RESEARCH ARTICLE

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Identification of Circular RNAs as Biomarker Candidates in Lung Cancer Treatment

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Abstract

Objective: Lung cancer is the most common malignancy and among the leading cause of cancer death worldwide. Therefore, there is an important need for biomarkers that can be used in the early diagnosis of the disease and in the follow-up of treatment. Circular RNAs (circRNAs) have a covalently closed circular structure that lacks 3′ and 5′ polar ends and is resistant to RNAase enzymes. Due to these properties, they can be stably found in body fluids. Therefore, they can serve as potential biomarkers in the diagnosis, monitoring of therapeutic response and prognosis of cancer. In our study, we aimed to investigate the expression levels of circRNA molecules in the treatment of lung cancer and to determine those that have the potential to be biomarkers. **Methods:** In this in vitro study, expression levels of 163 circRNAs were investigated in A549 cells, a non-small cell lung cancer cell line, before and after treatment with carboplatin and pemetrexed. Total RNA isolation and cDNA synthesis were performed after treatments. Expression levels of circRNA genes were determined by RT-qPCR method with the designed divergent primer sequences. **Results:** The study revealed the characterisation of differentially expressed circRNAs by treatment in lung cancer cells. Of them, hsa_circ_0001320 is not expressed in cancer cells, is expressed only after treatment, and increased the level of its expression in response to combination therapy. **Conclusion:** As a result, while carboplatin, pemetrexed, and combined drug applications changed the expression levels of some circRNAs in lung cancer cells, some circRNAs were expressed only after treatment. In treatment follow-up and management, hsa_circ_0001320 has been identified as potential biomarker candidate.

Keywords: Non-small cell lung cancer- circular RNA- carboplatin- pemetrexed- biomarkers

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Introduction

According to the report of the World Health Organization (WHO), lung cancer is the leading cause of cancer-related deaths all over the world and causes approximately 1.6 million deaths each year (WHO|World Health Organization https://www.who.int). Small cell lung cancer (SCLC) accounts for 15% of lung tumors [1] and non-small cell lung cancer (NSCLC) accounts for 85% [2, 3]. It includes different histological tumor types; adenocarcinoma, squamous cell carcinoma, and large cell carcinoma [4, 5]. Lung cancer is usually diagnosed at an advanced stage and rarely at the early stages. At the time of diagnosis, a significant subgroup of patients have already developed distant metastasis [6]. Clinical findings such as cough, chest pain, hemoptysis, weight loss and dyspnea are present in 75% of lung cancer patients. Chest radiography and imaging tests are performed in patients with these findings. However, the results can be found to be normal in 3% of patients with lung cancer [7]. For this reason, all patients with clinically and radiologically suspected lung tumors are screened with contrast-enhanced computed tomography (CT) of the chest and upper abdomen. After the lesion is detected by imaging techniques, the diagnosis is confirmed pathologically with the biopsy sample taken.

Due to the difficulties in the diagnosis of lung cancer, there is a significant need for non-invasive diagnostic tools or biomarkers. Some peripheral blood-derived biomarkers are being tested for cancer diagnosis, but their low sensitivity and specificity limit their widespread application in cancer screening of the general population [8]. Recently, circRNAs, which can be found in tumor tissues and body fluids, have started to be of great interest due to their biomarker capacity [9].

CircRNAs are described as a new type of non-coding RNA (ncRNA), characterized as a covalently closed, circular molecule without 3′ and 5′ polar ends [10, 11]. They arise from ligation of introns, exons, or both [9] and have potential functions in regulating gene expression. CircRNAs are expected to become a new molecular marker for cancer diagnosis and treatment. In recent years, circRNAs have been detected in peripheral blood,

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especially in serum exosomes [12]. It has been found that the expression of specific circRNAs in exosomes of cancer tissue is significantly higher than in normal tissues [13].

Significant subsets of circRNAs were associated with cell proliferation, histological subtype or genotype in a panel of 60 lung cancer patients and non-transformed cell lines [14]. She et al. [15] showed in their study that hsa_circ_0062389 circRNA reduced the proliferation of NSCLC cells and arrested the cells in the G0/G1 phase. They also reported that hsa_circ_0062389 sponges miR-103a-3p to promote CCNE1 expression in NSCLC [16]. Qiu et al. stated in their study that proto-oncogenic circPRKCI increased the proliferation and tumorigenesis of lung adenocarcinoma by sponging both miR-545 and miR-589, thus eliminating the suppression of the oncogenic transcription factor E2F7 [16]. All these and other reports from literature implies that circRNAs are potential promising biomarkers for both diagnosis and prognosis of lung cancer. In this preliminary in-vitro study, we analyzed the expression of 163 lung cancer associated circRNAs in carboplatin and pemetrexed treated A549 cell line.

Materials and Methods

Cell Culture

The non-small lung cancer cell line, A549 (ATCC, CCL-185) used in the study was were very kindly provided by Department of Bioengineering, Ege University, Izmir, Turkey. A549 cell was checked with a mycoplasma PCR Detection Kit (Sigma Aldrich, USA). Cells were sustained in RPMI 1640 culture medium with stable L-Glutamine (Biological Industries, USA), 10% FBS (Sigma Aldrich, USA), and 1% Penicilline/Streptomycin (Gibco, USA) at 37°C and with 5% CO_2. Subculturing was performed when the cells reached 90% confluency.

Cytotoxicity Test

Determination of the cytotoxic effect after carboplatin (Koçak Farma, Türkiye, 8699828770213) and pemetrexed (Koçak Farma, Türkiye, 869982879022) treatment to A549 cells was carried out by using Resazurin (Grisp, Portugal, GTC20.0025). A549 cells were seeded in 96-well plates at a concentration of 2 x 104 cells/ml. After 24 hours of incubation at 37°C and 5% CO_2 environment, the medium was replaced with the drug containing one. Carboplatin and pemetrexed were applied with fresh medium at different doses (carboplatin: 1µM, 2µM, 10µM, 20µM, 30µM, 60µM, 80µM, 100µM, 150µM; pemetrexed: 0.5µM, 1µM, 2µM, 5µM, 10µM). Following 24 hours of incubation, the wells were washed with PBS and 90 µl of fresh medium was added, together with 10 µl of Resazurin solution. After 4 hours of incubation at 37 °C and 5% CO_2, absorbance was measured at 570 nm and IC50 values were calculated.

