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Research Article

Exosomal MicroRNA -142-5p in Serum is a Prognostic Factor in Cutaneous Squamous Cell Carcinoma

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Abstract

Purpose: Exosomal microRNAs in tumor microenvironment can have a significant impact on the plasticity of cancer cells leading to the promotion of metastasis and angiogenesis. MicroRNAs expressed in tumor cells, fibroblasts, immune cells, and endothelial cells are known to target mRNAs in origin cells to promote tumor growth and metastasis, and induce changes in cell phenotype and expression and secretion of cytokines. MicroRNA expression patterns, which change depending on the tumor type, provide clues that can serve as a biological marker for diagnosis, determining the outcome of the disease, and treatment responses. However, there has been no definitive study on the relationship between exosomal microRNAs and prognosis in cutaneous squamous cell carcinoma. Here, we studied the association between prognosis of cutaneous squamous cell carcinoma patients and microRNAs detected in blood serum.

Patients and methods: We enrolled 43 patients with various stages of cutaneous squamous cell carcinoma. Blood samples from patients were obtained and serum was separated by the using centrifuge. microRNAs were purified from serum exosomes, and microRNA sequencing was performed. The microRNA expression profiles and copy number variations of 43 serum samples were obtained using next-generation sequencing (Hiseq, Illumina). We analyzed the relationship between the microRNA changes and patient prognosis.

Results: miR-142a-5p cluster expression levels in serum exosomes were most correlated with the poor prognosis of cutaneous squamous cell carcinoma. Exosomal miR-142-5p expression levels in serum were significantly higher ($P< 0.01$) in patients with late-stage disease than in those with early-stage disease. In addition, patients with recurrent tumors showed high levels of miR-142-5p.

Conclusion: miR-142-5p expression level may be an essential marker for predicting patient prognosis and to help decide whether to undergo additional systemic chemotherapy after surgery.

Keywords: MicroRNA; Exosome; miR-142-5p; Cutaneous squamous cell carcinoma

Introduction

Cutaneous squamous cell carcinoma (CSCC) is the second most common type of non-melanoma skin cancer after basal cell carcinoma [1]. Early-stage CSCC is generally treated locally, such as surgical resection, dermal ablation, using topical agents, or radiation. In the case of advanced inoperable CSCC, palliative chemotherapy or radiation therapy is mainly conducted [2]. In many cases, CSCC relapses due to insufficient adjuvant treatment after surgery [3]. Until now, there is no standard biomarker that can precisely predict postoperative recurrence in CSCC. MicroRNAs (miRNAs) are small non-coding RNAs that control

gene expression. They affect various cells and are released into the extracellular space and transported to body fluids such as the blood and urine [4]. Recent research found evidence that miRNA is carried into body fluids within exosomes, which are small vesicles of cellular origin that function in the out-of-cell communication processes [5]. Exosomes in the extracellular fluid fuse with other cells to transfer cargo to receptor cells. Exosomal miRNAs play essential roles in intercellular communication and may have potential as biomarkers for detecting and monitoring diseases [6]. In particular, miRNAs have been demonstrated to play a key role in cancer pathogenesis. The overexpression of certain miRNAs in cancer cells has been previously thought to be oncogenic and lead to tumor development [7]. Exosomal miRNA signatures change depending on specific conditions and diseases. For example, miRNA-15a and miRNA-16-1 are often deleted in B cell chronic lymphocytic leukemia and other cancers [8]. miRNA-21 has been found to influence the proliferation, apoptosis, migration, invasion, and survival of cancer cells. miRNA-21 is overexpressed in various cancers such as breast, colon, lung, and pancreatic cancers [9]. V-myc, avian myelocytomatosis viral oncogene homolog (c-Myc), regulates the miRNA-7-92 cluster, consisting of miRNAs 17, 18a, 19a, 20a, 19b-1, and 92a-1, located at chromosome 13q31 to modulate E2F1 expression and inhibit the P53 pathways. Expression of the miRNA-7-92 cluster is significantly increased in lung cancer and lymphoma [10,11]. On the other hand, tumor suppressor miRNAs inhibit oncogenes and control apoptosis, thus preventing cancer development. For example, the increase in let-7 miRNA expression suppresses the growth of lung cancer cells [12]. miRNA-34 controls the expression of P53. A lack of miRNA-34 expression is related to prostate cancer development by decreasing p53 activity, which is involved in DNA damage repair [13,14]. In many cases, CSCC relapses due to insufficient adjuvant treatment after surgery. To date, there is no standard biomarker that can precisely predict the postoperative recurrence of CSCC. In this study, we attempted to identify miRNAs that might predict recurrence by analyzing miRNAs in cancer tissues and micro exosomes in plasma at different stages of cancer in patients with CSCC.

