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RESEARCH ARTICLE

CFAP418-AS1 Gene Amplification is Associated with Decreased Pancreatic Cancer Patient's Survival Rate

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ABSTRACT: Identifying specific biomarkers associated with pancreatic ductal adenocarcinoma (PDAC) patients' survival at an early stage is essential. After analyzing 1,206 pancreatic cancer patients' genomic data provided by cBioPortal database, an open web-based platform for cancer genomics, fourteen genes were found to be significantly amplified in the deceased patient group compared to the living patient group. Among those fourteen genes, it was found that *CFAP418-AS1* amplified patients (n= 9) are significantly associated with decreased median overall survival month (9.14 months) when compared with the median overall survival month (24.9 months) of *CFAP418-AS1* non-amplified patients (n= 457). To determine how *CFAP418-AS1* amplification decreased patients' survival rates, an *in vitro* assay was performed to overexpress *CFAP418-AS1* in ASPC1 pancreatic cancer cell line. In addition, Prestoblue assay indicated that overexpression of *CFAP418-AS1* increased cell proliferation. Therefore, *CFAP418-AS1* may function as an oncogene and decrease survival rates of pancreatic cancer patients by increasing cancer cell proliferation. Further investigation of the mechanisms affecting *CFAP418-AS1* dysregulation will provide insights into the molecular differences underpinning pancreatic cancer's survival rate.

KEYWORDS: Biology; Cancer Biology; Genetics, CFAP418-AS1, Gene Amplification.

Introduction

Pancreatic cancer is a relatively uncommon cancer; approximately 60,000 new diagnoses are expected in 2021 in the US.[1] However, the incidence of pancreatic cancer is increasing about 0.7% per year, and it is expected to become the second-leading cause of cancer-associated mortality by 2030.[2] The most common type of pancreatic cancer is pancreatic ductal adenocarcinoma (PDAC). This cancer is late stage, which is when pancreas cancer patients are often diagnosed.[3]

Pancreatic adenocarcinoma heterogeneity makes it difficult to predict patients' survival rates due to poor tumor cellularity and genomic instability.[4] To address this, a whole genome was analyzed from pancreatic tumors. In this research, cBioPortal, a web-based open-source cancer genomic database, was used to analyze the large-scale cancer genomics data.[5]

Lines of evidence have shown copy number variation (CNVs) of certain genes are involved in cancer progression.[6] CNV is defined as an increasing or decreasing number of DNA segments (larger than 1kb) in the human genome.[7] Currently, research focuses on somatic CNV in cancer, which provides a biological function and human disease on the genomic level. CNV is highly associated with the development and progression of many cancers by altering gene expression levels.[8]

CFAP418 Antisense RNA 1 (*CFAP418-AS1*) is a long noncoding RNA (lncRNA) gene of unknown function.[9] lncRNA regulates gene expression through interacting with nucleic acids and proteins in the cells.[10] Therefore, lncRNAs have been shown to play an important role in many different types of cancers. For example, MALAT1 is overexpressed in various cancer cells, and knockdown potently reduces both proliferation and metastasis *in vivo* in mouse model assays.[11] On the other hand, lncRNAs may also function as a tumor suppressor. p53 mediates lncRNA-p21 to induce apoptosis in cancer cells.[12]

In this study, a meta-analysis of patient data from cBioPortal database was performed to find a novel genetic marker that predicts poor outcomes in pancreatic cancer patients. The amplification of *CFAP418-AS1* was evaluated as a potential prognostic biomarker in pancreatic cancer. Herein, a possible role of *CFAP418-AS1* as a prognostic biomarker in deceased patients was discovered. Furthermore, the role of *CAFP418-AS1* by ectopic overexpression in ASPC1 (human pancreatic cancer cell line) was investigated.

Methods

Patient survival Analysis with cBioPortal:

The cBioPortal provided visualization and analyzing tools for more than 6,000 tumor samples from 290 cancer studies in TCGA database. The database provided researchers with an opportunity to analyze genetic alterations across samples from other cancer studies with specific genes. *CFAP418-AS1* was searched in cBioPortal database; a total of 1,206 samples from 10 pancreatic cancer studies were obtained. Genetic alterations such as amplifications, deep deletions, and mutations can be identified. Overall survival (OS) was calculated using cBioPortal's survival tab.