Treatments and RNA Isolation

After determining the IC_{50} values of the drugs, 4 experimental groups were formed; A549 cell line with no treatment, A549 cancer cell line treated with Carboplatin, A549 cell line treated with Pemetrexed, and A549 cell line treated with the combined drug (Carboplatin and Pemetrexed). A549 cells were seeded into 75-cm^2 flasks at $2x10^4$cells/ml concentration. When the cells reached 80% confluency, IC_{50} doses of carboplatin and pemetrexed were added to the flasks. At the end of 48 h of incubation, total RNA from all cell lines was extracted using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, USA). RNA concentrations were measured using NanoPhotometer® N60 and stored at -80°C until cDNA synthesis was performed.

Complementary DNA Synthesis

After total RNA isolation of cells in all experimental groups, complementary DNAs (cDNAs) were synthesized using the OneScript Plus cDNA synthesis kit (Applied Biological Materials, Canada) following the instructions provided by the manufacturer. All procedures were carried out in ice. The reaction components were mixed so that the total volume per sample was 20 µL. Then, the reaction was carried out in a PCR Thermal Cycler device at 50°C for 15 minutes. The mixture was then incubated at 85°C for 5 minutes. Obtained cDNA products were stored at -20°C until qPCR experiments were performed.

Primer Design

Circrna associated with lung cancer has been identified from the relevant articles using the terms ("NSCLC", "Circular RNA", "Lung Cancer" and "Cancer") in PubMed and Web of Science databases. Only the articles written in English between 2016 and 2021 were included. In total, 163 circRNA molecules associated with lung cancer were found. The IDs of the circRNAs determined from the literature were checked from http://www.circbase.org. These circRNA ID numbers were entered into CircInteractome web tool (http://circinteractome.irp.nia.nih.gov) which designs "Divergent Primers" that bind and amplify circRNAs but fail to amplify linear counterparts (Figure 1). All primers were synthesized and purified by Macrogen Europe (Netherlands). The sequences of all circRNA primers were listed in supplementary (Table S1).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Expression levels of circRNAs were determined by RT-qPCR. cDNAs were diluted 1:20 with distilled water. The total reaction volume for each circRNA gene was 10 µl and PCR mix included 5 µl 2X FastStart Essential DNA Green Master (Roche, USA), 1 µl of 10 µM each primer, 1 µl diluted cDNA, and 2 µl H_2O. PCR condition was carried out in 45 cycles, 95°C 10 sec denaturation, 60oC 10 sec annealing, 72°C 10 sec elongation; preceded by an initial cycle of 95°C 10 min pre-denaturation, in a 96-well plate. LightCycler® 480 System was used to evaluate the expression level of target genes. The specificity of PCR amplification products was confirmed with electrophoresis on 2% agarose gels. Beta Actin (ACTB) was used for normalisation. Changes in Ct values obtained from the device were analyzed using the ΔΔCT method.

Bioinformatic Analysis

The expression levels of circRNAs that showed

significant differences among the experimental groups and that had biomarker potential were evaluated together with bioinformatics data. For this reason, circRNAs which have statistically significant expression levels were analyzed using the CircNET platform (https://awi.cuhk.edu.cn/~CircNet/php/index.php) in lung cancer and other cancer tissues. In addition, molecular pathways in which these circRNAs play a role were analyzed using KEGG platform. RNA-sequence data and clinical features of lung cancer patients were downloaded from The Cancer Genome Atlas (TCGA) and Clinical Proteomic Tumor Analysis Consortium (CPTAC) cohort. The overall survival analysis of the gene was demonstrated by Kaplan-Meier survival analysis (with 95% confidence interval and hazard ratio by Cox PH Model).

Statistical Analysis

The difference between the control group and the treatment group was analyzed using the GraphPad Prism program (Software Inc., CA, USA). One-way analysis of variance (ANOVA) test was used to analyze all the data. Significant changes in the ANOVA test were evaluated with Tukey or Bonferroni posttest. $p < 0.05$ was considered significant (ns; not significant, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0, 0001$). All the experiments were performed in triplicate.

Results

Four circRNAs expressed in all groups

We performed dose determination experiments for both carboplatin (CP) and pemetrexed (PX), for which IC50 values were 80μM and 2μM respectively. Following drug treatments for 48h, expression of a total of 163 cirRNAs was evaluated by qPCR. Of these, 79 were found to be expressed in at least one group, whereas 84 circRNAs lacked expression in A549 cell line either with or without treatment. The characteristics of the circRNAs expressed in any experiment group were given Table 1.

While 4 of the circRNA genes were expressed in all experimental groups, 75 of them were found to be expressed in some of the control and/or drug-administered experimental groups (Figure 2A). We determined that hsa_circ_0001346, hsa_circ_0002130, hsa_circ_0001900, and hsa_circ_0002346 genes were expressed in both control and treated cancer cell line. There was no significant change in the expression level of the hsa_circ_0002346 circRNA gene among experimental groups. In contrast, there was a significant increase in the expression level of the hsa_circ_0002130 gene after carboplatin and combined treatment ($p=0.0002$, $p<0.0001$, respectively). A significant decrease was detected in the hsa_circ_0001346 gene after carboplatin and combined therapy ($p=0.0024$, $p=0.0054$, respectively). No significant difference was found in either gene after pemetrexed treatment. While there was a significant decrease in the expression level of the hsa_circ_0001900 gene with carboplatin treatment ($p=0.0029$), no statistically significant change was found with pemetrexed and combined treatment. In conclusion, pemetrexed did not have a significant effect on the expression levels of these four circRNAs (Figure 2B).

