. The unique genetic features and structural properties of extrachromosomal circular DNA (eccDNA) offer a promising approach for early cancer diagnosis. **Objective:** This study aims to investigate the potential of eccDNA as a diagnostic marker by identifying its distinguishing characteristics using a two-dimensional cell culture model. **Methods:** EccDNA was isolated from the supernatant of HepG2 and THLE-2 cell cultures. Long-read sequencing and bioinformatic analysis were employed to detect and characterize eccDNA. The size distribution, chromosomal origin, and genomic annotation of eccDNA from both cell types were compared. PCR was performed to validate DNA fragments at the junctions of the closed-circular forms. **Results:** The total number of cell-free eccDNA derived from HepG2 cells averaged 2,669,673 bases, encompassing 2,542 sequences, while THLE-2 cells averaged 857,718 bases with 975 sequences. Size distribution analysis revealed that most cell-free eccDNA from both cell types ranged from 351 to 600 bp. Bioinformatic analysis revealed that approximately 80% of eccDNAs corresponded to gene coding regions. Ten eccDNAs were the most frequently detected in HepG2 cells, with four eccDNAs harboring *CDC27P11, RAC1P3, LOC112268123*, and *LOC124902279* genes successfully validated and uniquely detected in HepG2 cells, suggesting their potential as biomarkers for HCC diagnosis. **Conclusion:** Distinct eccDNA types were identified in HepG2 cells, which may serve as promising biomarkers for HCC diagnosis.

**Keywords:** hepatocellular carcinoma- extrachromosomal circular DNA- long-read sequencing- biomarkers

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## Introduction

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer and a leading cause of cancer-related mortality worldwide. Key risk factors for HCC include viral hepatitis B and C infection, alcoholism, and metabolic syndrome [1]. Surveillance for HCC is essential for early detection, enabling prompt treatment and ultimately improving survival rates. Currently, HCC diagnosis typically involves several methods, including computed tomography (CT) scans, magnetic resonance imaging (MRI), and liver biopsy. While tissue biopsy remains the gold standard for HCC diagnosis, it carries a significant risk of complications. There is an urgent need for non-invasive biomarkers to facilitate screening and early diagnosis of HCC. Liquid biopsy, which obtains genetic information, holds promise for clinical applications in screening, diagnosis, prognosis, and treatment monitoring [2, 3]. However, its sensitivity and specificity for HCC diagnosis remain limited. Thus, identifying genetic markers with high sensitivity and specificity is crucial for improving HCC diagnosis.

EccDNA is a circular DNA molecule derived from chromosomes, reflecting the genome's plasticity and instability. In eukaryotic cells, eccDNA can be categorized into organelle eccDNA, such as mitochondrial DNA, and non-organelle eccDNA, which includes more structurally flexible forms such as double minute chromosomes (DMs), episomes, small polydispersed circular DNA (spcDNA), and microDNA [4-6]. EccDNA is present in a wide range of eukaryotic cell types and has recently been identified in circulation as cell-free eccDNA under both normal and diseased conditions. Advanced high-throughput sequencing technologies have revealed that

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eccDNA is a multifunctional molecule with diverse characteristics. For instance, DMs serve as carriers of extrachromosomal gene amplification, often harboring oncogenes or drug-resistance genes, thus increasing gene dosage and providing growth advantages to tumor cells [7]. SpcDNA, ranging from approximately 100 bp to 10 kb, can contain either repetitive or unique sequences and is closely associated with chromosomal instability [8-10]. MicroDNA, a newly identified class of eccDNA, is predominantly derived from exon, 5' UTR and CpG islands [6]. It exhibits lineage specificity in human ovarian and prostate cancer cell lines [11], as well as cell type specificity in human fibroblast cells and granulocytes [12].

EccDNA, a form of cell-free DNA, can be released into biological fluids from both healthy and diseased cells under various conditions. This phenomenon presents an opportunity to utilize eccDNA as a novel biomarker for early cancer diagnosis, monitoring, and recurrence detection [13, 14]. In a study involving patients with lung adenocarcinoma (LUAD), unique eccDNA was identified in plasma samples from LUAD patients but was absent in healthy individuals, suggesting that cancer-specific eccDNA may serve as a promising diagnostic biomarker [15]. Additionally, a subtype of small eccDNA known as microDNA, derived from uniquely mapping regions of the genome, has been detected in plasma and serum samples from both mice and humans. Notably, tumor-derived microDNA has been identified in the circulation of mice in a xenograft model of human ovarian cancer. Comparative analysis of microDNA from paired tumor and normal lung tissues reveals that tumors harbor longer microDNA. Moreover, serum and plasma samples collected before surgery exhibit enrichment for longer cell-free microDNA compared to samples obtained post-tumor resection. This suggests that the analysis of microDNA may serve as a biomarker for cancer detection and monitoring throughout treatment and follow-up periods. Furthermore, an increase in circulating microDNA length may indicate cancer recurrence [13].

EccDNA originated from tens of thousands of unique genomic loci, and its distinct genetic characteristics hold significant promise as effective biomarkers for cancer diagnosis [16]. Compared to linear cell-free DNA, eccDNA exhibits greater stability, making it a more desirable biomarker for cancer. Investigating the characteristics of eccDNA associated with cancer could unlock its potential for early diagnosis, particularly in HCC. However, the in vivo characterization of cell-free eccDNA is challenging due to its low abundance and the substantial heterogeneity of blood samples. Consequently, in vitro studies focusing on the characterizing cell-free eccDNA using two-dimensional cell culture models are emerging as a crucial approach. Utilizing in vitro models to study the biological properties of cell-free eccDNA under both normal and pathogenic conditions allows for a better understanding and paves the way for the development of diagnostic biomarkers. This study aims to identify eccDNA in the cell culture supernatants of hepatoma (HepG2) and normal human liver (THLE-2) cell lines, with a focus on its potential applications in HCC diagnosis.

