Hsa_circ_0001438 and hsa_circ_0000417 are circular RNAs downregulated and upregulated respectively in hepatocellular carcinoma

（肝細胞癌では、環状 RNA の hsa_circ_0001438 と hsa_circ_0000417 はそれぞれ発現が減少もしくは増加している）

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Abstract

Hepatocellular carcinoma (HCC) is the most predominant type of liver cancer and is highly fatal. Alpha-fetoprotein, alpha-fetoprotein-L3, and protein induced by vitamin K absence or antagonist-II are biomarkers used to diagnose HCC but are not highly specific, especially for diagnosing early-stage HCC. Therefore, more specific biomarkers are needed. Recently, circular RNA (circRNA) biomarkers have been used to diagnose several intractable diseases. In this study, we sought to identify circRNA biomarkers to specifically diagnose HCC. We analyzed circRNA expression in publicly available RNA-seq data of primary HCCs and normal tissues, and measured circRNA expression in both HCC cell lines and normal hepatocytes. We identified eight circRNAs whose expression levels were changed in primary HCC compared with normal tissues. We confirmed that expression of hsa_circ_0001438, one of the circRNAs downregulated in primary HCC, was lower in both poorly and well-differentiated HCC cell lines compared with normal hepatocytes. By contrast, the expression of hsa_circ_0000417 was strongly upregulated in a well-differentiated HCC cell line compared with normal hepatocytes. Thus, hsa_circ_0001438 and hsa_circ_0000417 might be potential biomarkers to specifically diagnose HCC. In addition, the experimental strategy using publicly available RNA-seq data will be useful and cost-effective to find circRNA biomarkers.

Keywords: circular RNA; liver cancer; hepatocellular carcinoma; HCC; biomarker

Introduction

GLOBOCAN, a database provided by the International Agency for Research on Cancer [1], recorded 18.1 million new cancer patients and 9.6 million new cancer deaths worldwide in 2018. Among these were 0.84 million new liver cancer patients and 0.78 million new deaths due to liver cancer. Liver cancer is the third most fatal cancer (of 38 kinds of cancer) [1], and hepatocellular carcinoma (HCC) is the main type of liver cancer [2]. HCC is caused by chronic infection by either hepatitis B virus or hepatitis C virus [2]. Viral HCC was predominant in Western countries and Japan until the late 1990s. However, the number of non-viral HCC cases caused by either non-alcoholic fatty liver disease or non-alcoholic steatohepatitis has been increasing since the early 2000s [3,4]. By contrast, in developing countries, viral HCC is still predominant.

To diagnose liver cancer, clinicians use either alpha-fetoprotein (AFP) or CA19-9 biomarkers [5], or (more recently) AFP-L3 and protein induced by vitamin K absence or antagonist-II. However, these biomarkers are not sensitive enough to detect early-stage HCC [5]. Because of advances in diagnostic imaging sensitivity, in 2018 the American Association for the Study of Liver Diseases stopped recommending the use of AFP as a liver cancer biomarker [6]. Thus, more sensitive biomarkers are needed to diagnose liver cancer, especially early-stage HCC.

Circular RNAs (circRNAs) are non-coding RNAs produced by back-splicing of precursor mRNAs (pre-mRNAs); the 5′ and 3′ ends of back-spliced pre-mRNA segments covalently connect to form circRNAs [7]. CircRNAs maintain cellular homeostasis and can either promote or inhibit

tumorigenesis [8]. CircRNAs are also used as cancer biomarkers. For example, circ_ZKSCAN1, downregulated in liver cancer tissues, is a potential HCC biomarker [9]. CircRNAs may be detected by liquid biopsy because they are secreted into blood, saliva, and urine [10]. In this study, we sought to identify other circRNAs biomarkers for specifically diagnosing HCC. We identified hsa_circ_0001438 and hsa_circ_0000417 as potentially useful biomarkers.

Materials and Methods

Screening of circRNAs

Data of fragments per million mapped fragments (FPM) from GSE77509 [11], a GEO RNA-seq dataset deposited at NCBI (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE77509), were downloaded from CIRCpedia v2 (https://www.picb.ac.cn/rnomics/circpedia/). FPM data were analyzed to identify circRNAs whose expression levels change in HCC. Details of the analysis are shown in Supplementary Figure S1. CircRNA identification numbers were confirmed by using circBase (http://www.circbase.org/). Wilcoxon signed-rank test of R version 4.0.3. (https://www.r-project.org/) was used to compare expression in normal tissues with that in HCCs ($p < 0.05$).