Materials and Methods

Patient characteristics and sample collection

Patients diagnosed with cutaneous squamous cell carcinoma were enrolled in this study. Data regarding age, sex, cancer TNM stage, pathology, and ECOG status were recorded (Table 1, n=43). All serum and cancer tissue samples were obtained from patients at the Konyang University Hospital between 2017 and 2018. Informed consent was obtained from all patients enrolled in this study. Serum samples were collected in EDTA tubes and centrifuged at 2500 rpm for 10 min, and microsomes were extracted within one day of serum collection. Each patient received appropriate treatment according to the guidelines of the Korean Cancer Study Group guidelines. All experimental methods were approved by the institutional review boards (IRB) of the Konyang University Hospital [15-18].

Patient characteristics		Number(%)	High MiRNA142-5p expression(Blood) Number (%)
Total		43 (100)	9 (20)
Sex	Male	31 (72)	6 (28)
	Female	12 (28)	3 (13)
Age	< 60years	7 (16)	4 (28)
	> 60years	36 (84)	5 (17)
TNM Stage	I	22 (51)	0 (0)
	II	9 (20)	1 (7)
	III	7 (16)	5 (71)
	IV	5 (12)	3 (60)
Tumor location	Extremity	18 (42)	4 (20)
	Trunk	21 (49)	3 (27)
	Head and Neck	4 (9)	2 (16)
Human papilloma virus	Positive	13 (30)	3 (50)
	Negative	26 (61)	4 (15)
	Unknown	4 (9)	2 (18)

Table 1: Clinical characteristics and miRNA- 142-5p expression in cutaneous cell carcinoma.

Isolation of exosomes from serum

Blood samples (20 mL) were collected in EDTA-coated tubes from patients with cutaneous squamous cell carcinoma and allowed to sit at room temperature for 30 min. Then, whole blood was centrifuged for 15 min at 3000 × g at 4 ˚C to separate the plasma. Exo2D precipitation was carried out according to the manufacturer's instructions (Exosomeplus Guideline). Briefly, the sample was carefully mixed with a vortex and then re-centrifuged for 15 min at 3000 × g at 4 ˚C to separate the cells and cell debris. The supernatant was filtered through a 0.2 μm syringe filter (Sartorius, Goettingen, Germany) and transferred to another tube. The sample was mixed with the Exo2D kit (ExosomePlus, Gyeonggi-do, Korea) at a 10:5 ratio. The sample mixed with the kit was centrifuged for 10 min at 3000 g at 4 ˚C. After centrifugation, the interphase was visually checked to determine if the phases were divided, and the supernatant was removed as much as possible. Approximately 100 μL of phosphate-buffered saline (PBS) was added to the remaining phase and was vortexed. After dilution, the isolated exosome vesicles were aliquoted and stored at −80 ˚C until use [19-21].

Extraction of total RNA and miRNA

Total RNA was isolated using a miRNeasy Mini Kit (Qiagen, Hilden, Germany). The samples were stored at 2–8 ˚C for up to 6 h or used directly in the procedure. To process the frozen lysates, the samples were incubated at 37 ˚C in a water bath until they were completely thawed and the salts were dissolved. Approximately 200 μL of serum or plasma was transferred into a 2 mL microcentrifuge tube, the 60 μL of Buffer RPL was added. The tube caps were closed and vortexed for at least 5 s. Then, the tube was left at room temperature (15–25˚C) for 3 min. After drying, 20 μL Buffer RPP was added to the tubes. The tube caps were then closed and mixed vigorously by vortexing for at least 20 s and incubated at room temperature for 3 min. The tubes were then centrifuged at 12000 × g for 3 min at room temperature. The supernatant (~230 μL for 200 μL serum/plasma) was transferred to a new microcentrifuge tube. One volume of isopropanol was added. The solution was mixed well via vortexing. Then, the entire contents of the tube were transferred to an RNeasy UCP MinElute column. The columns were then centrifuged for 15 s at ≥8000 × g. After centrifugation, 700 μL Buffer RWT was pipetted onto the column, and it was centrifuged again for 15 s at ≥8000 × g. This time, 500 μL Buffer RPE was added, and the column was centrifuged again for 15 s at ≥8000 × g. Then, 500 μL of 80% ethanol was added to the column. The lid was closed gently and centrifuged for 2 min at ≥8000 × g (≥10,000 rpm) to wash the spin column membrane. The column was then placed in a new 2 mL collection tube. The lid of the spin column was opened and centrifuged at full speed for 5 min to dry the membrane. The spin column was placed in a new 1.5 mL collection tube, and RNase-free water (20 μL) was added directly to the center of the spin column membrane and incubated for 1 min. The lid was closed and centrifuged for 1 min at full speed to elute the RNA.