## Materials and Methods

*Cell culture preparation*

HepG2 (HB-8065™) and THLE-2 (CRL-2706™) cells were obtained from the American Type Culture Collection (ATCC). HepG2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (Gibco). THLE-2 cells were cultured in Bronchial Epithelial Growth Basal Medium (BEGM®, Lonza) supplemented with the BEGM® SingleQuots™ kit, which includes bovine pituitary extract (BPE), hydrocortisone, epidermal growth factor (EGF), insulin, triiodothyronine, transferrin, and retinoic acid. Additional supplements included 5 ng/mL human recombinant EGF (Corning), 70 ng/mL phosphoethanolamine (Sigma-Aldrich), and 10% FBS. Before seeding, THLE-2 cells required a pre-coated culture flask with a mixture of 0.01 ml/mL fibronectin (Corning), 0.03 mg/mL bovine collagen type I (Corning), and 0.01 mg/mL bovine serum albumin (Sigma-Aldrich) dissolved in BEGM medium. For the experiment, approximately $2x10^7$ cells were seeded in triplicate into 75 $cm^2$ flasks containing 12 ml of culture medium. After 12 h, the culture medium was replenished, and the cells were incubated for a total of 56 h, corresponding to the time of the maximum cell-free DNA release by HepG2 cells [17]. Both THLE-2 and HepG2 cells were maintained in a humidified atmosphere with 5% CO2 at 37°C. After 56 h, the growth medium was collected into 15 ml tubes and centrifuged at 1,000 g for 10 min. The supernatant was then transferred to fresh 15 ml tubes and stored at -80ºC until further extraction.

*Isolation of cell-free eccDNA*

Cell-free eccDNA was isolated from the cell culture supernatant using the GenepHlowTM Gel/PCR Kit (Geneaid, Cat. No. DFH100) according to the PCR clean-up protocol. The growth medium was mixed with buffer QG buffer at a ratio of 1:2 (growth medium: QG buffer), vortexed, and transferred to a spin column for centrifugation. Following a washing step, the samples were dissolved in TE buffer. The total concentration of cell-free DNA was measured using a Nanadrop spectrophotometer (Thermo Scientific). To isolate cell-free eccDNA, linear DNA was digested with 0.4 U/µl of plasmid-safe DNase (Lucigen®, Epicentre, UK, Cat. No. E3101K) for 5 days. The cell-free eccDNA was then precipitated using the isopropanol precipitation. The concentration of cell-free eccDNA was measured using a Qubit® fluorometer 3.0 (Invitrogen, Life Technologies). Mitochondrial and globin genes were used to confirm the presence of circular DNA and to eliminate linear DNA, respectively. PCR amplification was performed under the following conditions: initial denaturation at 94ºC for 5 min, followed by 35 cycles of denaturation at 94ºC for 30 s, annealing at 55ºC for 30 s, and extension at 72ºC for 30 s. The list of primers is shown in Table 1.

*Amplification of Cell-free eccDNA by Rolling Circle Amplification (RCA)*

To enhance the yield of cell-free eccDNA, RCA was performed using REPLI-g® Mini Kit (QIAGEN, Cat. No. 150023) according to the manufacturer's protocol. Approximately 20 ng of cell-free eccDNA was mixed with buffer D1 and incubated for 3 min at room temperature. The reaction was then stopped by adding buffer N1, followed by the addition of Repli-g mini reaction buffer and Repli-g mini DNA polymerase. The mixture was incubated at 30°C for 16 h, after which the reaction was terminated by heating at 70°C for 30 min. Enriched cell-free eccDNA was precipitated with isopropanol and dissolved in TE buffer. DNA quality was assessed by gel electrophoresis, and the DNA concentration was measured using a Nanodrop spectrophotometer.

*Identification of cell-free eccDNA from cell culture supernatant*

Purified RCA products of the cell-free eccDNA were used to prepare DNA libraries using the Rapid Barcode Sequencing Kit 24 (SQK-RBK114-24, Oxford Nanopore). The libraries were loaded into the MinION flow cell R10.4.1 and subjected to a machine, MinION (Oxford Nanopore). Sequencing was performed until approximately 100 Mbases per sample were reached. Nucleotide sequence data in FASTQ format were generated from FAST5 files using Dorado (v0.5.1) (https://github.com/nanoporetech/dorado) with a super-accurate model. Reads with a mean q-score below 10 or a length less than 100 bp were filtered out using Chopper version (v0.7.0) [18].To identify eccDNA sequences, the cleaned reads were mapped to a human reference genome (GRCh38.p14) using Minimap2 (v2.26) [19]. The mapped reads were then sorted and indexed to generate a BAM file using Samtools (v1.20) [20]. EccDNA was detected from the BAM file using the FLED program (v1.7.0) [21]. After localizing, general characteristics of eccDNAs, including length distribution, chromosomal origin, and genomic biotypes, were analyzed using Bedtools (v2.31.1) [22] and SeqKit (v2.6.1) [23].

*Confirmation of circular DNA by end-joining amplification*

To verify the presence of circular DNA, putative eccDNAs were validated using PCR, gel electrophoresis, and Sanger sequencing. Outward-facing primers were designed from the 3' and 5' ends of the linear dsDNA fragment to ensure that PCR amplification would yield amplicons exclusively from the corresponding eccDNA sequence. Enriched circular DNA was used as the template for PCR amplification, generating fragments that contained nucleotide sequences indicative of the closed end-joining region of circular DNA. PCR was performed using the One-Taq™ PCR reaction kit (NEB, USA, Cat. No. M0484S) with the following protocol: an initial denaturation step at 94ºC for 5 min, followed by 35 cycles of denaturation at 94ºC for 30 s, annealing at 55ºC for 30 s, and extension at 72ºC for 1 min. The PCR primers used in this study are listed in Table 1. The PCR products were analyzed by agarose gel electrophoresis and visualized using the Bio-Rad GelDoc EZ Gel Imaging System. Subsequently, these products were extracted from the gel and cloned into the pGEM-T® Easy Vector System (Promega, Cat. No. A1360), with selected clones screened by *Eco*RI digestion. Finally, recombinant clones were subjected to Sanger sequencing using T7/SP6 primers (Ward Medic, Thailand).