Expression of circRNAs dependent on treatment response

According to the results of RT-qPCR analysis, it was determined that some circRNAs were not expressed in A549 cell line, but were expressed following drug treatment. Of these, 4 circRNA genes (hsa_circ_0077837, hsa_circ_0000003, hsa_circ_0001320, and hsa_circ_0087862) were found to be expressed in all three treatment groups (Figure 3A). For the expression levels of hsa_circ_0001320 and hsa_circ_0087862 genes, a synergistic increase was detected in the combined treatment, along with an increase in the treatments applied alone. On the other hand, a decrease in expression level of hsa_circ_0077837 was detected with combination therapy. These differences were found to be statistically significant.

In addition, hsa_circ_0001492, hsa_circ_0008193, hsa_circ_0000064, and hsa_circ_00014235 genes were found to be expressed when carboplatin and

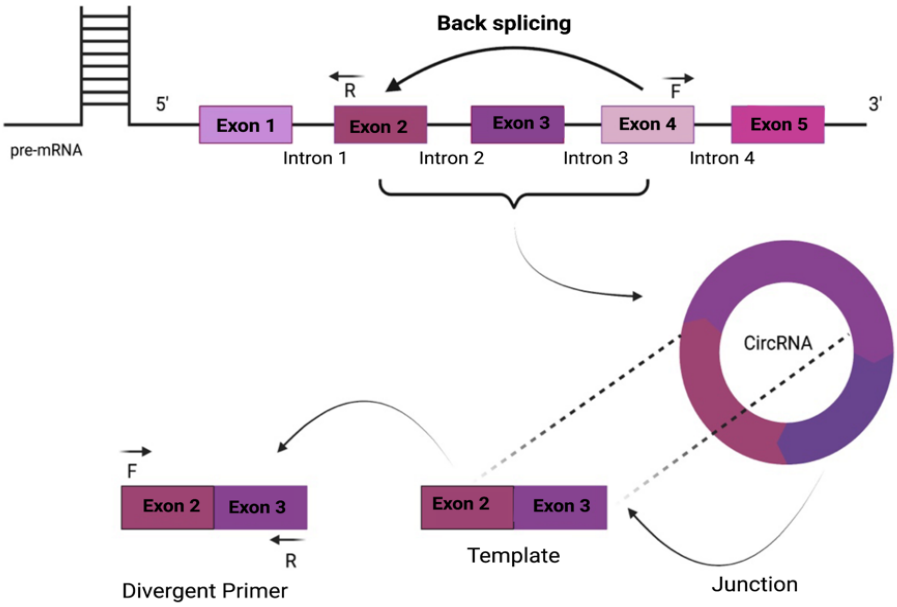

Figure 1. Primer Design of circRNAs. F, forward; R, reverse

Table 1. Characteristics of the circRNAs Expressed in Experiment Groups

circRNA-ID	Chromosomal Localization	Genomic Length (bp)	Gene Symbol
hsa_circ_0001875	chr9:96233422-96261168	27,746	*FAM120A*
hsa_circ_0007836	chr19:13919649-13920034	385	*ZSWIM4*
hsa_circ_0003998	chr7:27668989-27672064	10,343	*ARFGEF2*
hsa_circ_0003958	chr7:27668989-27672064	3,075	*HIBADH*
hsa_circ_0000003	chr1:1423242-1459777	36,535	*ATAD3B*
hsa_circ_0011385	chr1:32691771-32692131	360	*EIF3I*
hsa_circ_0008305	chr8:141799572-141840625	41,053	*PTK2*
hsa_circ_0008975	chr4:148800382-148803083	2,701	*ARHGAP10*
hsa_circ_0008133	chr9:36197547-36199093	1,546	*CLTA*
hsa_circ_0014235	chr1:153536209-153536363	154	*S100A2*
hsa_circ_0018818	chr10:75264607-75265184	577	*USP54*
hsa_circ_0017247	chr1:243736227-243859018	122,791	*AKT3*
hsa_circ_0087862	chr9:110068659-110081160	12,501	*RAD23B*
hsa_circ_0006867	chr4:151388824-151412187	23,363	*LRBA*
hsa_circ_0002702	chr9:35546426-35548532	2,106	*RUSC2*
hsa_circ_0102533	chr14:71502781-71522279	19,498	*PCNX*
hsa_circ_0137287	chr8:92301363-92307931	6,568	*SLC26A7*
hsa_circ_0106705	chr17:35608930-35646430	37,500	*ACACA*
hsa_circ_0072309	chr5:38523520-38530768	7,248	*LIFR*
hsa_circ_0001320	chr3:71064699-71102924	38,225	*FOXP1*
hsa_circ_0001287	chr3:44434346-44442826	8,480	*C3orf23*
hsa_circ_0049657	chr19:13183860-13186485	2625	*NFIX*
hsa_circ_0007385	chr2:32142994-32157204	14,210	*MEMO1*
hsa_circ_0067934	chr3:170013698-170015181	1,483	*PRKCI*
hsa_circ_0020123	chr10:119042605-119049859	7,254	*PDZD8*
hsa_circ_0060937	chr20:52779255-52782372	3,117	*CYP24A1*
hsa_circ_0000284	chr11:33307958-33309057	1,099	*HIPK3*
hsa_circ_0077837	chr6:131247744-131277639	29,895	*EPB41L2*
hsa_circ_0000064	chr1:44446997-44447136	139	*B4GALT2*
hsa_circ_0001936	chrX:79962925-79975155	12,230	*BRWD3*
hsa_circ_0022812	chr11:65055186-65063461	8,275	*POLA2*
hsa_circ_0030998	chr13:113963957-113964177	4,249	*LAMP1*
hsa_circ_0008193	chr9:96233422-96238620	8,206	*FAM120A*
hsa_circ_0001492	chr5:65284462-65290692	6,230	*ERBB2IP*
hsa_circ_0008274	chr13:96485180-96489456	4,276	*UGGT2*
hsa_circ_0000043	chr1:31465236-31468067	2,831	*PUM1*
hsa_circ_0046264	chr17:79813017-79817263	4,246	*P4HB*
hsa_circ_0037516	chr16:2369581-2369841	260	*ABCA3*
hsa_circ_0001346	chr3:149563797-149639014	75,217	*RNF13*
hsa_circ_0128332	chr5:149607617-149610943	3,326	*CAMK2A*
hsa_circ_0001900	chr9:138773478-138774924	1,446	*CAMSAP1*
hsa_circ_0086414	chr9:16435552-16437522	1,970	*BNC2*
hsa_circ_0072309	chr5:38523520-38530768	7,248	*LIFR*
hsa_circ_0002130	chr19:6702137-6702590	453	*C3*
hsa_circ_0007534	chr17:61869771-61877977	8,206	*DDX42*
hsa_circ_0039411	chr16:55523562-55540586	17,024	*MMP2*
hsa_circ_0001821	chr8:128902834-128903244	410	*TCONS00015354*
hsa_circ_0002346	chr2:36623756-36669878	46,122	*CRIM1*
hsa_circ_0008003	chr4:16587544-16900052	312,508	*LDB2*