Heatmaps

Heatmaps were prepared by using R v4.0.3. (https://www.r-project.org/). Briefly, the gplots package heatmap.2 function was used, and clustering was conducted by using the average mode.

Receiver operating characteristic (ROC) curves

ROC curves were prepared by using R v4.0.3. (https://www.r-project.org/) with the default parameter settings.

Cell lines

The hepatoblastoma cell line HepG2 [12] was obtained from the RIKEN BRC through the National Bio-Resource Project of the MEXT/AMED, Japan. The HCC cell lines, JHH-4 and JHH-5 [13, 14, 15], were obtained from the JCRB Cell Bank (Osaka, Japan). HepG2 was cultured in Dulbecco's modified Eagle's medium (Wako, Tokyo, Japan), which contained fetal bovine serum (FBS), 10% (v/v; Nichirei Biosciences, Inc., Tokyo, Japan), and penicillin–streptomycin (Sigma-Aldrich, St. Louis, MO, USA). JHH-4 was cultured in Eagle's minimal essential medium (Wako), which contained FBS, 10% (v/v), and penicillin–streptomycin. JHH-5 was cultured in Williams' Medium E medium (Thermo Fisher Scientific, Waltham, MA, USA), which contained heat-inactivated FBS, 10% (v/v), and penicillin–streptomycin. All cell lines were cultured in a humidified incubator with 5% CO_2 at 37°C. Human hepatocytes (pooled from ten mixed-gender donors) were purchased from Lonza (HUCS10P; Basel, Switzerland), and total RNA was extracted without culturing cells.

Preparation of RNA

Cultured hepatocellular carcinoma cells were collected on three separate days. Total RNA was extracted from cells by using the RNeasy Mini Kit (QIAGEN, Hilden, Germany). Total RNA (5 μg) was incubated in the presence or absence of RNase R (Lucigen, Madison, WI, USA) for 30 min at 37°C, and then purified by using the RNeasy Mini Kit (30 μl eluate).

Reverse transcription (RT) followed by quantitative PCR (qPCR)

Purified RNA (6 μl) was reverse transcribed using ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan). Template cDNA was amplified by using GoTaq qPCR Mater Mix (Promega, Madison, WI, USA) and the following cycling conditions: 1 cycle of 95°C for 2 min; 40 cycles of 95°C for 15 sec and 60°C for 1 min. To calculate the number of target circRNA-derived cDNAs, DNA fragments corresponding to target cDNA were used as references. If necessary, amplicons were analyzed by sequencing (Eurofin, Kanagawa, Japan). The primer sequences used in these experiments are shown in Supplementary Table S1.

Results

Screening of circRNAs for potential biomarkers of liver cancer

To identify circRNA biomarkers of liver cancer, we compared circRNA expression levels in primary HCC with those in adjacent normal tissues by analyzing publicly available RNA-seq data from 16 HCC patients (GSE77509; clinical features summarized in Supplementary Table S2). We measured expression of 271 circRNAs commonly expressed in these patients. On the basis of these circRNA expression patterns, we classified patients into three groups, which comprised either only normal tissue samples (Group I), only HCC samples (Group III), or mainly HCC samples but also some normal tissue samples (N3, N6, N8, and N10; Group II; Supplementary Figure S2 and Supplementary Table S3).