## Results

*Presence of eccDNA in cell culture supernatants*

Total cell-free DNA was extracted from the culture supernatants of HepG2 and THLE-2 cells and treated with Plasmid-Safe™ DNase for 5 days. Digestion efficiency was confirmed using the globin gene as a linear DNA marker, demonstrating successful degradation of linear DNA compared to the untreated control. Mitochondrial DNA (mtDNA) was used as a positive control for circular DNA. The results showed that mtDNA was detected in all samples, confirming the presence of circular DNA in the culture supernatants (Figure 1). These findings suggest that eccDNA is released from both cell types and resists exonuclease digestion. Following purification, approximately 20 ng of eccDNA was amplified using the RCA method, yielding 10-30 µg of RCA products

Table 1. List of Primers

| Name of primer | Primer sequence (5'-3') | length (bp) |
|---|---|---|
| globin-F | GTGCACCTGACTCCTGAGGAG | 102 |
| globin-R | CCTTGATACCAACCTGCCCAG | |
| mtDNA-F | ACCCACCAATCACATGCCTA | 184 |
| mtDNA-R | GTGTTACATCGCGCCATCAT | |
| LOC1249-748-F | CATCTCTGCTCTCGGTGTGA | variable |
| LOC1249-748-R | AGTGACCCTGACCTCCTAGA | |
| LOC112-2221-F | TCACGCTACTCAGAACAGCA | variable |
| LOC112-2221-R | ATGAGCAGCTTAGACAGGAC | |
| CDC-F | ACAGGTCGCCAGTAAGAACAAG | 572 |
| CDC-R | TCAACACCATCCTCACCG TT | |
| RAC1P3-F | AGCGCCTTCTCACTCAATGC | 426 |
| RAC1P3-R | AGGCACTAAGGCAAGAAGTG | |

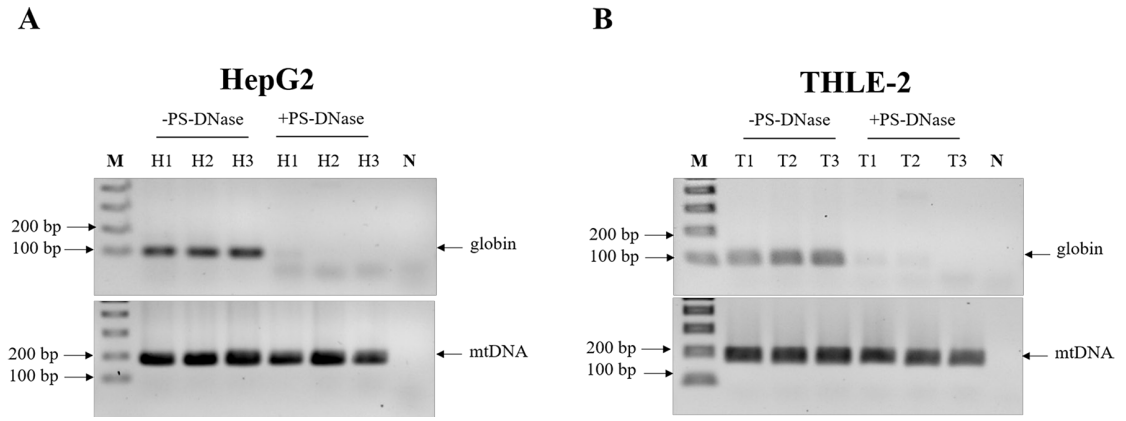


Figure 1. Validation of Cell-Free eccDNA Isolation.

per sample, corresponding to a 500- to 1500-fold increase. Agarose gel electrophoresis further confirmed successful amplification, showing a dominant band over 10 kb (Supplementary Figure 1). The purity of the RCA products, assessed prior to DNA library preparation, showed absorbance ratios of 260/280 and 260/230 within the ranges of 1.8-2.0 and 2.0-2.2, respectively.

*Characterization of eccDNA isolated from the cell culture supernatants*

We quantified the total eccDNA in the cell culture supernatants of HepG2 and THLE-2 cells. The results revealed that HepG2 cells contained an average of 2,669,673 bases from 2,542 eccDNA sequences, with minimum and maximum sequence lengths of 153 bp and 5,983 bp, respectively. In comparison, THLE-2 cells contained 857,718 bases from 975 eccDNA sequences, with minimum and maximum sequence lengths of 101 bp and 6,286 bp, respectively. A summary of cell-free eccDNA characteristics is provided in Table 2. The size distribution of cell-free eccDNA ranged from 100 to 10,000 bp in both cell types. A similar distribution pattern was observed, with the majority of eccDNA falling between 351 and 600 bp, while larger eccDNAs were less frequent. Notably, smaller eccDNAs (100 and 350 bp) were more abundant in THLE-2 cells compared to HepG2 cells (Figure 2A).

To investigate the chromosomal origins of eccDNAs, we mapped the putative eccDNAs to the human genome. Our results indicated that eccDNAs from the supernatants