Table 1. Continued

circRNA-ID	Chromosomal Localization	Genomic Length (bp)	Gene Symbol
hsa_circ_0003028	chr14:66028054-66028484	430	*FUT8*
hsa_circ_0001869	chr9:88920106-88924932	4,826	*ZCCHC6*
hsa_circ_0001439	chr4:129913321-129925031	11,710	*SCLT1*
hsa_circ_0079530	chr7:19155090-19155754	664	*TWIST1*
hsa_circ_0053958	chr2:36623756-36744685	120,929	*CRIM1*
hsa_circ_0008717	chr1:229665945-229678118	12,173	*ABCB10*
hsa_circ_0134501	chr7:64437862-64438282	420	*ZNF117*
hsa_circ_0023404	chr11:71668272-71671937	3,665	*RNF121*
hsa_circ_0031250	chr14:23395341-23396023	682	*PRMT5*
hsa_circ_0001073	chr2:148653869-148657467	3,598	*ACVR2A*
hsa_circ_0006571	chr2:200188525-200213896	25,371	*SATB2*
hsa_circ_0015278	chr1:173726114-173744981	18,867	*KLHL20*
hsa_circ_0092857	chr10:120809312-120810833	1,521	*EIF3A*
hsa_circ_0007534	chr17:61869771-61877977	8,206	*DDX42*
hsa_circ_0005927	chr8:42259305-42260979	1,674	*VDAC3*
hsa_circ_0000211	chr10:7318853-7327916	9,063	*SFMBT2*
hsa_circ_0007766	chr17:37864573-37866734	2,161	*ERBB2*
hsa_circ_0013958	chr1:147131074-147131890	816	*ACP6*
hsa_circ_0003645	chr16:19656207-19663412	7,205	*C16orf62*
hsa_circ_0006404	chr6:108984657-108986092	1,435	*FOXO3*
hsa_circ_0043265	chr17:35620587-35722691	102,104	*ACACA*
hsa_circ_0018534	chr10:70252888-70253327	439	*SLC25A16*
hsa_circ_0004050	chr4:88959402-88987031	27,629	*PKD2*
hsa_circ_0046263	chr17:79813017-79813462	445	*P4HB*
hsa_circ_0010235	chr1:19201875-19216599	1,724	*ALDH4A1*
hsa_circ_0000079	chr1:62908829-62914337	5,508	*USP1*
hsa_circ_0001724	chr7:92462409-92463134	725	*CDK6*
hsa_circ_0002360	chr21:36206706-36231875	25,169	*RUNX1*
hsa_circ_0020123	chr10:119042605-119049859	7,254	*PDZD8*
hsa_circ_0002874	chr9:4286037-4286523	486	*GLIS3*

pemetrexed were administered alone, but interestingly their expressions were completely inhibited by combined treatment (Figure 3B).

circRNAs silenced by CP or PX or combination therapy

Furthermore, some circRNAs were found to be affected by only single drug treatment. Of these, hsa_circ_00072309, hsa_circ_0067934, hsa_circ_0008274 and hsa_circ_0022812 genes were found to be completely inhibited by pemetrexed treatment (Figure 4A). Similarly, the expressions of hsa_circ_0003998, hsa_circ_00049657, hsa_circ_0018818 and hsa_circ_0000043 genes were inhibited by carboplatin (Figure 4B).

While significant changes were detected in the expression levels of some circRNAs expressed in the cancer cell line after single treatment, no expression was detected in the combined treatment. In particular, the expression levels of hsa_circ_0102533 and hsa_circ_0008305 genes decreased with both treatments, but completely disappeared with the combined treatment. The reductions in the expression levels of these circRNAs were found to be statistically significant when compared with non-treated control (CP; p=0.0450 and PX; p=0.0124, CP; p=0.0027 and PX; p=0.0136; respectively). On the other hand, the expression levels of hsa_circ_0001875 and hsa_circ_0007385 genes increased in single treatment. This increase was significant with carboplatin, but not with pemetrexed (CP; p=0.0203, p=0.0013, respectively). The expression level of the hsa_circ_0060937 gene increased significantly only after pemetrexed treatment (p=0.0074) (Figure 4C).

hsa_circ_0001320 as a potential biomarker

As hsa_circ_0001320 is not expressed in cancer cells, is expressed only after treatment, and increased the level of their expression in response to combination therapy, it has been identified as a potential biomarker candidate (Figure 5A). Therefore, the expression levels of this circRNAs was investigated in more detail in lung cancer and other cancer tissues by using the CircNet platform and TCGA data. Consistent with our RT-qPCR result, the expression of hsa_circ_0001320 gene was not detected in lung cancer