Cell line and culture:

Human pancreatic cancer cell line ASPC1 was purchased from the Korean Cell Line Bank. ASPC1 cells were cultured with RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum (Thermo Science) and 1% penicillin and streptomycin in a 5% CO2 atmosphere at 37°C.

cDNA synthesis:

Total cellular RNA was extracted with Total RNA extraction spin kit (Intron) and treated with DNase I (Invitrogen). According to the manufacturer's protocol, the cDNA was

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reverse transcribed from 1 µg of total RNA using oligo (dT) primers (Enzynomics)

Cloning CFAP418-AS1 overexpression vector :

The cDNA of *CFAP418-AS1* was amplified with the forward primer 5′- ATAGAATTCGAGTGAAGAGGTG-CCAGAAT -3′ with EcoRI restriction site, and the reverse primer 5′- CACTCCAGCCTGGGTGACAA -3′ with XhoI restriction site. After PCR purification (Bioneer), the amplified product was digested with EcoRI and XhoI restriction enzyme. After PCR purification, the amplified DNA was cloned into pcDNA3 (addgene) vector.

Cell transfection :

Lipofectamine 2000 (Invitrogen) was used to transfect *pcDNA3-CFAP418-AS1* in ASPC1 cell line. Diluted Lipofectamine to the diluted DNA (1:1 ratio) was used to transfect the cells. After incubating for 15 min at room temperature, DNA-lipid complexes were added to the cultured cells drop-wise. The cell culture media was replaced after three hours. Downstream experiments were performed 48 hours post-transfection.

Polymerase Chain Reaction :

20 µL of Polymerase Chain Reaction (PCR) was performed with PCR-Premix (Bioneer). The primers were designed to amplify CAFP418-AS1 and GAPDH. The cycles of 94˚C for 15 s, 55˚C to 60˚C for 15 s, and 74˚C for 30 s were used to amplify the target gene. The forward primer 5′- TGGCCATGAGGGATTCAAGG -3′ and reverse primer 5′- GAACACACTGTGCTGTCCCT -3′ yielded a 172-base pair (bp) product from CFAP418-AS1 using an annealing temperature 60˚C. For GAPDH amplification, 176 bp of amplified product was synthesized using forward primer 5′- TGGAGAAGGCTGGGGCTCAT -3′ and reverse primer 5′- GACCTTGGCCAGGGGTGCTA -3′.

Cell transfection :

RedSafe nucleic acid staining reagent (Intron) was used to stain 1.3% agarose gels. After samples were loaded, agarose gels were run at 100 volts for 25 min. The gel electrophoresis images were captured with a digital camera, and the Image J program was used to quantify band intensities. The relative intensities of *CFAP418-AS1* bands were normalized by the GAPDH band.

Statistical analysis :

All statistical analysis was performed using Prism 7 program. Unpaired t-test was used to calculate the statistical significance. $p < 0.05$ was considered statistically significant.

Results and Discussion

Table 1: The list of amplified genes that are enriched in deceased pancreatic patient's group.

Gene	Cytoband	Number of living patients (%)	Number of decreased patients (%)	Log Ratio	p-Value	q-Value	Enriched in
TP53	17p13.1	88 (34.92%)	128 (59.81%)	-0.78	5.83E-08	1.226E-03	DECEASED
CFAP418-AS	8q22.1	0 (0.00%)	9 (9.09%)	<-10	8.614E-06	0.0325	DECEASED
LINC02894	8q22.1	0 (0.00%)	9 (9.09%)	<-10	8.614E-06	0.0325	DECEASED
DHX36	3q25.2	0 (0.00%)	13 (6.53%)	<-10	1.917E-05	0.0325	DECEASED
DNAI1	9p13.3	0 (0.00%)	13 (6.53%)	<-10	1.917E-05	0.0325	DECEASED
PMEPA1	20q13.31	0 (0.00%)	13 (6.53%)	<-10	1.917E-05	0.0325	DECEASED
C8ORF34-AS1	8q13.2	0 (0.00%)	8 (8.08%)	<-10	3.247E-05	0.0325	DECEASED
C8ORF88	8q21.3	0 (0.00%)	8 (8.08%)	<-10	3.247E-05	0.0325	DECEASED
C8ORF89	8q21.11	0 (0.00%)	8 (8.08%)	<-10	3.247E-05	0.0325	DECEASED
LINC01030	8q21.3	0 (0.00%)	8 (8.08%)	<-10	3.247E-05	0.0325	DECEASED
LINC01111	8q21.13	0 (0.00%)	8 (8.08%)	<-10	3.247E-05	0.0325	DECEASED
LINC01298	8q22.1	0 (0.00%)	8 (8.08%)	<-10	3.247E-05	0.0325	DECEASED
LINC01592	8q13.2	0 (0.00%)	8 (8.08%)	<-10	3.247E-05	0.0325	DECEASED
MIR2052HG	8q21.11-q21.13	0 (0.00%)	8 (8.08%)	<-10	3.247E-05	0.0325	DECEASED