Next, we selected eight circRNAs that were expressed at least 3-fold more or 3-fold less in HCCs than in normal tissues, and whose expression in HCCs was significantly different from that in

normal tissues ($p < 0.05$; Figure 1). Five of these circRNAs were downregulated in tumor tissues whereas three of these circRNAs were upregulated. Downregulation of one of the circRNAs, circ_ZKSCAN1, was reported for HCC, and circ_ZKSCAN1 is a potential liver cancer biomarker [9]. Hsa_circ_0001438, another downregulated circRNA, is derived from the *La-related protein 1 B* (*LARP1B*) gene. *LARP1A*, a gene encoding a member of the LARP protein family, is an oncogene often upregulated in cervix, liver, breast, prostate cancers [16], and HCC [17]. LARP1B is a paralog protein of LARP1A and possesses the La motif, a protein domain common to members of the LARP protein family [16]. Although involvement of *LARP1B* in cancers remains unclear, we focused on hsa_circ_0001438 because of its potential role in HCC oncogenesis. Hsa_circ_0000417, one of three upregulated circular RNAs, is derived from the *cleavage and polyadenylation specific factor 6* (*CPSF6*) gene. Because CPSF6 has been reported to progress HCC by alternative polyadenylation [18] and promote glycolysis for suppression of apoptosis in HCC [19], we also focused on hsa_circ_0000417.

To measure the specificity and sensitivity of hsa_circ_0001438, circ_ZKSCAN1, and hsa_circ_0000417, we prepared ROC curves (Figure 2). Area under the curve (AUC) of hsa_circ_0001438, circ_ZKSCAN1, and hsa_circ_0000417 were 0.941, 0.965, and 0.738, respectively. Because diagnostic tests with $AUC > 0.9$ and those with $0.7 < AUC < 0.9$ are considered highly and moderately accurate respectively [20], hsa_circ_0001438 and hsa_circ_0000417 might be effective diagnostic biomarkers. We noted that the downregulated circRNAs were more sensitive and specific HCC biomarkers ($AUC \geq 0.9$) than the upregulated circRNAs ($AUC < 0.9$; Supplementary Figure S3).

Expression of hsa_circ_0001438 and hsa_circ_0000417 in liver cancer cells

To measure expression levels of hsa_circ_0001438 in liver cancer cells, we used divergent primers. We extracted total RNA from hepatoblastoma HepG2 cells [21], treated the RNA either with or without RNase R, and performed RT-PCR. Hsa_circ_0001438 stably and specifically amplified by using the divergent primers (Supplementary Figure S4A and B). We confirmed that the divergent primers amplified the intended target sequence by sequencing the amplicon (Supplementary Figure S4B–D). We also confirmed specific amplification of hsa_circ_0000417 with designed divergent primers (Supplementary Figure S5). Thus, these primers could be used to detect hsa_circ_0001438 and hsa_circ_0000417 by qPCR. Although we designed some divergent primers to detect other circRNAs, only primers for circ_ZKSCAN1 successfully amplified the target, as reported previously (Supplementary Figure S4A) [9].

Next, we compared expression levels of hsa_circ_0001438 among commercially available normal hepatocytes (mixed hepatocytes from ten donors) and the non-viral HCC cell lines JHH-5 (Edmondson-Steiner grade I) and JHH-4 (grade III) [13, 14, 15]. By Giemsa staining, we observed that the normal hepatocytes had typical phenotypes (e.g., well-stained, uniformly sized, round, with either one or two round nuclei; Supplementary Figure S6), although the average body mass index of these donors was slightly high (Supplementary Table S4) [22]. Therefore, we concluded that these normal hepatocytes were non-pathological and did not comprise many fatty hepatocytes. We found that expression of hsa_circ_0001438 was lower in JHH-4 and JHH-5 than in normal hepatocytes

(Supplementary Figure S7A). Normalizing circRNA expression levels to those of an internal control RNA (*GAPDH* mRNA) [9], we confirmed that hsa_circ_0001438 expression was reduced in the HCC cell lines (Figure 3A). We obtained similar results for circ_ZKSCAN1 (Supplementary Figure S7B and Figure 3B), consistent with a previous report [9]. On the other hand, hsa_circ_0000417 was strongly and slightly upregulated in JHH-5 and JHH-4 respectively (Supplementary Figure S7C and Figure 3C). Thus, hsa_circ_0001438 and hsa_circ_0000417 might be potentially biomarkers to specifically diagnose HCC.

