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Original Research

Gene expression of *ING4* gene in pancreatic cancers and splice forms of *ING4* gene

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Abstract: Pancreatic cancer is characterized by rapid metastasis and resistant to medical treatments. As the other cancers, mutations of tumor suppressor genes that involved in suppression of cell growth are observed in pancreatic cancers. ING4 protein is one of the proteins involved in the regulation of *p53* tumor suppressor gene functions. ING4 involved in suppression of cell proliferation, chromosome rearrangement, cell migration, and angiogenesis. In this study, gene expressions and splicing variants of *ING4* gene were investigated. Fresh tumor and normal specimens of the same pancreatic cancer patients were used. Gene expression study carried out by calculating the brightness of the bands on agarose gel and splicing variants were detected by direct sequencing. According to the results, three splice forms of *ING4* and a decrease in gene expression of *ING4* were determined. Splicing type of *ING4* affects the translocation of ING4 proteins into the nucleus. To determine the gene expression of each splicing variant, will further clarify the role of ING4 in pancreatic cancers.

Key words: ING4; Alternative splicing; Gene expression; p53; Pancreatic cancer.

Introduction

Pancreatic cancer is a very malignant type of cancer with a 5-year survival rate of less than 1% (1). Pancreatic cancer, which is the first cause of cancer-related deaths in developed countries, is distinguished from other cancers by the fast systemic spread and extraordinary regional tumor development (1). Pancreatic cancer is also called "silent killer" because it does not give any symptoms until the advanced stage (2). Metastasis that develops immediately after the cancer formation in pancreas is the cause of approximately 90% of deaths due to pancreatic cancer (1).

Studies have shown that tumor suppressor genes involved in cell cycle control such as *p53*, *p16*, and *SMAD4* have been mutated at high often in pancreatic cancers (3,4). It has been reported that there are errors in cell cycle control mechanisms due to the loss of function of the p53 gene or its side mutations in more than 50% of pancreatic cancers (3,4). One of the genes that also reported to have genetic alterations in pancreatic cancers is the *ING1* gene (5). The INhibitor of Growth (ING) genes were identified during hybridization studies and were named as Genetic Suppressor Elements (GSE) (6). Cell growth is inhibited by transfection with sensitive strains of ING genes, while growth and proliferation were promoted in cells transfected with antisense sequences of *ING* genes (6). ING proteins also affect the change of chromosomal structures based on the gene transcription by introducing histone acetyltransferase (HAT) and histone deacetylase (HDAC) enzymes into the phosphoinositol pathway through PHD (Plant Homodomain) domains (7). ING4 is a member of the ING family and like other members of the family plays role in cell cycle and programmed cell death (7). In contrast to other members of the family, *ING4* has been reported to be effective on angiogenesis and cell migration (7).

In a study performed in pancreatic cancer, it was reported the loss of heterozygosity on chromosome 12p that the *ING4* gene is also located (8). In another study conducted in head and neck cancers, the frequency of loss of heterozygosity in the same chromosomal region has been reported (9). In our study, gene expression and mutations of *ING4* in the chromosome region of 12p, in which loss of heterozygosity was reported in pancreatic cancer, were studied. Both in terms of angiogenesis and proliferation and metastasis, the presentation of the *ING4* gene expression and possible genomic mutations in pancreatic cancers will be useful in explaining the role of *ING4* in pancreatic cancer.

Materials and Methods

Patients and sample preparation

This study was approved by the decision of Gaziantep University Medical Faculty Ethics Committee No: 06-2009/221. Seven male patients (mean age 61.18 ± 14.3 years) who were diagnosed with pancreatic cancer were included in this study. Small pieces of tumor tissues and normal tissues were excised during the operations of these patients and placed quickly into the liquid nitrogen. This study was performed by comparing the data obtained from tumor tissues and non-tumor normal tissues of the same patients. Total RNA was extracted by using High Pure RNA Tissue Kit (Roche, Cat. No. 12 033 674 001, Mannheim, Germany) after the surgical specimens were disrupted with a mechanical homoge-

Table 1. Primer pairs used for amplification of *ING4* cDNA and annealing temperatures (9).

Name of study		Sense and Anti-sense primers	PCR Temperature °C
ING4 sequencing	S1	5'-CTTTGTTTTGCTTCGAGATGG	58
	AS1	5'-AGGGGATGTGGAAGAAACTGT	
ING4 gene expression	S2	5'-CACAAGTCCTGAGTATGGGAT	57
	AS1	5'-AGGGGATGTGGAAGAAACTGT	

Table 2. Results of *ING4* expression in tumor and normal tissues.