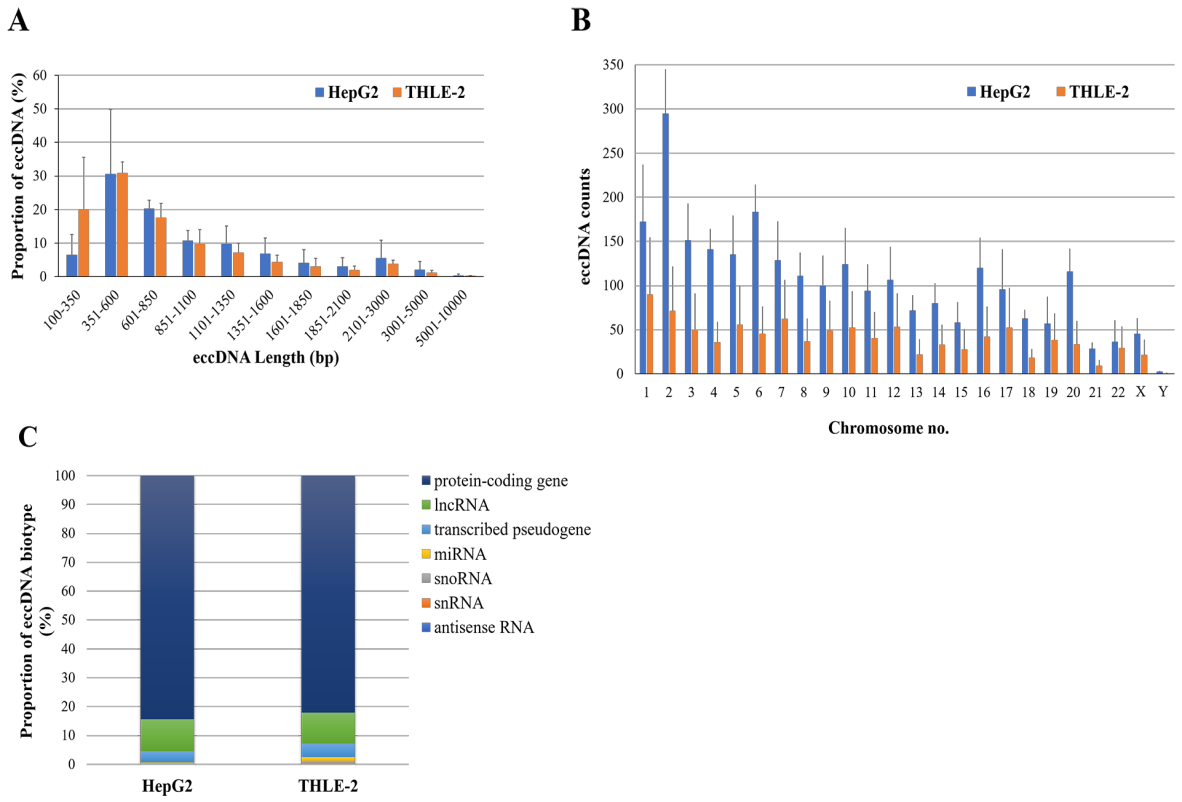


Figure 2. Characteristics of Cell-Free eccDNA Identified from the Cell Culture Supernatant of HepG2 and THLE-2 Cell Lines.

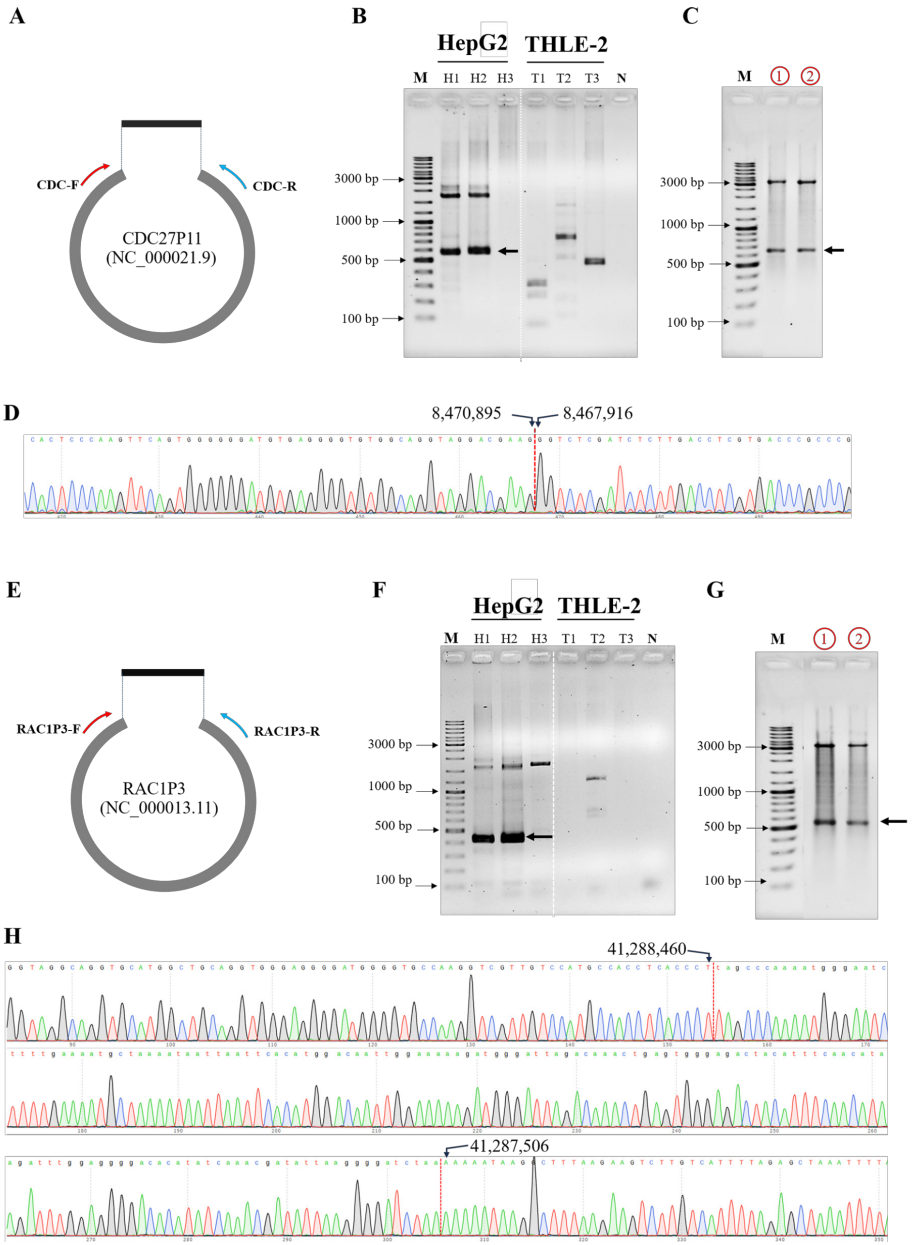


Figure 3. PCR Analysis of Cell-Free eccDNA Homologous to *CDC27P11* and *RAC1P3* Genes Using Adjacent Primers.