miRNA RNA sequencing

To evaluate the miRNAs, library construction was performed using an Illumina SMARTer® smRNA-Seq kit (TAKARA Korea Biomedical Inc., Seoul, Korea) according to the manufacturer's instructions. SMART technology is used in a ligation-free workflow to generate sequencing libraries for Illumina platforms. The polyadenylation Master mix was prepared without adding ATP. Twenty microliters of the supernatant was transferred for the next steps. Sequencing libraries were generated from an equimolar pool of 963 synthetic miRNAs (miRXplore Universal Reference) using a SMARTer® smRNA-Seq Kit for Illumina® (1 ng input). Library fragments from 148 to 185 bp were size-selected on a Sage Science Pippin Prep instrument using a 3% dye-free agarose gel, as recommended in the manufacturer's protocol. Library preparation was performed in one day without the use of stopping points. Sequencing libraries were generated in parallel from the indicated input amounts of human brain total RNA using the same sequencing kit and was size-selected using a BluePippin system. Illumina-ready sequencing library preparation, specifically for small RNAs ranging from 15-150 nucleotides (nt). High-throughput sequences were produced using a HiSeq 2500 system using single-end 75 sequencing (Illumina, San Diego, CA, USA).

Statistical analyses

Statistical analysis was performed using SPSS software (version 13.0; SPSS, Inc., Chicago, IL). The data consisting of log2-transformed proportions followed a normal distribution, so Student's t-test was chosen to determine whether the difference between the two variables was statistically significant. Kaplan–Meier analysis and the log-rank test were used to determine whether the survival rates of 43 patients with CSCC correlated with clinic pathologic factors and the expression of specific miRNAs. Furthermore, Cox proportional hazard modeling was performed to identify potential prognostic factors for the patient cohort. Statistical significance was set at $P < 0.05$.

Results

Patient Characteristics

The patient characteristics and miR-142-5p expression status in this study are shown in Table 1. The cohort included 31 (72%) men and 12 (28%) women with ages between t 42 and 85 years. Almost 29% of the patients had never smoked, and 28% were already in the advanced clinical stages (III or IV). The sites of tumor origin were the extremities (42%), trunk (49%), and the head and neck (9%). miR-142-5p was highly expressed in 9 out of 43 patients, all of whom were with stage 3/4 disease.

Exosomal miRNA clusters exhibited genomic alternation in the serum of patients with cutaneous squamous cell carcinoma

Of the 963 miRNAs analyzed, 19 had correlation coefficients greater than 0.5. Thirteen miRNAs were amplified, and six miRNAs showed deletions. Among the amplified miRNAs, miR-142-5p showed the highest correlation coefficient, and miR-106-3p showed the highest correlation coefficient among the deleted miRNAs (Table 2).

miRNA	chromosome	Correlation	Amplification or Deletion
miR-451a	17	0.812	Amplication
Let-7c-5p	21	0.862	Deletion
miR-106a-3p	X	0.923	Deletion
let-7a-5p	22	0.635	Deletion
miR-1-3p	15	0.746	Amplification
miR-101-3p-	9	0.879	Deletion
miR-10a-3p	17	0.914	Amplification
miR-1246	2	0.753	Amplification
miR-126-5p	9	0.694	Amplification
miR-1273g-3p	X	0.784	Amplification
miR-142-5p	17	0.951	Amplification
miR-144-5p	17	0.796	Deletion
miR-146a-5p	11	0.653	Deletion
miR-150-5p	19	0.782	Amplification
miR-15b-3p	3	0.612	Amplification
miR-192-5p	11	0.934	Amplification
miR-25-3p	7	0.845	Amplification
miR-30e-3p	1	0.692	Amplification
miR-374b-5p	X	0.723	Amplification

Table 2: Exosomal miRNA clusters in serum that reflect the genomic alternations of patients with cutaneous squamous cell carcinoma.