Meta-analysis of 1,206 pancreatic cancer patients' genomic data provided by cBioPortal database, 14 genes were found to be significantly amplified in deceased patient group compared to living patient group (Table 1). Among 14 genes, ten genes were located on chromosome 8. Since amplification of CFAP418-AS was analyzed as one of the top significant alterations enriched in deceased pancreatic patient group, CFAP418-AS was the focus of amplification for downstream analysis.

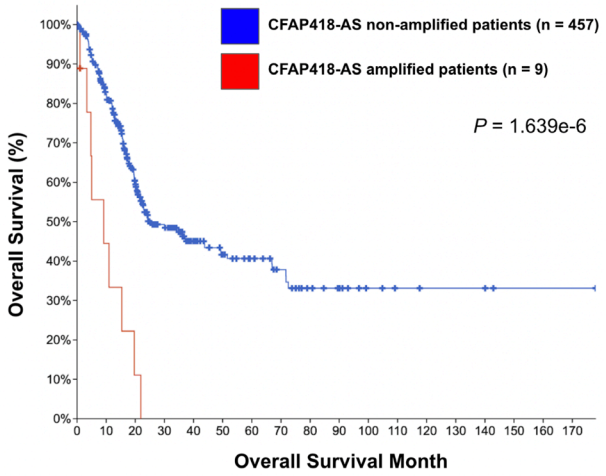

Figure 1: Kaplan-Meier survival plot of patients with pancreatic cancer, assessing overall survival percentage in regard to CFAP418-AS amplification. The patient samples were split into two groups according to CFAP418-AS amplified status (n= 9) and non-amplified (n= 457). The overall survival curve of gastric cancer patients between two groups was analyzed by Kaplan–Meier survival plot (p=1.639e-6).

To determine if the amplification of CFAP418 can be predictive for pancreatic cancer patient survival, 466 patient samples were analyzed using cBioPortal to validate the gastric cancer survival biomarker candidates. The patient samples were divided into two groups according to the amplification status of CFAP418-AS. After performing the patient survival analysis using Kaplan-Meier Plot, two patient cohorts (amplified vs. non-amplified of CFAP418-AS) were compared. As expected, patients with CFAP418-AS amplification showed a statistically significant decrease in overall survival (p = 1.639e-6) (Figure 1). Overall, CFAP418-AS amplification is significantly associated with a low survival rate of pancreatic patients.

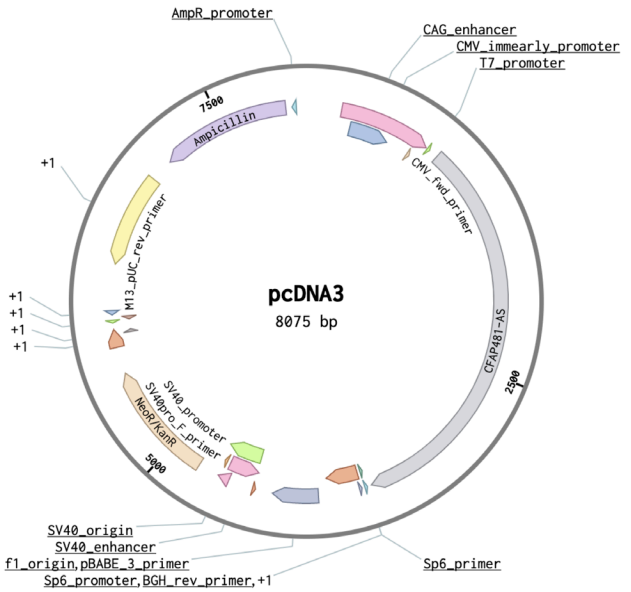

Figure 2: pcDNA3 plasmid with CFAP418-AS gene (pcDNA3-CFAP418-AS).

CFAP418-AS gene (2659bp) was cloned into pcDNA3 plasmid to investigate the functional role of CFAP418-AS gene in pancreatic cancer. pcDNA3 is a mammalian expression vector with the CMV promoter. It is a widely used vector for overexpressing the specific gene of interest. The human gene CFAP418-AS was successfully cloned into pcDNA3 plasmid (Figure 2).