Discussion

In this study, we found that hsa_circ_0001438 and hsa_circ_0000417 are downregulated and upregulated respectively in HCC. Hsa_circ_0001438 may be a useful biomarker to diagnose either early-stage HCC or non-viral HCC, since hsa_circ_0001438 expression is downregulated in the early-stage, Edmondson-Steiner grade I HCC cell line, JHH-5, and since both JHH-4 and JHH-5 are non-viral HCC cell lines. Hsa_circ_0001438 may perform as well as other potential HCC circRNA biomarkers (such as hsa_circ_0005075 and cSMARCA5 [hsa_circ_0001445]) [23, 24] because the hsa_circ_00001438 AUC was 0.941 (Figure 2A), which is comparable to those of hsa_circ_0005075 (0.94) [23] and cSMARCA5 (0.938) [24]. Hsa_circ_0001438 may even be superior for diagnosis because its expression was downregulated (3-fold) in HCC more than that of cSMARCA5 (2-fold; Supplementary Table S3), and because hsa_circ_0005075 expression was undetectable in our dataset, contrary to a prior report (Supplementary Table S3) [23]. Thus, hsa_circ_00001438 may be a superior

diagnostic biomarker for HCC than alternative biomarkers (such as cSMARCA5 or hsa_circ_0005075). Hsa_circ_0000417 was strongly upregulated in the Edmondson-Steiner grade I HCC cell line, JHH-5, but just slightly upregulated in the Edmondson-Steiner grade III HCC cell line, JHH-4 (Figure 3B). These results suggest that it is expressed at an earlier stage of carcinogenesis and might be useful to distinguish undifferentiated or highly differentiated HCC by combinatorial use with hsa_circ_0001438.

Absolute quantification of hsa_circ_0001438 and hsa_circ_0000417 (i.e., quantitative comparison without normalization) requires only amplification primers for each target. By contrast, relative quantification requires additional primers to amplify an internal control (such as *GAPDH* mRNA). Relative quantification of circRNA biomarkers may more accurately diagnose HCC than absolute quantification because normalization addresses sample-to-sample variation due to differences in RNA quality or technical error. Both absolute quantification and relative quantification of has_circ_0001438 and hsa_circ_0000417 consistently demonstrated that hsa_circ_0001438 and hsa_circ_0000417 are downregulated or upregulated respectively in the HCC cell lines (Figure 3 and Supplementary Figure S7).

CircRNA expression patterns in some normal tissue samples (N3, N6, N8, and N10) were similar to those in some HCC samples; these normal tissue samples and similar HCC samples were classified together (Group II; Figure 1 and Supplementary Figure S2). The normal tissue samples in Group II might have contained malignant or pre-malignant cells. We expect that the detection of abnormal cells in regions adjacent to HCCs may help to develop treatment strategies or to predict patient

outcome. It may be useful to measure hsa_circ_0001438 and hsa_circ_0000417 expression in normal tissues taken from regions adjacent to HCCs during surgical treatment of liver cancer. We expect that these normal tissues collected during biopsy in fact contain abnormal cells. Further experiments are required to measure hsa_circ_0001438 and hsa_circ_0000417 levels in normal tissue samples and by liquid biopsy.

In this study, we identified potentially useful biomarkers for diagnosing HCC. Our experimental strategy using a public database was simpler and more cost-effective than the strategy of performing RNA-seq and bioinformatically analyzing the data. Our strategy is useful and reliable, since we identified circRNAs that were previously reported to be useful HCC biomarkers (such as circZKSCAN). Our strategy can inform and help efforts to identify circRNA biomarkers for other diseases. On the other hand, we used limited kinds of normal hepatocytes and HCC cell lines to confirm expression of identified circRNAs in HCC. We could not perform some statistical analyses because the commercial normal hepatocytes used were a mixture of hepatocytes from ten different donors. Thus, further studies using unmixed normal hepatocytes (from distinct, healthy donors) would be an interesting future issue to more accurately evaluate expression of identified circRNAs.

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Author contributions

S.I. conceived the project, designed experiments, performed experiments, analyzed data, and wrote the manuscript. S.N. performed the experiments. T.F. and H.F. supervised the project, designed experiments, and wrote the manuscript. All authors approved the final manuscript.

Competing interests

The authors declare no competing interests.