No	Normalized tumor tissue value	Normalized normal tissue value	Expression rate
1	0.931	1.065	0.874
2	1.189	1.413	0.841
3	1.138	1.224	0.930
4	1.134	1.406	0.807
5	0.933	1.17	0.797
6	1.22	1.374	0.888
7	0.826	0.857	0.964

nizer. The cDNAs were synthesized by using oligo dT primers with TaKaRa One Step RNA PCR Kit (Cat No; RR024B, Japan).

Gene expression studies

Gene expression studies carried out in a semi-quantitative manner. The *ING4* expression study was performed with the S2-AS1 primer pairs that replicating exons 5, 6, 7 and 8 (Table 1). *β-actin* was used for normalization. *ING4* and *β-actin* cDNAs were amplified in triplicate by 32 cycles of PCR. The PCR products obtained from normal and tumor tissues were run on the same agarose gel. Analyzes were done with the size and brightness of the bands on the agarose gel by "Image J" program. The results were obtained from the average of three PCRs.

Sequencing of ING4 gene and splicing variants identification

In this study, S1-AS1 primer pairs were used to replicate the entire 8 exons of *ING4* (Table 1). PCR amplified samples were run on an agarose gel electrophoresis before sequencing. By the way, bands thought to be splicing variants were obtained. These bands were excised from the agarose gel, purified and DNA sequencing of these samples were performed.

The results were given as percentage, statistical evaluation was done in SPSS 21.0 program with Wilcoxon Signed Rank Test with $p<0.05$ considered as the statistically significant difference.

Statistical Analyzes

The results were given as percentage, statistical evaluation was done in SPSS 21.0 program with Wilcoxon Signed Rank Test with $p<0.05$ considered as the statistically significant difference.

Results

Gene expression results of *ING4*

In order to determine the *ING4* gene expression level, PCRs of the 5, 6, 7 and 8 exons of the *ING4* gene and the β-actin gene were performed in three different experiments. PCR products were run on the same agarose gel and the brightness of the bands was evaluated by the Image J program. Gel image was given in Figure 1. Analyzes were done by calculating the average of the obtained band densities/brightness from three different PCRs. According to findings, the gene expression level of *ING4* gene in pancreatic tumor tissues was determined as 87% when compared to normal tissues. The comparison results are shown in Table 2. According to the statistical analysis performed between normal and tumor tissues, a statically difference was found in the expression of *ING4* between tumor and normal tissues (p=0,018, Wilcoxon Signed Rank Test).

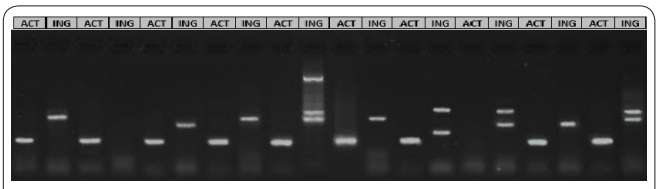

Figure 1. Agarose gel image of *ING4* and *β-actin*. The brightness of each band calculated by Image J program and evaluated.

Results of *ING4* cDNA sequence analysis

S1-AS1 primer pairs were used to amplify all exons of the *ING4* gene and in some samples, more than one band determined during the agarose gel electrophoresis. These samples were run on polyacrylamide gel electrophoresis, DNA bands excised from the gel and sequenced. Sequence analysis results showed that there were 9 and 12 nucleotide deletions at the boundary of the exon 4 and exon 5.

We compared our results with other studies that identified splicing variants of *ING4* gene. Results from a study performed on cell lines and tissues, splicing variants of *ING4* due to the nucleotide changes at the exon 4-5 boundary site, were found to confirm our results (10). The longest sequence identified in these studies was accepted as *ING4_v1* and the other variants were evaluated as *ING4_v2* (3 nucleotides AAG were deleted), *ING4_v3* (9 nucleotides GCAAAAAGA were

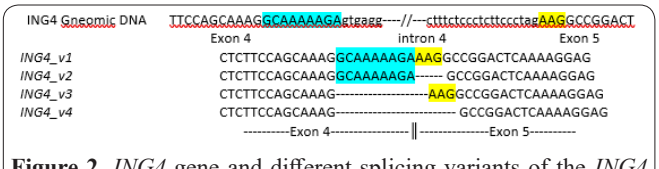

Figure 2. *ING4* gene and different splicing variants of the *ING4* gene (10).

Table 3. Splicing variants obtained in pancreatic tumors.

No	*ING4_v1*	*ING4_v2*	*ING4_v4*
1	+	+	-
2	+	-	-
3	+	-	-
4	+	+	-
5	+	+	+
6	+	+	-
7	+	-	+

deleted) and *ING4_v4* (12 nucleotides GCAAAAAGAAAG were deleted). A representation of the *ING4* splicing variants determined by Unoki et al. is shown in Figure 2.

