Small Nuclear RNA 64 (snoRNA64): A novel Tumor Biomarker for Pancreatic Cancer

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Abstract:
The pancreatic ductal adenocarcinoma (PDAC), which represents over 90% of pancreatic cancer cases, has the highest proliferative and metastatic rate in comparison to other pancreatic cancer compartments. This study is designed to determine whether small nucleolar RNA, H/ACA box 64 (snoRNA64) is associated with pancreatic cancer initiation and progression. Gene expression data from the Gene Expression Omnibus (GEO) repository have shown that snoRNA64 expression is reduced in primary and metastatic pancreatic cancer as compared to normal tissues based on statistical analysis of the in Silico analysis. Using qPCR techniques, pancreatic cancer cell lines include PK-1, PK-8, PK-4, and Mia PaCa-2 with different levels of snoRNA64, including PK-1, PK-8, PK-4, and Mia PaCa-2. The level of expression is correlated with the cell line epithelial or mesenchymal characteristics. Cell lines displaying epithelial characteristics such as PK-1, PK-8 show high levels of snoRNA64 meanwhile, cell lines displaying mesenchymal characteristics such as PK-4, Mia PaCa-2 show low levels of snoRNA64. The level of expression is correlated with the cell line epithelial or mesenchymal characteristics. After knocking down the PK-8 with high snoRNA64 expression, the epithelial markers E. cadherin (E-cad) and Cytokeratin-8 (CK-8) are decreased, while mesenchymal markers Vimentin (Vim), Cytokeratin-19 (CK-19), Metalloprotease -2 (MMP-2), and Metalloprotease-3 (MMP-3) are activated. Those changes suggest that PK-8 responding to the snoRNA64 knock down protocol and increase in mesenchymal function. Together, snoRNA64 expression may participate in epithelial to mesenchymal transition (EMT) and mesenchymal to epithelial transition (MET), in which during metastasis these processes are crucial. In addition, snoRNA64 may be considered as a potential diagnostic biomarker for both early and invasive stages of PDAC. And due to its gradual expression decreases, it may be considered a barrier in tumor progression.

Keywords: Biomarker, EMT, MET, Metastatic Cancer, PDAC, snoRNA64

Introduction:
There are several types of RNA molecules produced by the cell after transcription processes, including messenger RNA (mRNA), ribosomal RNA (rRNA), transport RNA (tRNA), and small nuclear RNAs (snRNAs). The latter are non-coding RNAs mainly found in the nucleolus and contain 60-300 nucleotides. In the cellular context, snoRNA takes part in post-transcriptional modification and maturation of rRNA. In general, two types of snoRNAs are involved: C/D box snoRNAs are involved in methylation process, and H/ACA box snoRNA are involved in Pseudo uridylation process. In fact, Pseudo uridylation is the process of converting the nucleoside uridine to a different isomeric form. By using pseudo uridine rather than uridine, three hydrogen bonds can be formed with adenine as opposed to two. This enables rRNA to become mature, functional and tertiary structure.

It is known that snoRNAs are associated with various genetic diseases such as Prader-Willi syndrome and . snoRNAs play both tumor-promoting (oncogenic) and a tumor suppressing functions that are critical in the progression and development of the tumor. A number of snoRNAs participate in the cell functions that promote lung cancer onset and growth. They affect cellular proliferation and viability as well as the epithelial to mesenchymal transition (EMT).
In both in vivo and in vitro, SnoRNA overexpression shows a crucial role in carcinogenesis. SnoRNA overexpression has been observed in various cancer types, such as leukemia, carcinoma, and even sarcoma. Additionally, murine and human cancers such as breast cancers, prostate cancers, all overexpress snoRNAs. A study on prostate cancer proposed that snoRNA55 might act as a biomarker and therapeutic target. The expression of snoRNA42 is related to growth factor signaling and inflammatory cytokines where a significant association has been found between snoRNA42 expression and growth factor signaling. Upregulation of snoRNA42 was detected throughout tumor progression. Thus the molecule predicting tumor progression and suppressing its expression has a diverse effect on cell proliferation and metastatic capacity.