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Figure legends

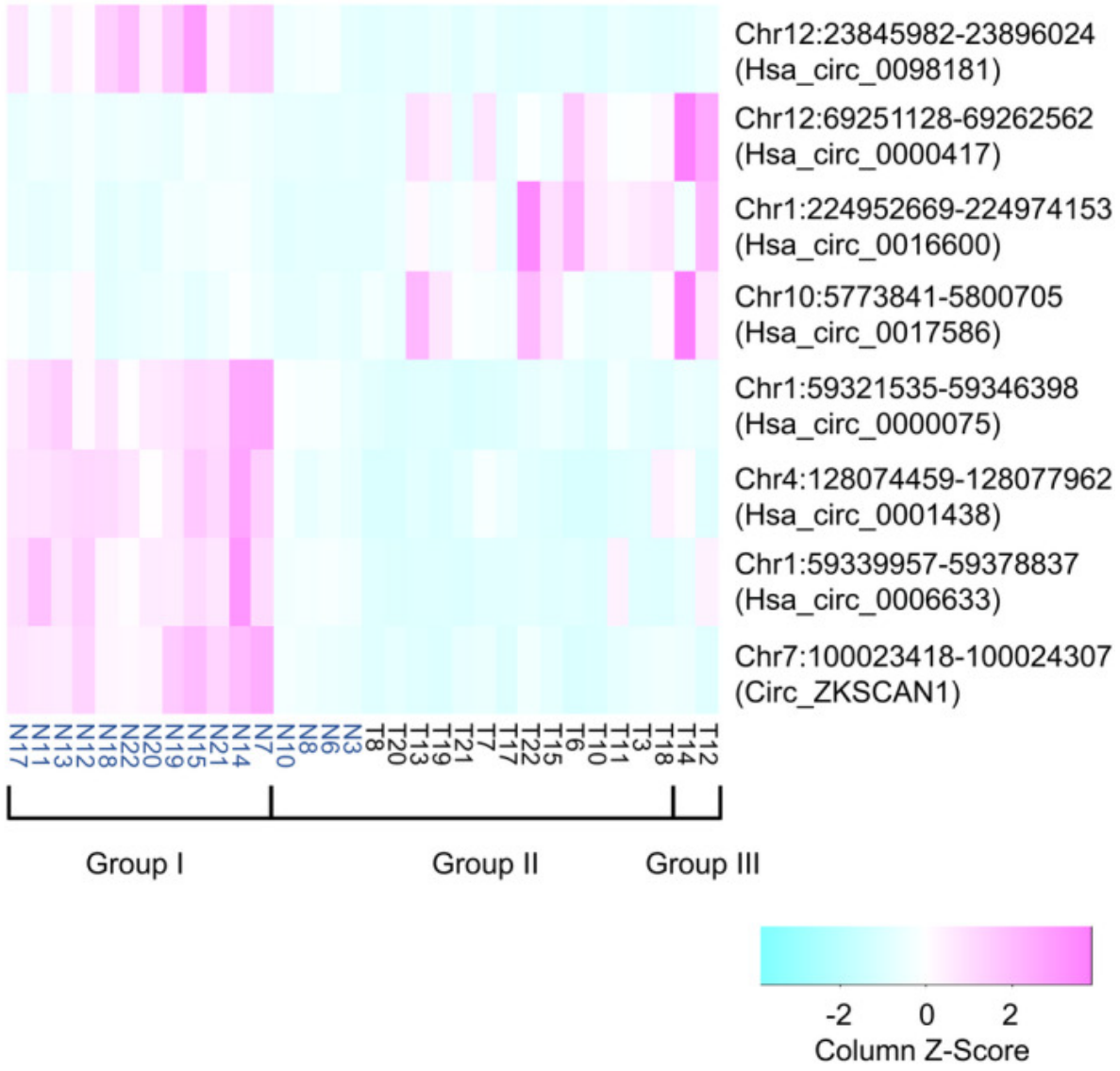

Figure 1. Heatmap of circRNA expression in hepatocellular carcinomas (HCCs) and adjacent normal tissues. Expression of eight selected circRNAs commonly expressed in 16 HCCs was measured by analysis of RNA-seq data. Both the chromosomal location and the circBase identification numbers of each circRNA gene are shown. Circ_ZKSCAN1 is also known as hsa_circ_0001727.