Table 2. General Description of eccDNA Isolated from the Cell Supernatant of HepG2 and THLE-2 Cells

| Description | HepG2 | THLE-2 |
|---|---|---|
| Average number of sequences | 2,542 | 975 |
| Average number of bases | 2,669,673 | 857,718 |
| Minimal sequence length | 153 | 101 |
| Maximal sequence length | 5,983 | 6,286 |
| Average sequence length | 988 | 791 |
| N50 | 1,222 | 998 |
| GC (%) | 42.58 | 49.1 |

of both THLE-2 and HepG2 cells were ubiquitously distributed throughout the genome (Figure 2B). We further analyzed the eccDNA sequences and categorized their biotypes using BlastN analysis. The results revealed that eccDNAs isolated from both HepG2 and THLE-2 cell supernatants belonged to various biotypes, including protein-coding genes, transcribed pseudogenes, long non-coding RNAs (lncRNAs), and non-coding RNAs (ncRNAs). Approximately 80% of the cell-free eccDNAs in both cell types were derived from gene coding regions. In the HepG2 cell culture supernatant, eccDNA sequences derived from 1,047 protein-coding genes, whereas in the THLE-2 supernatant, they were derived from 526 protein-coding genes. Additionally, about 10% of eccDNA sequences in the HepG2 supernatant were homologous to lncRNAs, with the remaining sequences corresponding to transcribed pseudogenes, microRNAs (miRNAs), small nucleolar RNAs (snoRNA), and antisense RNAs, similar to those found in the THLE-2 supernatant (Figure 2C).

To identify HCC-specific eccDNA as potential diagnostic biomarkers, eccDNA with high abundance isolated from the HepG2 cell culture was selected. The ten eccDNAs with the highest frequency in the HepG2

Table 3. The Most Abundant eccDNA Identified in the Cell Culture Supernatant of HepG2 Cells

| Gene name | eccDNA position | Length (bp) | References |
|---|---|---|---|
| *CDC27P11* | Chr21: 8467927-8470663 | 2737 | NC_000021.9 |
| *CDC27P9* | Chr21: 8239234-8243077 | 3844 | NC_000021.9 |
| *LOC124902279* | Chr9: 127852203-127852950 | 748 | NC_000009.12 |
| *CDC27P10* | Chr21:8417660-8426101 | 8442 | NC_000021.9 |
| *LOC124903289* | Chr14: 23530014-23532372 | 2359 | NC_000014.9 |
| *LEFTY1* | Chr1:225886282-225889146 | 2865 | NC_000001.11 |
| *RAC1P3* | Chr13: 41287506-41288460 | 955 | NC_000013.11 |
| *LOC124904056* | Chr17: 74464008-74467774 | 3767 | NC_000017.11 |
| *LOC112268123* | Chr14: 34541234-34543454 | 2221 | NC_000014.9 |
| *SOCS1* | Chr16: 11254417-11256204 | 1789 | NC_000016.10 |

cell culture are listed in Table 3. Of these 10 eccDNAs, six were located in known gene coding regions, including three isoforms of CDC27 (cell division cycle 27 pseudogene); *CDC27P11*, *CDC27P9* and *CDC27P10*; *LEFTY1* (left-right determination factor 1), *RAC1P3* (Rac family small GTPase 1 pseudogene), and *SOCS1* (suppressor of cytokine signaling 1). Additionally, four eccDNAs were located in long non-coding RNA (lncRNA)