Recently, there has been increased interest in using snoRNAs (snoRNA25, snoRNA74A) for the early detection of pancreatic cancer in serum from patients.

Reports have stated that pancreatic ductal adenocarcinoma (PDAC) is the head of all pancreatic cancers with an estimated median survival as short as four months. Pancreatic cancer is the third leading cause of cancer deaths in the United States. Currently, there are not many treatment options for pancreatic cancer, and progress in drug development is hindered by the complexity of the cancer’s genome, its epigenetics, and its metabolism, with several pathways actively involved and mutual crosstalk.

Approximately 90% of pancreatic malignancies belongs to PDAC subtypes, which arise from epithelial cells lining the pancreatic duct. Intraductal papillary mucinous neoplasms (IPMNs) are hyperplastic lesions that develop in PDACs. Precancerous IPMN can progress into cancer and manifest as a metastasis to the liver or lymph nodes. As a result of the absence of symptoms in the early stages of PDAC, the cancer is only diagnosed when it has already spread. Hence, anti-cancer therapies have a limited impact on cancers that have developed drug-resistance at this stage. Within individual primary tumors as well as among patients with PDAC, there is a wide variety of genetic heterogeneity. Consequently, there is an increase in gene instability, which may play a significant role in the growth of PDAC tumors and resistance to drugs. PDAC arises mainly from intraductal papillary mucinous adenoma (IPMA), which is a premalignant lesion arising from the large pancreatic duct. The progression of PDAC can be divided into several stages based on several characteristics, such as the production of mucin, aggression, and metastasis rate. A common example is (IPMNs), which is characterized by the production of thick fluid called mucin within the pancreatic ducts. If left untreated, some of them can evolve into invasive cancers. Therefore; Intraductal papillary carcinoma (IPMC) is a condition in which IPMNs are associated with minimally an invasive carcinoma.

The present study examines the role of small nucleolar RNA, H/ACA box 64 (snoRNA64) in initiating, developing, and disseminating pancreatic cancer. A member of the H/ACA class, snoRA64 is located on 16p13.3, a chromosome on the 16p13.3 chromosome. SnoRNA64 is not known to have a specific function. According to some researchers, it could be involved in telomerase modulation through interaction with several proteins. Furthermore, it can be served as a potential biomarker for Osteoarthritis.

### Material and Methods:
#### Data retrieved from the GEO profiles database

Gene Expression Omnibus (GEO) is a database repository of high throughput gene expression data and hybridization arrays, chips, microarrays. By reviewing the GEO profiles database, each profile appears as a graph depicting a specific level of expression of RNA for a specific gene for each condition. Subsets of experimental variables are represented in the boxes at the bottom of the graph to simplify the data. The GDS3836 database was used in our study, which consisted of 7 specimens consisting of the normal main pancreatic duct, 6 specimens consisting of an intraductal papillary mucinous adenoma (IPMA), 6 specimens consisting of a (IPMC), and 3 specimens consisting of an invasive cancer having originated in the intraductal papillary mucinous neoplasm (IPML).

The researchers showed the multistep pancreatic carcinogenesis by using epithelial neoplastic cells at 3 stages of tumor development and normal cells from the main pancreatic duct for the best comparison. The value of expression of each specimen is used as mean of the gene expression measurements for comparison and the rank within the sample is used as standard error of the replications. Excel sheet program has been used to analyze the data.