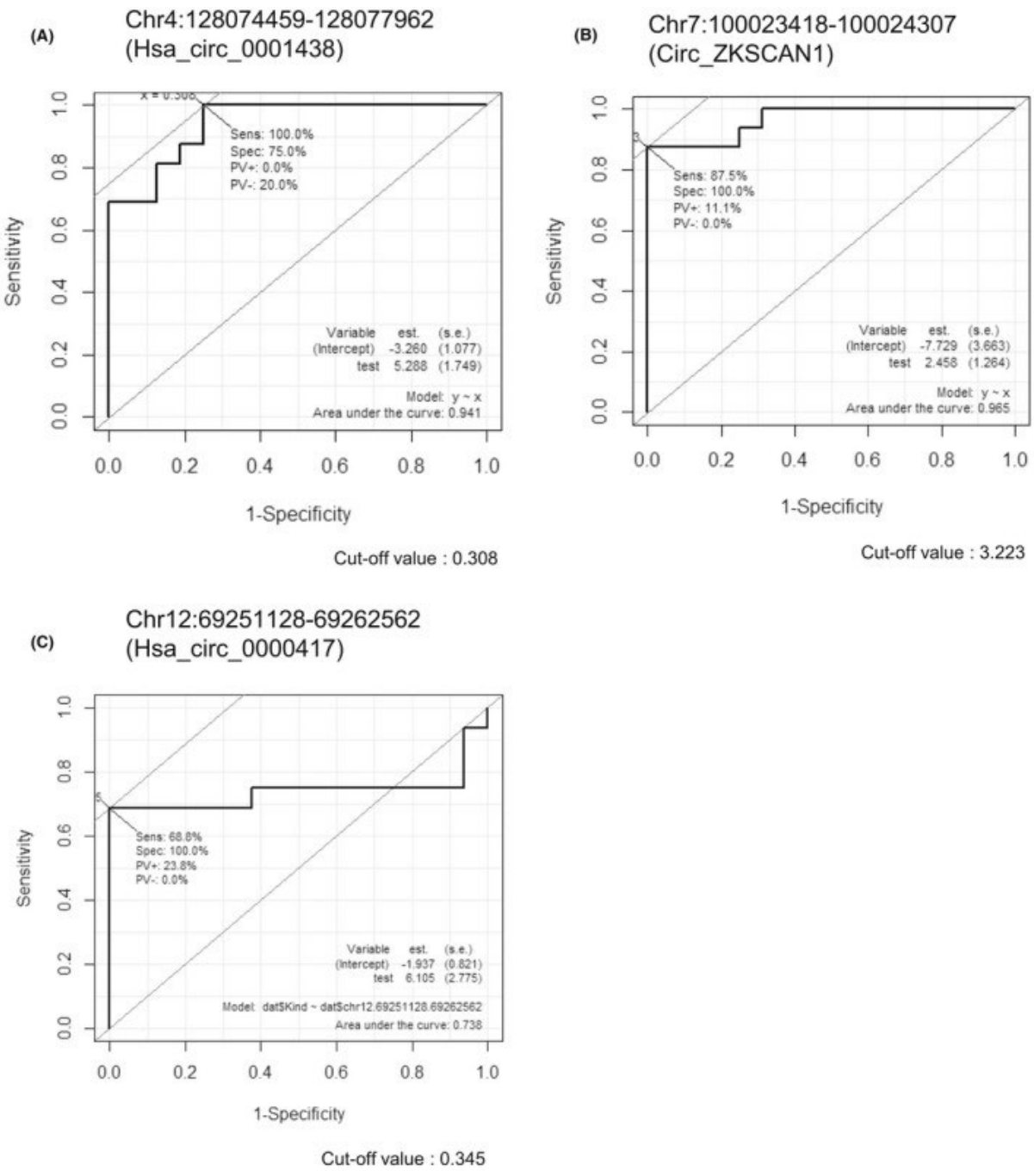

Figure 2. Receiver operating characteristic (ROC) curves for HCC circRNA biomarkers. ROC curves were prepared for hsa_circ_0001438 (**A**), circ_ZKSCAN1 (**B**), and hsa_circ_0000417 (**C**). Cut-off values are shown under each graph.