Univariate and multivariate Cox-regression analyses of overall survival of patients with cutaneous cell carcinoma

Cox regression analyses were performed using the following covariates: age, sunlight exposure time per day, T stage, N stage, M stage, TNM stage, and relative exosomal miRNA expression levels. In the univariate analysis, N stage, M stage, TNM stage, and miR-142-5p were found to be significant factors for BCR (HR: 1.972 [95% CI 1.126–2.988], HR: 1.847 [95% CI 1.121–3.125], HR: 2.231 [95% CI 1.417–3.261], and 1.845 [95% CI 1.138–2.977], respectively), while age, sunlight exposure time per day, miRNA-10a-3p, miRNA-106a-3p, and miRNA-192a-5p expression were not. In the multivariate analysis, M stage and miR-142-5p expression were factors significantly associated with OS in patients with cutaneous cell carcinoma (HR: 2.853 [95% CI 1.145–3.871] and HR: 2.668 [95% CI 0.939– 3.573], respectively) (Table 3).

Factor	Univariate Cox regression		Multivariate Cox regression	
	Hazard ratio (95% CI)	P value	Hazard ratio (95% CI)	P value

Age (>60Vs ≤60)	1.13 (0.624-1.914)	0.254		
Sunlight exposure time per day (>6 hr Vs ≤6 hr)	0.741 (0.417-1.478)	0.162		
T stage (T3/T4 Vs T1/T2)	1.623 (0.982-2.559)	0.072		
N stage (N1 Vs N0)	1.972 (1.126-2.988)	0.026	1.568 (1.117-2.551)	0.077
M stage (M1 Vs M0)	1.847 (1.121-3.125)	0.035	2.853 (1.145-3.871)	0.013
TNM stage (4/3 Vs 2/1)	2.231 (1.417-3.261)	0.045	2.173 (1.352-3.192)	0.053
miRNA- 10a-3p (high/low)	1.313 (0.782-2.126)	0.069		
miRNA- 106a-3p (high/low)	0.864 (0.385-1.737)	0.074		
miRNA- 142-5p (high/low)	1.845 (1.138-2.977)	0.041	2.668 (0.939-3.573)	0.047
miRNA- 192-5p (high/low)	1.249 (0.622-2.352)	0.058		

Table 3: Cox regression analysis of various progression factors of the overall survival of 43 patients with cutaneous squamous cell carcinoma enrolled in this study.

Summary of small RNA composition

For each sample, the processed reads were sequentially aligned to the reference genome, miRbase v21, and non-coding RNA databases (RNAcentral release 10.0) to classify known miRNAs and other types of RNA such as piRNAs, tRNAs, snRNAs, and snoRNAs. Genome mapping was performed through Bowtie and STAR using RSEM. Bowtie was subsequently used for miRDeep2 analysis using a genomic sequence. Known/novel miRNAs predicted by miRDeep2 and other smRNAs matching RNAcentral were also aligned using Bowtie (target smRNA, <50 nt) and Bowtie2 (target smRNA, ≥50 nt). The composition differed for each sample; rRNA and genome accounted for the largest proportion in most samples (Figure 1).