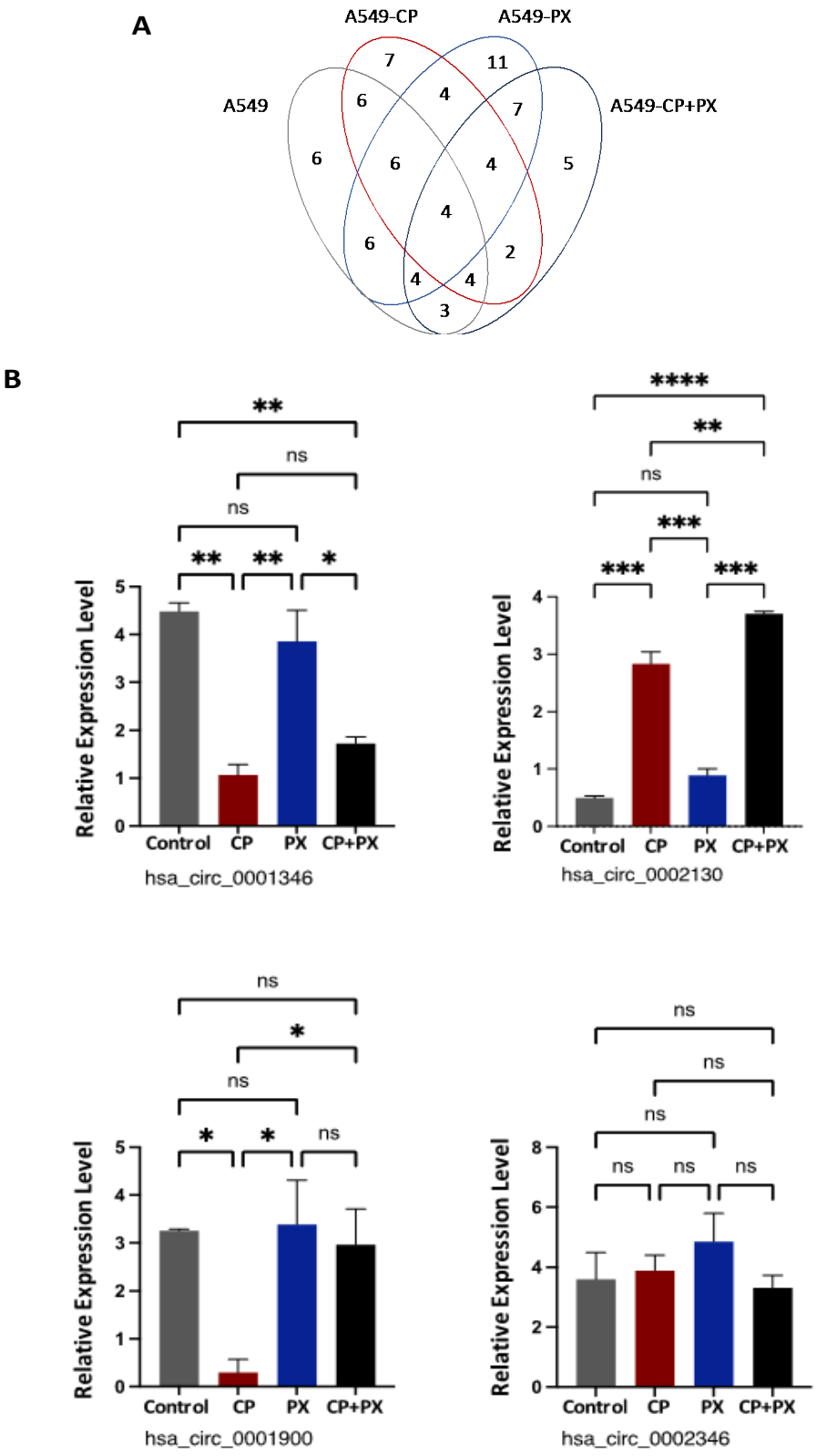

Figure 2. circRNA Genes Expressed in All Experimental Groups. A) All circRNAs expressed were shown in the venn diagram. B) Expression levels of circRNAs detected in all experimental groups. Gene expression level was calculated using the ΔΔCt method. Statistics were performed using one-way analysis of variance (ANOVA) test for comparisons * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. ns: not significant. Analyzes was performed in RT-qPCR as triplicate. (carboplatin; CP, pemetrexed; PX).

patients, with TCGA data. Similary, CircNet platform also showed that hsa_circ_0001320 gene was not expressed in lung cancer patients. However, this circRNAs has been found to be expressed in some cancer tissues (Figure 5B). In addition, we detected that FOXP1, parent gene of hsa_circ_0001320, was nonsignificantly decreased in lung cancer patients, with TCGA data (Figure 5C). The overall survival analysis showed that expression level of FOXP1