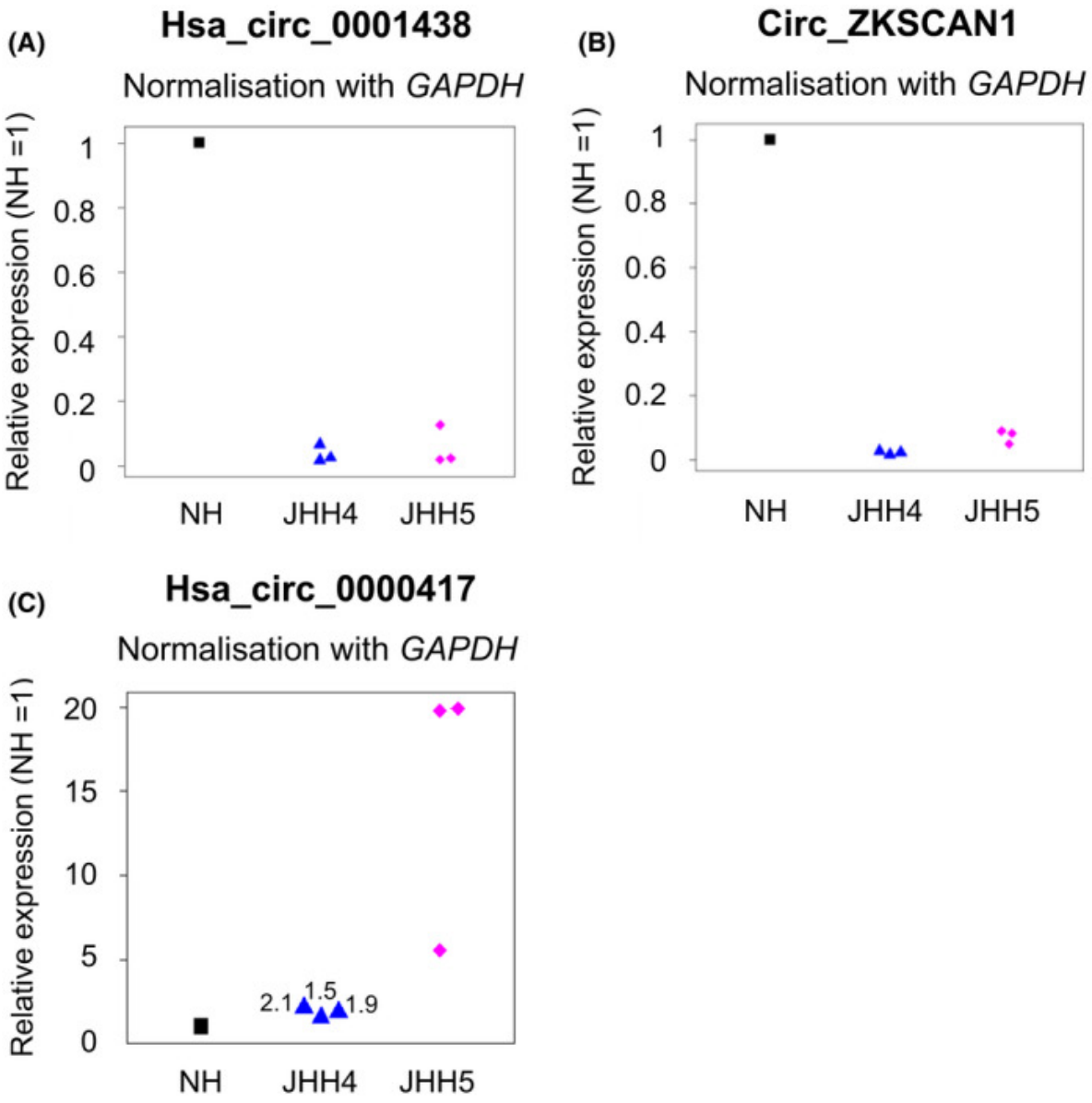

Figure 3. Expression levels of hsa_circ_0001438, circ_ZKSCAN1, and hsa_circ_0000417 in HCC cell lines. Normalized expression levels of hsa_circ_0001438 (**A**), circ_ZKSCAN1 (**B**), and hsa_circ_0000417 (**C**) are shown. *Glyceraldehyde-3-Phosphate Dehydrogenase* (*GAPDH*) mRNA was used for normalization. NH: Normal hepatocytes.