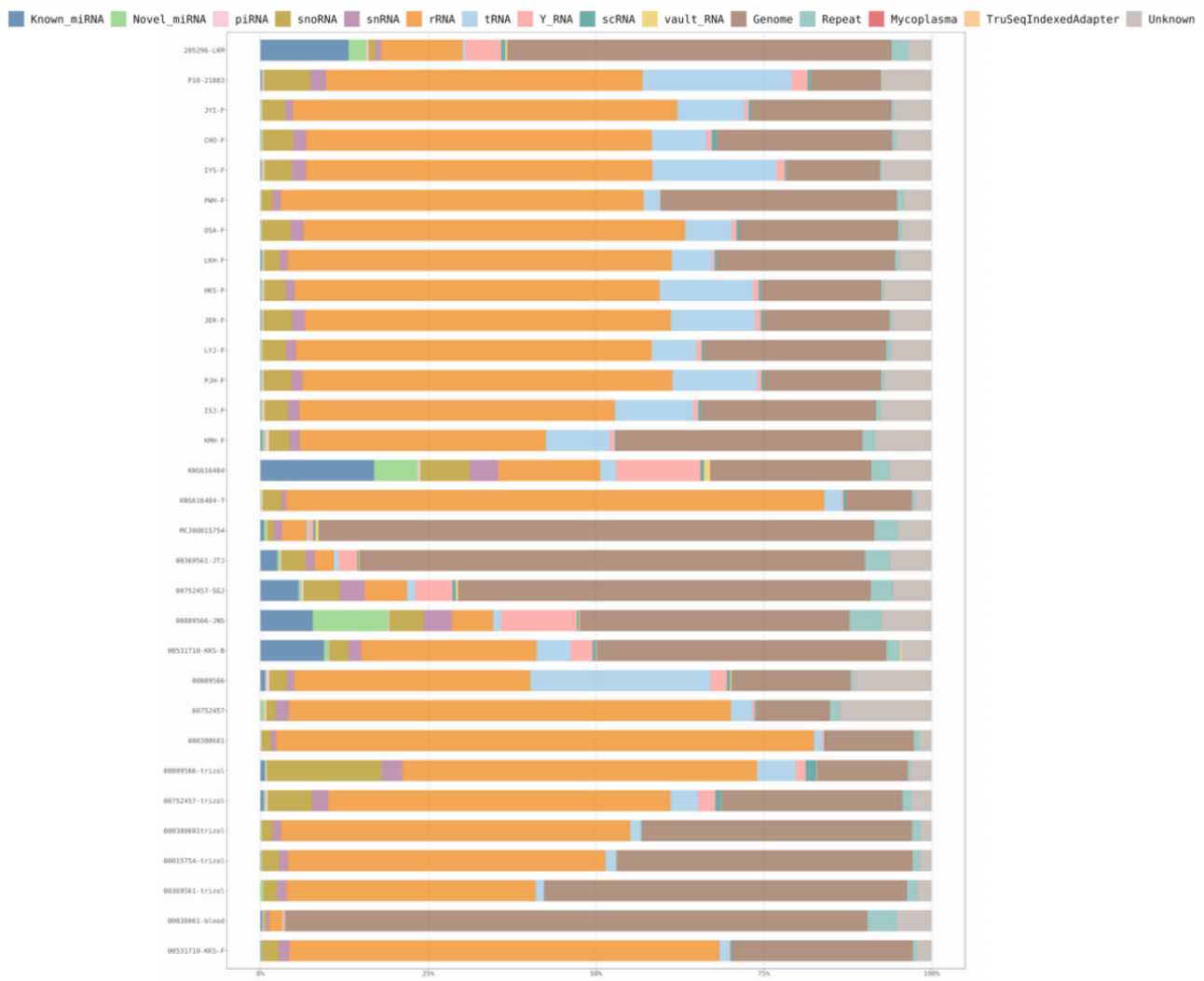

Figure 1: The smRNA composition of each sample, i.e., the ratio of smRNA class type. (such as known miRNA, candidate miRNA, rRNA, tRNA, sn RNA, etc.) Processed reads.

Relationship between the aberrant expression of selected miRNAs and clinical factors

miRNA-106a-3p expression levels were lower in patients with stage III/IV disease than those with stage I/II disease (P = 0.0113). On the other hand, the expression of miRNA-10a-3p/miR-142-5p/miRNA-192a-5p in the stage III/IV group was higher than that in the stage I/II group (P = 0.0072, P = 0.0012, and P = 0.0026, respectively) (Figure 2).