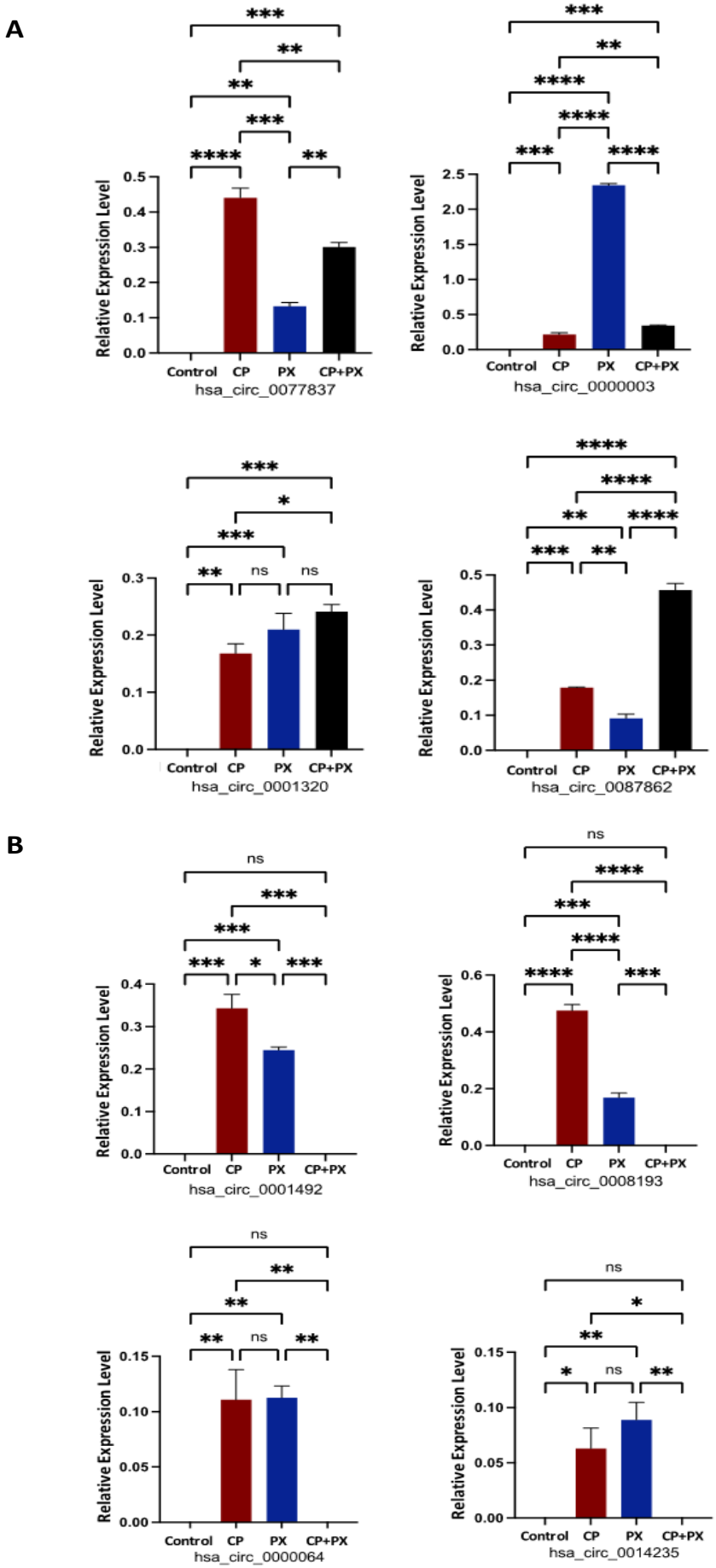

Figure 3. circRNA Genes Expressed Only in the Treatment Groups. A) Expression levels of circRNAs detected in the three treatment groups. B) Expression levels of circRNAs detected only in CP and PX treatments (carboplatin; CP, pemetrexed; PX).

was not associated with a prognosis in lung cancer patients ((Figure 5D). In addition, by KEGG analysis, the selected circRNAs are found to be mostly involved in PI3K-AKT signaling, and FOXO signaling in cancers (Figure 5E).

Discussion

CircRNAs have emerged as promising biomarkers in cancer because they exhibit tissue-specific expression patterns and are frequently dysregulated in cancer. Therefore, circRNAs have been increasingly studied for their potential as diagnostic, prognostic, and predictive biomarkers in lung cancer. CircRNAs may also serve as predictive biomarkers for treatment response and drug resistance in lung cancer, suggesting their potential utility in guiding treatment decisions [9, 12, 14].

In this study, as a result of the analysis of the expression levels of 163 circRNAs that we examined, it was found that 79 genes had statistically significantly different expression. Some of these circRNAs are thought