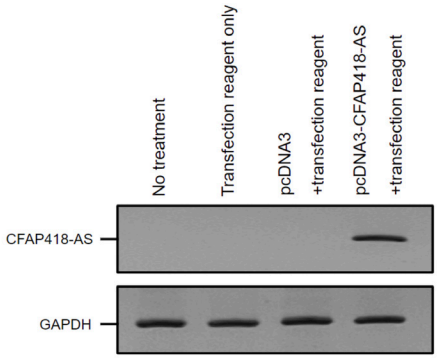

Figure 3: pcDNA3-CFAP418-AS transfected ASPC1 cells overexpress CFAP418. The amplified cDNA from each sample by PCR was analyzed with 1.3% agarose electrophoresis gel.

Four different conditions were used to verify the overexpression of CFAP418-AS gene on ASPC1 cells: no treatment (negative control), transfection reagent only, pcDNA3 + transfection reagent, pcDNA3-CFAP418-AS + transfection reagent. Overexpression of CFAP418 was expected only in pcDNA3-CFAP418-AS transfected samples. After DNA transfection on ASPC1 cells, RNA was extracted, and cDNA was synthesized by RT-PCR. cDNA was amplified with specific primer pairs that target CFAP418-AS and GAPDH genes by PCR. Then agarose gel electrophoresis was performed to check the amplified DNA. Agarose gel data indicates that CFAP418-AS was amplified only in pcDNA3-CFAP418-AS transfected cells showing a band on CFAP418-AS (Figure 3). GAPDH band showed a similar intensity of bands on all four conditions, indicating a similar total RNA and cDNA was used for each condition. Overall, pcDNA3-CFAP418-AS successfully overexpressed CFAP418-AS.

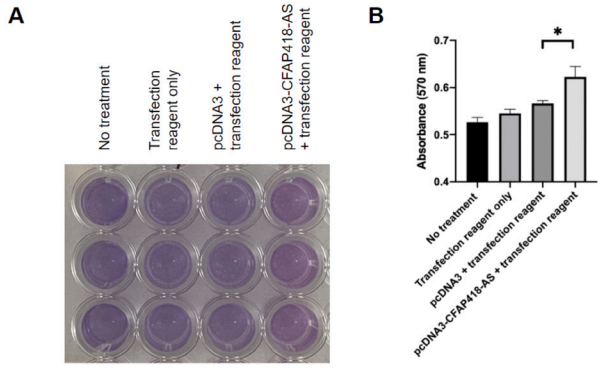

Figure 4: CFAP418-AS overexpression increased cell proliferation in the pancreatic cancer cell line, ASPC1. (A) Comparison of cell proliferation using Prestoblue reagent (B) Quantification of cancer cell proliferation based on the 570nm absorbance measurement. The number of samples = 3, Student's t-test. (*, p< 0.05)

Cell proliferation is how quickly a cancer cell replicates its DNA and divides into two cells.[13] Therefore, an increase in cell proliferation is associated with faster-growing tumors and is more aggressive. Prestoblue assay was performed to investigate the effect of overexpression of CAFAP418-AS on pancreatic cancer cell proliferation. pcDNA3-CFAP418-AS transfected cells showed the highest cancer cell proliferation (Figure 4). When the proliferation of pcDNA3 transfected cells was compared to the pcDNA3-CFAP418-AS transfected cells, the cell proliferation increased to about 120%. This result indicated that CFAP418-AS overexpression may positively regulate pancreatic cancer proliferation.

Conclusion

Antisense gene expression can be regulated either coordinately or independently of their neighboring genes.[14] Also, antisense transcripts can regulate the expression of their target genes from transcription and translation to RNA degradation.[15] CFAP418-AS gene is an antisense gene that may regulate CFAP418 gene expression level. CFAP418 encodes a protein of unknown function. CFAP418 is ubiquitously expressed in many organs such as the brain, heart, retina, and pancreas.[9] This study found that amplification of CFAP418-AS gene is significantly associated with decreased pancreatic patients' survival. Also, this *in vitro* experiment showed that overexpression of CFAP418-AS may enhance pancreatic cancer progression. In conclusion, this study indicates that CFAP418 amplification or overexpression can be used as a biomarker that predicts poor prognosis in pancreatic cancer. Also, this study can be used to develop a novel cancer treatment. However, further study is needed to investigate the detailed molecular mechanism of how CFAP418-AS functions as an oncogene in pancreatic cancer.

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