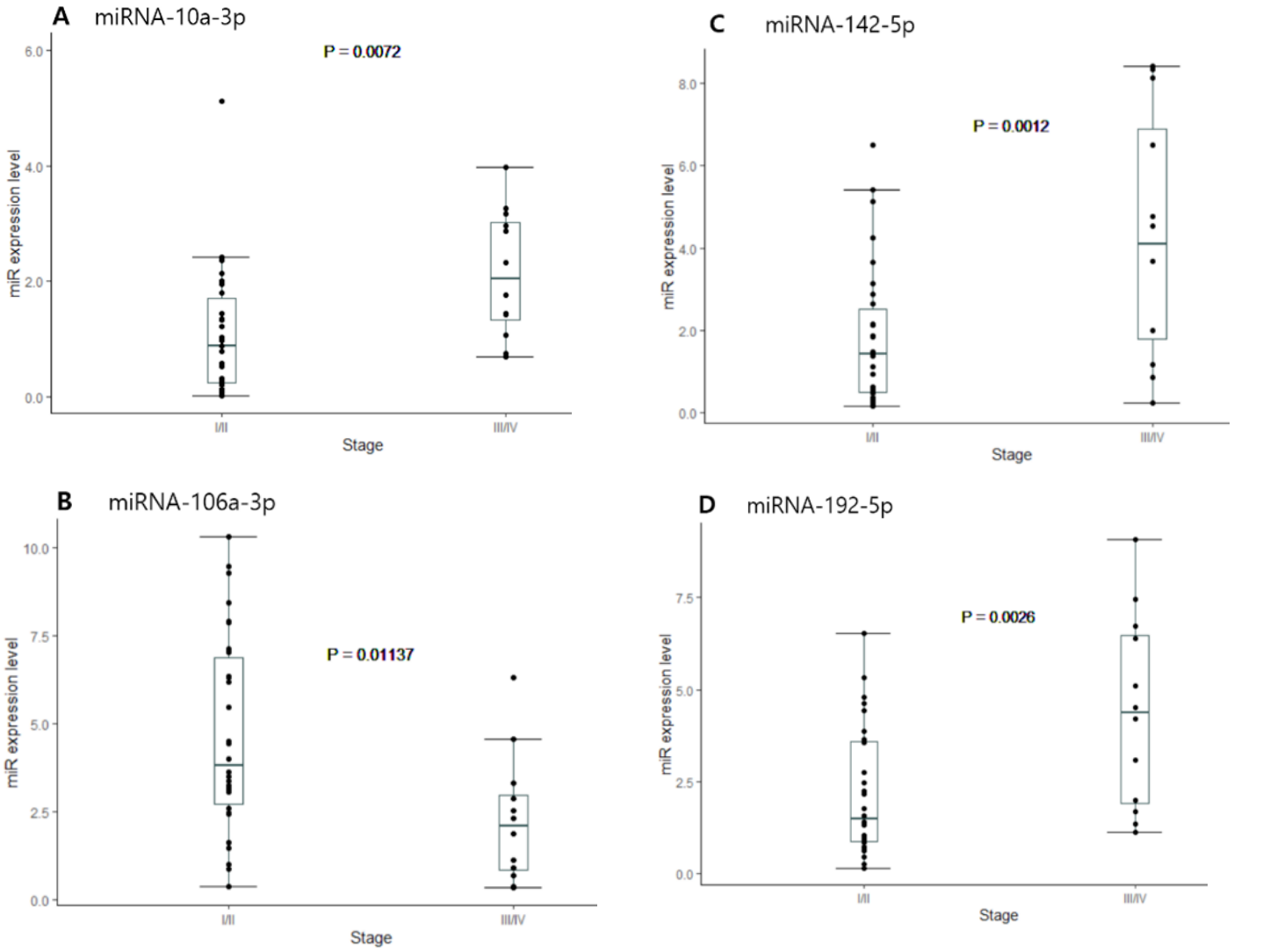

Figure 2: Relationships between miRNA expression and TNM stage differentiation in cutaneous cell carcinoma. Relationship between miRNA-10a-3p (A), miRNA-106a-3p (B), miRNA-142a-5p (C), and miRNA-192a-5p (D) expression with the degree of TNM stage differentiation in cutaneous cell carcinoma ($P < 0.05$ each).

Exosomal miRNA expression and overall survival

A survival assay was performed on a validation cohort of 43 patients to assess the clinical significance of the expression of exosomal miRNAs. Kaplan–Meier analysis demonstrated that patients with high miRNA-10a-3p/miRNA -192a-3p expression in their serum had shorter overall survival than patients with low miRNA-10a-3p/miRNA -192a-3p expression; however, this difference was not statistically significant (p = 0.056 and p = 0.067, respectively). Only patients with high miRNA-142-5p expression in their serum had significantly shorter overall survival than patients with low miRNA-142-5p expression (P = 0.036) (Figure 3).