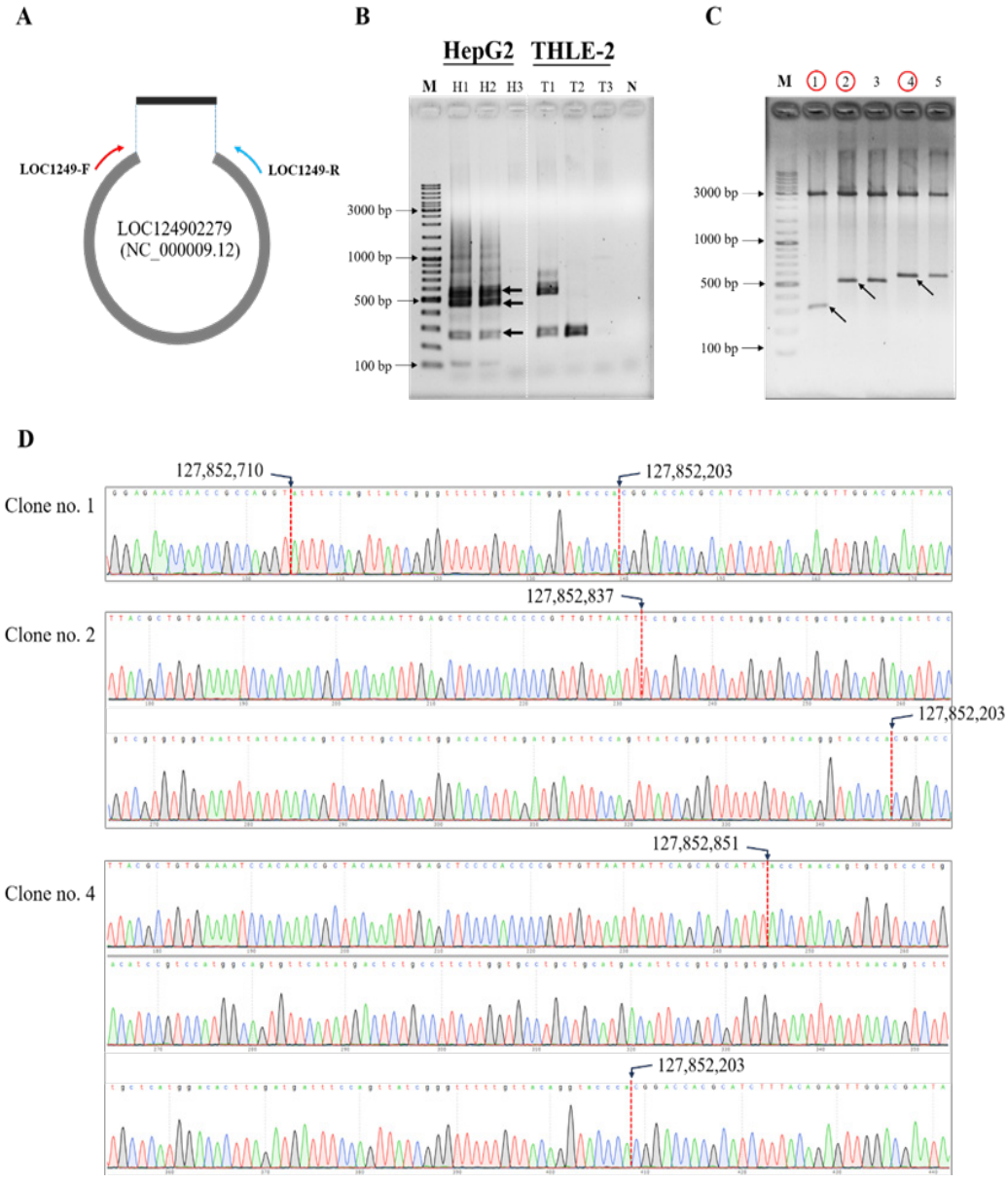


Figure 4. PCR Analysis of Cell-Free eccDNA Homologous to lncRNA (LOC124902279).

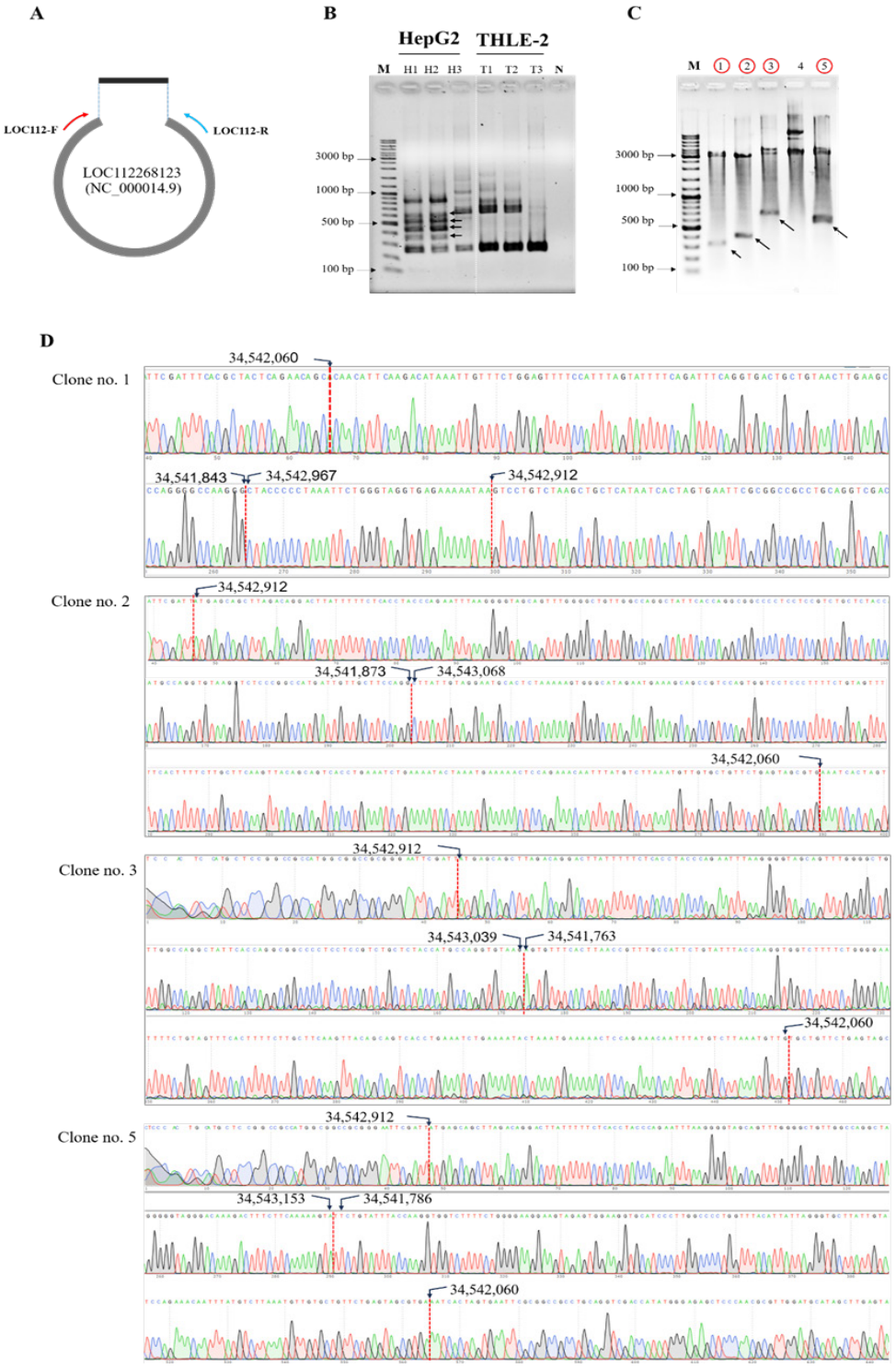


Figure 5. PCR Analysis of Cell-Free eccDNA homologous to lncRNA (LOC112268123).