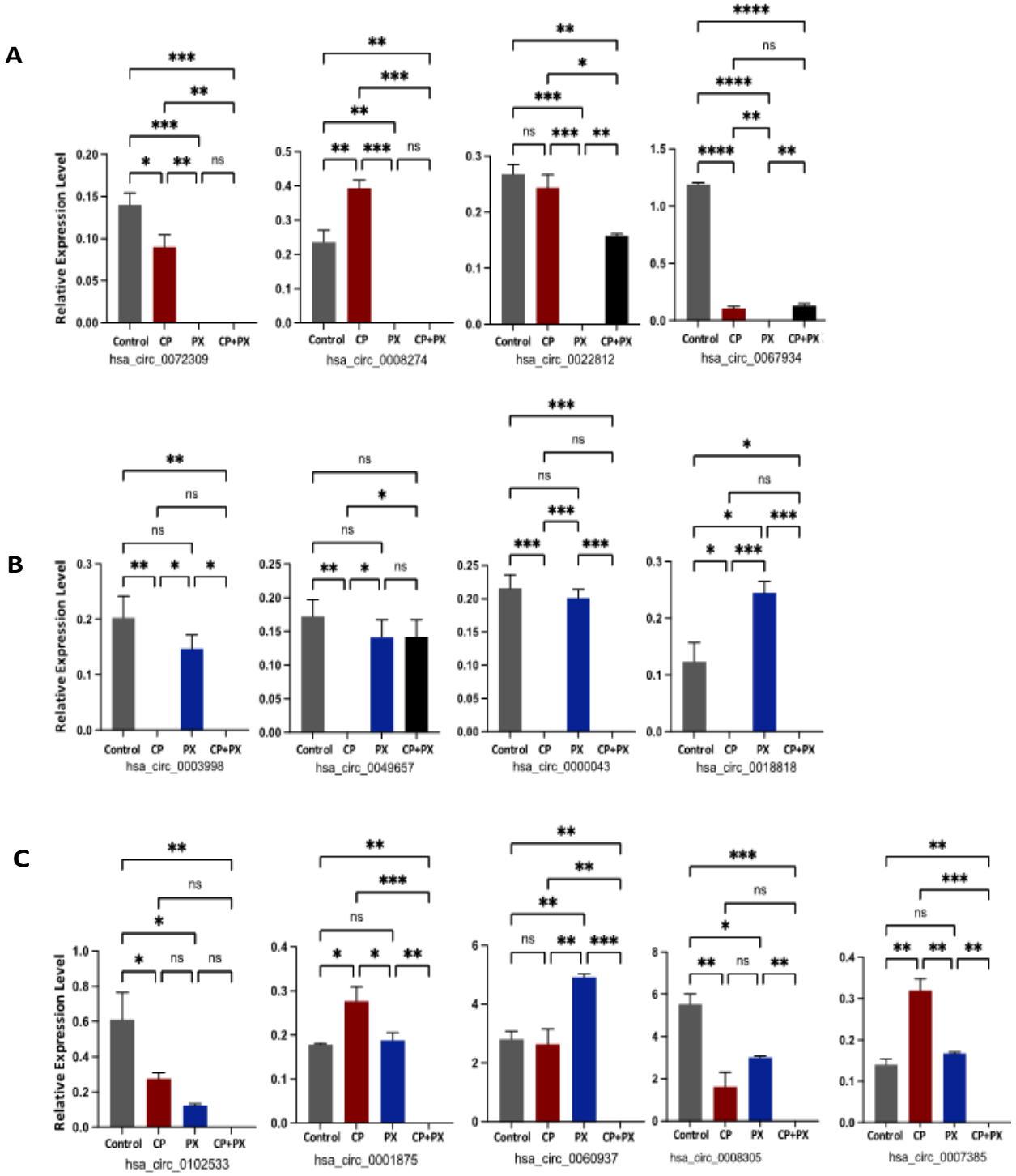

Figure 4. circRNAs Silenced by Treatment. A) The expressions of circRNA inhibited by only pemetrexed treatment. B) The expressions of circRNA inhibited by only carboplatin treatment. C) circRNAs affected by combination therapy (carboplatin; CP, pemetrexed; PX).