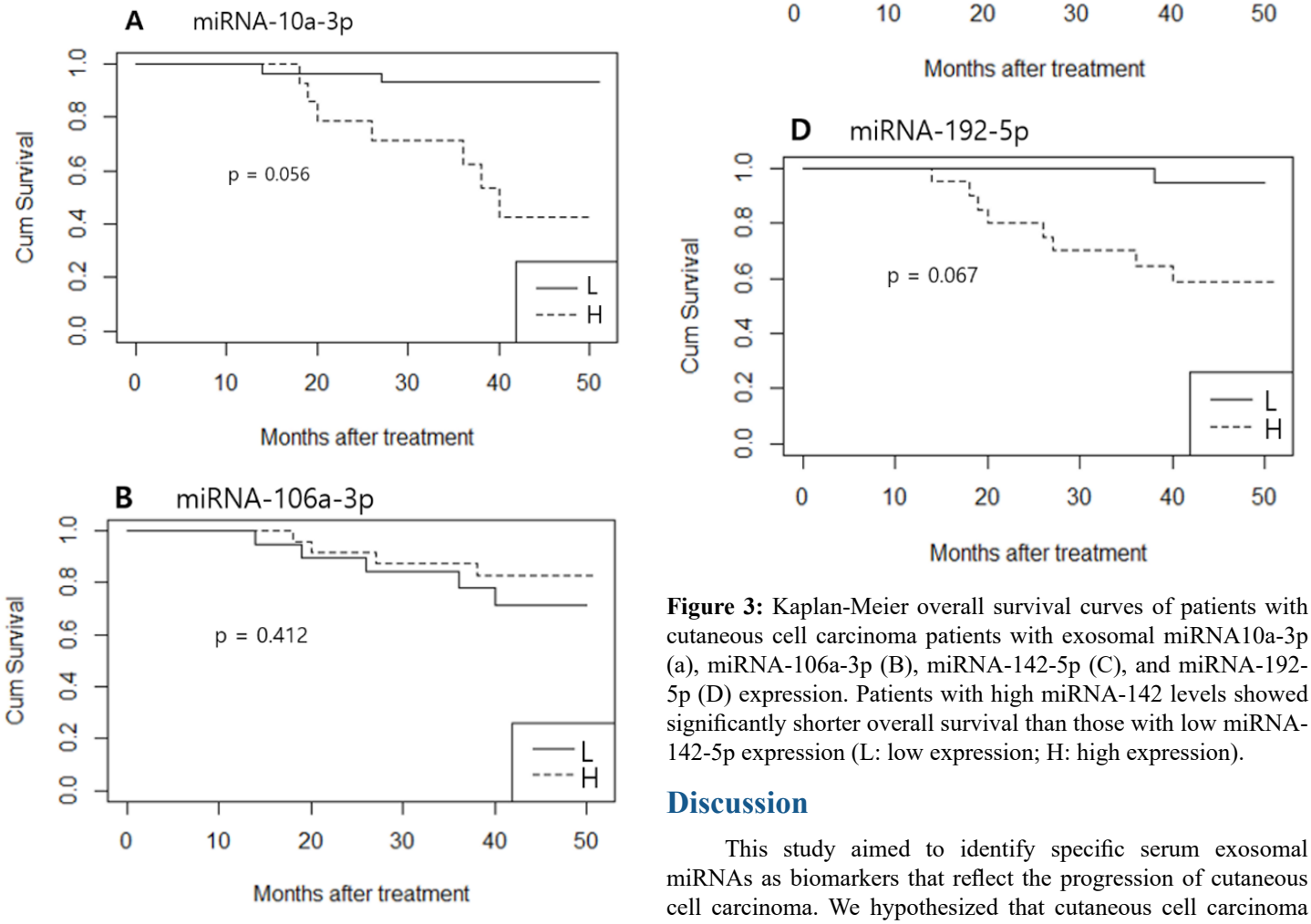

Figure 3: Kaplan-Meier overall survival curves of patients with cutaneous cell carcinoma patients with exosomal miRNA10a-3p (a), miRNA-106a-3p (B), miRNA-142-5p (C), and miRNA-192-5p (D) expression. Patients with high miRNA-142 levels showed significantly shorter overall survival than those with low miRNA-142-5p expression (L: low expression; H: high expression).