genes including *LOC124902279, LOC124903289, LOC124904056*, and *LOC112268123.*

*PCR confirmation of eccDNA junctions homologous to known gene coding regions*

To confirm that linear DNA fragments obtained by long-read sequencing correspond to eccDNA homologous to known gene coding regions, eccDNA isolated from the culture supernatant of HepG2 and THLE-2 cells were used as templates to verify the eccDNA junctions via PCR. Outward-facing primers were designed to amplify the ends of the linear fragments of the putative eccDNAs, specifically targeting the circular junctions (Figure 3A and 3E). EccDNA sequences homologous to *CDC27P11* and *RAC1P3* were successfully amplified using specific primers. Agarose gel electrophoresis confirmed the presence of positive clones containing the expected PCR fragments. For eccDNA homologous to the *CDC27P11*, circular junction analysis revealed that two eccDNA samples from HepG2 supernatants exhibited strong bands

with distinct patterns compared to those from THLE-2 supernatants, while a third HepG2 sample displayed a faint band. PCR amplification of eccDNA from HepG2 supernatants produced a major band of approximately 600 bp (Figure 3B). This 600 bp amplicon was purified, cloned and digested with the *Eco*RI enzyme (Figure 3C). Two clones were selected for plasmid sequencing, both of which revealed identical sequences. One representative sequence was analyzed and found to correspond to the expected amplified regions based on the reference genome NC_000021.9. Chromatogram analysis of these sequences indicated the position of nucleotide junction of the eccDNA, corresponding to chromosome 21 for the *CDC27P11* gene (Figure 3D). Similarly, for eccDNA homologous to the *RAC1P3*, two samples from HepG2 supernatants were successfully amplified, showing strong bands with distinct patterns compared to those from THLE-2 supernatants. PCR amplification using RAC1P3-specific primers produced a single, strong band of approximately 450 bp (Figure 3F). Two clones were verified by *Eco*RI digestion (Figure 3G), and sequencing of individual clones revealed identical sequences. One representative sequence was selected for further analysis. Chromatogram analysis pinpointed the position of the nucleotide junction of the eccDNA, aligning with chromosome 13 for the *RAC1P3* (Figure 3H). Junction analyses of the PCR fragments confirmed that the amplified sequences linked the two ends of the linear fragment to form circular structures, matching the expected sequences of *CDC27P11* and *RAC1P3*.

*PCR confirmation of circular DNA junction in eccDNA homologous to lncRNAs*

Next, we identified two linear DNA sequences corresponding to *Homo sapiens* uncharacterized *LOC124902279* (NCBI reference sequence: NC_000009.12) and *LOC112268123* (NCBI reference sequence: NC_000014.9), both of which showed homology to lncRNA sequences. Outward-facing primers LOC1249-F/R were designed to target the ends of the linear *LOC124902279* sequences (Figure 4A) using the templates described earlier. Among three samples from each group, two eccDNA samples from HepG2 supernatants exhibited three major fragments with distinct patterns compared to THLE-2, while the third HepG2 sample showed no amplification. (Figure 4B). The fragments were purified, cloned, and digested with *Eco*RI enzyme, and the digestion results are presented in Figure 4C. Variations in amplicons were observed among the three selected clones. These clones were subjected to plasmid sequencing. Sequencing analysis showed that the three amplified fragments represented the circular junction containing the target sites for primer LOC1249F/R at each end. These amplified sequences joined the two fragment ends to form a circular structure, consistent with sequence identities to the expected *LOC1202279* sequences. Chromatogram analysis revealed that the shorter fragments indicated the loss of portions of the original sequences, resulting in smaller circular forms (Figure 4D). Additionally, amplification using eccDNA derived from THLE-2 supernatant as a template revealed major fragments similar to those from HepG2 samples. These fragments were also cloned and sequenced, showing sequence similarity to eccDNA from HepG2 samples.

Similarly, primers LOC112-F/R were designed to target the two ends of the *LOC112268123* sequences (Figure 5A). PCR amplification of the circular junctions for *LOC112268123* produced multiple fragments of varying sizes, ranging from 200 to 700 bp (Figure 5B). Four clones, representing different amplified fragments of eccDNA derived from THLE-2 cells, were selected, cloned, and digested with the *Eco*RI enzyme (Figure 5C). Sequencing results of these fragments confirmed that all sequences matched the human reference genome NC_000014.9. Moreover, sequencing analysis showed that the amplified fragments could be joined at both ends to form a closed circular structure, exhibiting sequence identities with the expected *LOC112268123* sequences. Chromatogram analysis revealed sequences of varying lengths, indicating the loss of portions from the original sequences, resulting in diverse circular forms (Figure 5D). Fragments from the normal group were also cloned and sequenced, showing identity to *LOC112268123.* These findings suggest that cell-free eccDNAs are present in both normal and cancerous cells; however, specific types of eccDNAs were exclusively identified in cancerous cells.

## Discussion

EccDNA, a form of cell-free DNA, exhibits greater structural stability than linear cell-free DNA, making it a promising biomarker for early cancer diagnosis. Its distinct topological structure and unique genetic characteristics further enhance its potential for clinical applications. In this study, we characterized the differences in eccDNA released into the culture medium of cancerous HepG2 cells compared to normal THLE-2 cells. Our analysis focused on various properties of cell-free eccDNA, including length distribution, chromosomal origin, and biotype classification. These findings provide insights into the distinct eccDNA profiles of cancerous and normal cells, contributing to a better understanding of the potential role of eccDNA in cancer and its application in diagnostics.