Identified splicing variants of the *ING4* gene

In our study, *ING4_v1* was found in 7 patients, *ING4_v2* in 4 patients and *ING4_v4* in 2 patients. According to DNA sequence analysis results, *ING4_v3* was not detected. The distribution of the splicing variants determined in our study is given in Table 3.

Discussion

The results of this study show that the level of *ING4* gene expression in pancreatic cancer is reduced. Changes in *ING4* gene expression levels between tumor and normal tissues were studied in gastric adenocarcinoma, glioma, head and neck cancers, colorectal carcinoma and cervical cancer (9,11,12,13,14). In the study performed in the gliomas, it was determined that *ING4* expression was six times lower than in gliomas when compared to normal brain tissue, and there was a correlation between tumor grade and decreased *ING4* expression (11). In another study, it was shown that the level of *MMP-9* gene expression was increased due to decreased expression of the *ING4* gene (15). NF-κB acts as a transcription factor in the expression of MMP-9, and the acetylation and deacetylation of the promoter region of *MMP-9* play an important role in its expression (16). The ING4 protein has been shown to bind to the *MMP-9* promoter region in conjunction with NF-κB, thereby contributing to HDAC1 function by inhibiting p300 function (16). *ING4* gene expression in gastric carcinoma was found 75% when compared to normal tissues, and a relationship was determined between the decrease in gene expression and tumor grade (17). In studies conducted on breast cancer cell lines, it has been reported that *ING4* gene expression level was decreased and chromosomal deletions occurred in the gene region in which *ING4* is present (18). *ING4* expression is also decreased in pancreatic cancers as in gliomas. ING4 may indirectly contribute to cancer development by affecting the regulation of the transcription of proteins such as MMP-2, MMP-9, VEGF, and IL-8 via the NF-κB pathway due to decreased expression. Apart from these, it has also been shown that ING4 is directly involved in the promoter region of *p21* and it also plays a role in regulating the promoter region of the p16 protein by physically interacting with p300, a histone deacetylase. The decrease in *ING4* expression can also affect cell proliferation by affecting the p16 gene products INK4a, ARF and p21.

According to the DNA sequence analysis, we found the splicing form of *ING4_v1* in 7 samples, splicing form of *ING4_v2* in 4, and splicing form of *ING4_v4* in 2 of the studied samples. The different splicing forms of *ING4* originate from the boundary site of the exon 4 and exon 5. In *ING4_v1* the AAG sequence of 3 nucleotides enters the exon 4-5 fusion regions while in *ING4_v4* a 9 nucleotide GCAAAAAGA sequence in the same region is deleted. *ING4* splicing structures differ in their cellular functions as they are in different protein structures.

ING4 carries out its cellular functions through different splicing processes, by being found in relevant parts of the cell and by joining structures of large protein compounds or by interacting with proteins such as p53 (10). Splicing variants identified in this study lead to the alteration of the amino acid sequence of the NLS domain involved in the transport of the ING4 protein into the nucleus (10,17). ING4 protein is transported into the nucleus through the NLS domain and it is also associated with the p53 protein via this domain (7,19,20,21). ING4 plays a role in stabilizing the p53 protein by phosphorylating at the N-terminus of p53 and prevents degradation by the ubiquitin-proteasome pathway (10,19). The ability of p53 to function as a transcription factor is related to its ability to bind to DNA (20, 22). The binding of p53 to DNA is regulated by acetylation and phosphorylation of the C-terminus (20). The ING4 protein is involved in the regulation of the ability of p53 to bind to DNA by acting in the acetylation of the p53 protein through the lysine amino acid at position 382 (20). The p53 protein, which is phosphorylated from the N-terminus and functionalized by processing the C-terminus, encodes the p21 protein involved in stopping the cell cycle in G1 stage (23). The splicing variants lacking the NLS domain will not enter the nucleus and will not be able to perform the functions listed above.

In a study that investigated the effects of *ING4* splicing variants on cell migration, it was reported that *ING4_v1* was involved in actin polymerization but *ING4_v2* and *ING4_v4* could not polymerize actin filaments (10). The same study reports that *ING4_v1* plays a role in the regulation of foot-like membrane protrusions (filopodia/lamellipodia), which is important in cell migration, and *ING4_v4* reduces this effect of *ING4_v1* (10).

In conclusion, the decreased gene expression level of *ING4* in pancreatic cancer tissues suggests that these gene products function in the development of pancreatic cancer. ING4 is involved in many cellular events closely related to tumor development such as suppression of cell growth/proliferation, programmed cell death, neovascularization, and cell migration. The cellular events mentioned in the development and progression of pancreatic cancer are important and the expression of the gene expression levels and cellular location and function of each of these different fusion structures of ING4 in pancreatic cancer tissues or pancreatic cell lines will elicit the role of ING4 in pancreatic cancers due to the different cellular functions of the splicing variant structures of *ING4*.

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