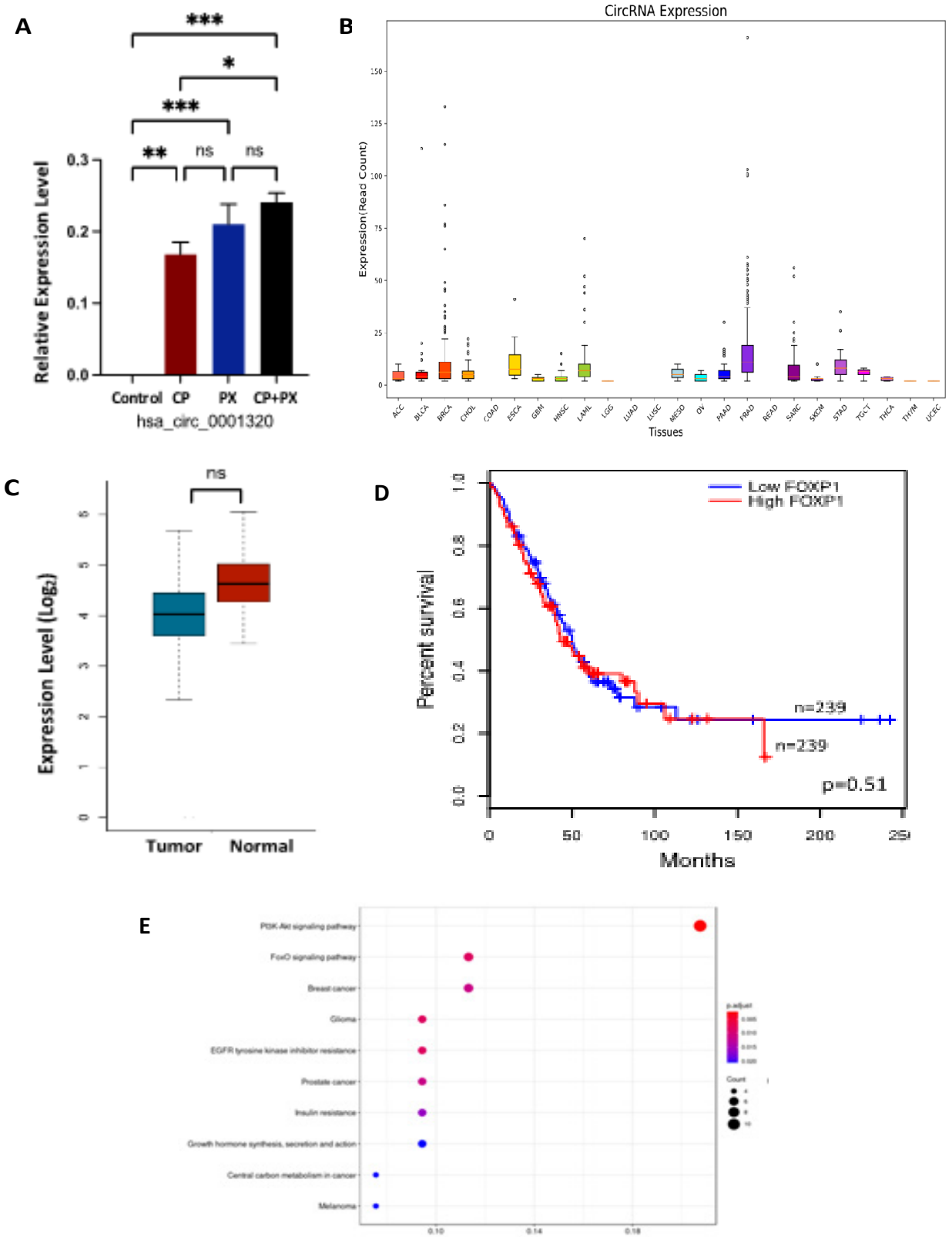

Figure 5. Upregulation of hsa_circ_0001320 in Treatment Groups. A) The expression level of hsa_circ_0001320 in all experimental groups by RT-qPCR performing in triplicate. B) The expression levels of hsa_circ_0001320 in different types of cancer tissues. C) The expression levels of FOXP1 gene in lung cancer patients (tumor n=483, normal n=347) in TCGA data. D) Kaplan-Meier survival curve of the overall survival in lung cancer patients from the CPTAC dataset. E) Functional prediction of hsa_circ_0001320 by KEGG analysis. (ACC: Adrenocortical carcinoma, BLCA: Bladder Urothelial Carcinoma, BRCA: Breast invasive carcinoma, CHOL: Cholangiocarcinoma, COAD: Colon adenocarcinoma, ESCA: Esophageal carcinoma, GBM: Glioblastoma multiforme, HNSC: Head and Neck squamous cell carcinoma, LAML: Acute Myeloid Leukemia, LGG: Brain Lower Grade Glioma, LUAD: Lung adenocarcinoma, LUSC: Lung squamous cell carcinoma, MESO: Mesothelioma, OV: Ovarian serous cystadenocarcinoma, PAAD: Pancreatic adenocarcinoma, PRAD: Prostate adenocarcinoma, READ: Rectum adenocarcinoma, SARC: Sarcoma, SKCM: Skin Cutaneous Melanoma, STAD: Stomach adenocarcinoma, TGCT: Testicular Germ Cell Tumors, THCA: Thyroid carcinoma, THYM: Thymoma, UCEC: Uterine Corpus Endometrial Carcinoma).

to be candidates for follow-up biomarkers in therapy due to their expression levels or changes in these levels.

It has been determined that some of these circRNAs are co-expressed in both the control cancer cell line and the treated cancer cell line, and there are changes in their expression levels. These genes were determined as hsa_circ_0001346, hsa_circ_0002346, hsa_circ_0002130 and hsa_circ_0001900. hsa_circ_0001346 (circRNF13) is reported to sponge and sequester miR-93-5p and thus suppress tumor invasion and metastasis in lung adenocarcinoma [17]. It was recently shown that circRNF13 plays a role in chemoresistance. Its knockdown sensitized colorectal cancer cells to oxaliplatin [18]. In our study, its expression markedly decreased following carboplatin treatment, whereas pemetrexed treatment did not alter it. Contrary to circRNF13, hsa_circ_0002130 expression was lower in A549 cells and it increased with platin based therapy. hsa_circ_0002130 has been implicated in osimertinib resistance in NSCLC [19]. Ma et al. also suggested that it may serve as a serum exosome-based biomarker. Although we observed low expression in A549 cells, we did not measure its exosomal levels.

In a study by Yang et al. [20] 15 circRNA genes were bioinformatically found to be downregulated in NSCLC tissues. By qPCR in A549 cell line, 7 of these circRNAs, including hsa_circ_0002346, were confirmed to be downregulated [20]. Although an increase in the expression level of this gene was detected with treatment in our study, this was not significant.

The significant decrease in expression levels of hsa_circ_0102533 and hsa_circ_0008305 genes with treatment suggests that they may be candidates for follow-up biomarkers. In our study, while these two genes decreased significantly with separate treatments, they showed a higher significant decrease with the synergistic effect of the combined treatment. Zhou et al. [21] found a significant increase in the expression of hsa_circ_0102533 gene in tumor tissues and blood samples from NSCLC patients. It has also been reported that high expression of hsa_circ_0102533 is associated with tumor type, TNM stages, lymph node metastasis, and distant metastasis or recurrence. Ultimately, the researchers thought that hsa_circ_0102533 in the blood could serve as an early tumor marker for NSCLC detection [21]. Taken together with our finding that its expression decreases significantly with treatment, hsa_circ_0102533 has a promising potential to be a follow-up biomarker.

Interestingly, we found that the expression of hsa_circ_0007385 increased after carboplatin treatment, while the expression was completely inhibited by combined treatment. This result suggests that the cells might respond to combined use of drugs by a completely different mechanism. Ye et al. showed that hsa_circ_0007385 is upregulated in NSCLC tissues and cells. Silencing hsa_circ_0007385 decreased NSCLC cell proliferation, migration, invasion, in vitro cisplatin resistance, and in vivo tumor growth [22]. Taken together with this, the increase in the expression levels of this gene after carboplatin treatment implies that it may be associated with the resistance to platinum based therapies.

Here we identify circRNAs hsa_circ_0001320 as potential biomarkers because they are not expressed in cancer cells, are expressed only after treatment, and increased their expression in response to combination therapy. RNA-Seq studies showed that hsa_circ_0001320 is downregulated in lung adenocarcinoma [23]. Mao et al. showed that overexpression of hsa_circ_0001320 inhibits the growth and invasion of lung cancer cells [24]. Consistent with these, our bioinformatics analysis results suggest that it may serve as a prognostic biomarker.

The current study is the first to evaluate the effects of pemetrexed and carboplatin on circRNA in lung cancer cells, suggesting basic information about the potential of biomarkers in monitoring treatment. Limitations of this study are the use of a single cell type for gene expression and the lack of patient samples.

In conclusion, the unique properties of circRNAs may serve as diagnostic biomarkers in lung cancer as well as predictive biomarkers for treatment response and drug resistance. A better understanding of the impact of chemotherapeutics on circRNA biology can reveal potential benefits in guiding treatment decisions. Therefore, further studies are needed in this area.

Author Contribution Statement

The authors confirm contribution to the paper as follows: AC contributed to the study of conception and design. Material preparation and data collection were performed by BA, UM, CM, MA, AC. Analysis was performed by BA, UM, AC. The first draft of the manuscript was written by UM and edited by AC. All authors read and approved the final manuscript.

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Conflict of Interest

The authors declare no conflict of interest.

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