EccDNA was extracted from the culture medium of HepG2 and THLE-2 cells following a 56-hour incubation. Total cell-free DNA was isolated, and linear DNA was digested using Plasmid-safe™ ATP-dependent DNase, an enzyme that specifically degrades linear DNA while preserving circular DNA. Complete digestion of linear DNA required at least 5 days, with enzyme replenishment every 24 h. This observation aligns with the findings of Tuns et al., who reported a similar digestion period for linear DNA in Kelly cells [24]. Despite the extended digestion time, circular DNA remained detectable, along with mtDNA, which is known to be released and stabilized in the extracellular milieu [25]. The purified RCA product was sequenced using nanopore technology and analyzed through a computational workflow for eccDNA detection [24]. Clean reads were mapped to the human reference genome to identify eccDNA sequences. The analysis revealed that cell-free eccDNA sizes ranged from 100 bp

to 10 kb, with the majority being under 1 kb. Most of the eccDNA in the supernatant of both HepG2 and THLE-2 cells was in the 351-600 bp range. As the size of eccDNA increased, their abundance decreased in both cell types, although larger eccDNAs were more prevalent in HepG2 cells. Notably, eccDNA in the 100-350 bp range, likely classified as microDNA, was more abundant in THLE-2. These findings are consistent with previous studies, which reported that eccDNA smaller than 1 kb predominates in healthy cells, while larger eccDNA is more frequent in cancer cells [26, 16]. Chromosomal mapping revealed that eccDNA was distributed throughout the genome, consistent with studies on eccDNA in the plasma of lung cancer patients and healthy individuals [15, 27]. In contrast, eccDNA in prostate and ovarian cancer cell lines has been reported to be dispersed across distant chromosomal regions, suggesting that eccDNA formation sites may be cell-lineage dependent [11].

EccDNA is not restricted to specific genomic regions but originates from diverse sites and is enriched in particular areas. In cancer cells, eccDNAs are often derived from the promoter, untranslated regions (UTRs), and exons [28], suggesting a preferential production of cancer-associated eccDNAs that may influence gene transcription. EccDNA contributes to genomic evolution by enhancing genetic diversity and is linked to the selection for growth or resistance to cellular proliferation, suggesting its influences on cell phenotype. Furthermore, eccDNA can drive oncogene amplification, increasing their copy number and promoting higher oncogenes expression compared to chromosomal amplification [26]. Some studies suggest that eccDNA enhances transcription in exonic regions, further boosting oncogene expression. Additionally, certain eccDNAs may be transcribed into regulatory RNAs that control the expression of target genes [29]. In this study, we analyzed eccDNAs from the supernatants of HepG2 and THLE-2 cells, focusing on the proportion of eccDNA associated with known genes. Notably, one of the most frequently detected eccDNA in the HepG2 supernatant harbored the CDC27, which plays a role in cell division and functions as both a tumor suppressor and oncogene. Overexpression of CDC27 has been linked to enhanced proliferation, invasion, and metastasis in colorectal, breast, and gastric cancers, as well as lymphoma [30-34]. CDC27 has been proposed as a prognostic biomarker, with potential utility in assessing treatment response and identifying patients at risk of treatment failure [35]. In our study, three isoforms of eccDNA containing CDC27 were identified in the HepG2 cell supernatant, each varying in length. This suggests that variations in the CDC27 sequences may influence its various transcription and contribute to its diverse roles in cancer. We also identified eccDNA containing the RAC1P3, a member of the Rho GTPase family implicated in tumorigenesis and proposed as a biomarker for early-stage metastasis in colorectal cancer [36]. The detection of eccDNA containing *RAC1P3* suggests a potential role in HCC development, making it a promising biomarker for HCC. Additionally, our study identified eccDNA homologous to lncRNAs, a class of ncRNAs longer than 200 nucleotides [37]. LncRNAs are known to regulate gene expression, mRNA splicing, and serve as precursors for other ncRNAs, including microRNAs (miRNAs) [38, 37]. They can function as oncogenes or tumor suppressors and are widely used in cancer diagnosis, prognosis, and treatment [39]. In this study, we identified several eccDNAs homologous to lncRNAs matching uncharacterized *Homo sapiens* loci, such as *LOC124902279*, *LOC124903289, LOC124904056*, and *LOC112268123*. However, their specific functions remain unknown. Furthermore, small eccDNAs, approximately 100 nucleotides in length, likely representing microDNA, were identified. These microDNAs were transcribed into non-coding RNAs, consistent with findings by Paulsen et al., who reported regulatory RNAs involved in gene expression control [29]. Among the small RNAs identified were SNORA71D (small nucleolar RNA H/ACA box 71D), SNAR-C3 (small nuclear ribonucleoprotein polypeptide pseudogene 3), miRNA-640, and miRNA-216a, some of which have been reported to regulate cancer cell proliferation, migration, and invasion [40-42].

In our study, we successfully screened for unique eccDNA in HepG2 cells and confirmed their circular forms through circular DNA junction fragment amplification. EccDNA located in known gene coding regions, including *CDC27P11* and *RAC1P3*, were specifically detected in HepG2 cells but not in normal liver cells. These results suggested that eccDNA (*CDC27P11* and *RAC1P3*) are uniquely present in HepG2 cells, indicating their potential as biomarkers for HCC diagnosis. Additionally, we identified several eccDNAs containing lncRNA sequences matching uncharacterized *Homo sapiens* loci. Our study confirmed the circular form of eccDNA homologous to *LOC124902279* and *LOC112268123*, which exhibited diverse forms. Notably, we found that while eccDNAs are present in both normal and cancerous cells, specific types were exclusively identified in cancerous cells, suggesting that eccDNA exhibits unique characteristics in diseased conditions and highlighting their potential as biomarkers for HCC diagnosis.

In conclusion, eccDNA plays a crucial role in cancer, being present in both normal and cancer cells, with greater specificity in the latter. In this study, we identified eccDNA released by HepG2 cell supernatants and defined a circular DNA junction fragment detectable by PCR, demonstrating sufficient sensitivity to identify eccDNA-encoded genes. These findings provide a foundation for the development of eccDNA-based diagnostics. Detecting these specific circular DNA fragments through liquid biopsy could offer greater sensitivity and accuracy for early cancer diagnosis compared to the short linear DNA currently used in clinical tests.

## Author Contribution Statement

All authors contributed equally in this study.

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*Conflicts of interest*

The authors declare that they have no conflict of interest.

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