Discussion

This study aimed to identify specific serum exosomal miRNAs as biomarkers that reflect the progression of cutaneous cell carcinoma. We hypothesized that cutaneous cell carcinoma

secretes exosomes containing miRNAs and transmits signals to their respective receiving cells. Our results show that the serum miRNA-142-5p cluster could be a potential biomarker for predicting the recurrence of cutaneous cell carcinoma. The TNM stage is typically already 3 or higher when lymphatic metastasis occurs. However, despite recurrence, no lymph node metastasis was observed at the time of diagnosis. An analysis of the serum external miRNA expression profile in this study showed that four serum exosomal miRNAs (miRNA-106a-3p, miRNA-10a-3p, miR-142-5p, miRNA-192a-5p) were adjusted to match cutaneous cell carcinoma progression. Among them, only miRNA-142-5p was found to be statistically related to overall survival. MiRNAs have been suggested for several years as a suitable prognostic and predictive biomarker, which greatly influences the ability to distinguish between normal and malignant phenotypes according to the tumor type [22-25]. Exosomal secretion from malignant tissue is greater than that of the corresponding normal tissue, as evidenced by high concentrations of biological fluids such as ascites, urine, and serum plasma [26,27]. Exosome-encapsulated miRNAs receive high protection from degradation even under optimal storage conditions and in the presence of RNAse [28,29]. miRNA-106a-5p, a member of the miRNA-17 family, has been reported to be a tumor regulator. It is significantly downregulated and has been proven to have tumor-suppressive effects in astrocytoma, osteosarcoma, and colorectal cancer [30-32]. The inhibition of miRNA-106a-5p expression is associated with increased cell migration, invasion, and wound healing. In addition, miRNA-106a-5p could function as an antitumor agent by directly targeting the tumor gene PAK5 since an inverse correlation between miRNA-106a-5p and PAK5 expression levels has been previously observed in renal cell carcinoma [33]. Huang, et al. showed that miRNA-10a is dysregulated in extranodal natural killer/T-cell lymphoma (ENKTCL). The expression level of miRNA-10a was inversely correlated with the protein expression level of T-lymphoma invasion and metastasis-inducing factor 1 (TIAM1) in ENKTCL tissues. miRNA-10a may be involved in the development of ENKTCL via the TIAM1 pathway [34,35]. Recent studies have shown that miR-192-5p is involved in several human diseases, especially various cancers, including lung cancer, liver cancer, and breast cancer. The expression of miR-192-5p is regulated by diverse factors, including p53 and TGF-β. Puppo, et al. showed that TGF-β represses the expression of components of the ribonucleoprotein complex, which controls the maturation of miR-192-5p and promotes the expression of some Epithelial-To-Mesenchymal Transition (EMT) factors.35 miRNA-142-5p has been reported as an oncogenic miRNA promoting colorectal cancer and renal cell carcinoma [36,37]. In a recent study, Seiichiro, et al. reported that miRNA-142-5p promotes tumor growth in oral squamous cell carcinoma via the PI3K/AKT pathway by regulating phosphatase and tensin homolog deleted on chromosome 10 (PTEN). PTEN expression levels were downregulated, and AKT expression levels were upregulated in miR-142-5p-overexpressing cells. miR-142-5p targets the PTEN gene and is involved in cancer progression [38].

Many cell signaling pathways are involved in the development of CSCC. Ultraviolet radiation-induced P53 mutations occur early in CSCC development and cause severe genomic instability [39]. The tumor suppressor P16 and the RAS oncogene are also frequently involved. The accumulation of genetic changes such as EGFR overexpression, NF-kB activation, and NOTCH inactivation significantly affect important signaling pathways that promote CSCC carcinogenesis [40-42]. MicroRNAs are implicated in various biological functions and diseases. Depending on the pathway in which they are involved, they can progress to either oncogenic miRNAs or tumor suppressor miRNAs. Natalia et al. reported that several miRNAs are dysregulated in CSCC, exhibiting oncogenic functions (such as mir-21, mir-205, mir-365, mir-31, mir-135b, mir-142, and mir-186) or suppressor functions (such as mir-20a, mir-203, mir-181a, mir-125b, mir-34a, mir-148a, mir-214, mir-124, mir-204, and mir-199a) [43]. MicroRNA profiling is a useful tool to identify predictive miRNA signatures related to tumor growth, progression, and prognosis. Recently, novel methods based on nanotechnology and enzymatic amplification have improved the sensitivity and specificity of miRNA detection [44]. These new technologies will allow miRNAs to improve the prediction of CSCC prognosis as a biomarker. This study was conducted with a relatively small sample size, making it difficult to identify miRNAs associated with a statistically significant prognosis. However, we believe that this study derived a meaningful miRNA signature that can predict prognosis and inform future therapeutic strategies.

Conclusion

In conclusion, for CSCC patients with miRNA-142-5p expression, additional chemotherapy should be considered even after surgery. To Prove the clinical usefulness of the miRNA signature obtained in this study, more patients will need to be tested in the future.

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