### Cell lines and constructs

In this study, cell lines representing a variety of pancreatic cancer characteristics have been used: Mia PaCa-2, PK-4, PK-8, and PK-1. Unless otherwise specified, cell lines are cultured with RPMI-1640 supplemented with 10% fetal bovine serum (FBS), penicillin (100 u/ml), and streptomycin (100 g/ml) at 37 °C with 5% carbon dioxide in a humidified incubator.
Establishment of a pancreatic cancer cell line with low expression of snoRNA64

Transfection is performed 12 hours after plating the cells into culture plates with 40 % confluency. Lipofectamine is used to transfet PK-8 cells with siRNA oligos carried by psiRNA-h7SK G1. Similarly, control siRNA is scrambled under the same conditions (Table1). qRT-PCR is used to determine the efficiency of transfection using snoRNA46 primers within 90 hours following transfection.

Table 1. siRNA oligo sequence carried by psiRNA-h7SK G1 to knockdown snoRNA64.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>siRNA oligo</td>
<td>5'-ACCTCGCCTTACTGTTGGCCTCTCTTTTCAAGGAAAGAGAGGAGGCCACAGTAAGGCTTTG-3'</td>
</tr>
</tbody>
</table>

Real-Time polymerase chain reaction (qPCR)

QPCR analysis is used to determine gene expression by using two sets of primers (forward and reverse) for each gene. E-cadherin (E-cad) and Cytokeratin-8 (CK-8) serve as major epithelial markers. On the other hand, Vimentin (Vim), Cytokeratin-19 (CK-19), Metalloprotease-2 (MMP-2), and Metalloprotease-3 (MMP-3) serve as mesenchymal markers (Table 2). Housekeeping protein Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) serve as internal control. A Trizol reagent method was used to obtain total RNA. cDNA was synthesized using the ProtoScript ® First strand cDNA synthesis kit (catalog # E6300S). qPCR Mastermix-LowROX (catalog # NBMastermix-LR) was used to amplify the CDNA using specific primers.

Table 2. Forward and reverse primer sequences used in qPCR analysis.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Forward primer sequence</th>
<th>Reverse primer sequence</th>
</tr>
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<tbody>
<tr>
<td>snoRNA64</td>
<td>5'-TAG TTG CAC TTG GCT TCA CC-3'</td>
<td>5'-ACC CCT CAA GGA AAG AGA GG-3'</td>
</tr>
<tr>
<td>E-cad</td>
<td>5'-TGC CCA GAA AAT GAA AAA GG-3'</td>
<td>5'-GTG TAT GTG GCA ATG GTG TC-3'</td>
</tr>
<tr>
<td>CK-8</td>
<td>5'-GTG GAG AGC TGG CCA TTA AG -3'</td>
<td>5'-TCA TGT TCT GCA TCC CAG AC -3'</td>
</tr>
<tr>
<td>Vim</td>
<td>5'-GAG AAC TTT GCC GTT GAA GC-3'</td>
<td>5'-TCC AGC AGC TTC CTA CTA GTG -3'</td>
</tr>
<tr>
<td>CK-19</td>
<td>5'-GGT CAG TGT GGA GGT TT-3'</td>
<td>5'-TCA GTA ACC TCG GAC CTG CT -3'</td>
</tr>
<tr>
<td>MMP-2</td>
<td>5'-GCT CAG ATC CTT GTT GGT GAG AT-3'</td>
<td>5'-GGT GCT GGC TGA GTA GAT CC-3'</td>
</tr>
<tr>
<td>MMP-3</td>
<td>5'-CTC CAG GAA GCT TGA ACC TG-3'</td>
<td>5'-GGG AAA CCT AGG GTG TGG AT-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-ACC CAG AAG ACT GTG GAT GG-3'</td>
<td>5'-TTC TAG ACG GCA GGT CAG GT -3'</td>
</tr>
</tbody>
</table>

Statistical analysis

The data were analyzed and significant differences in gene expression were determined by a two-tailed student's t test. P-value ≤ 0.05.

Results:

In Silico analysis revealed variation in snoRNA64 expression in normal tissue, primary cancer, and metastatic cancer.

As compared to normal pancreatic tissue, snoRNA64 is expressed significantly lower in all tumor types. On the other hand, there is a significant difference between invasive cancer originating from IPMN and the invasive cancer with IPMC showing less metastatic potential. Furthermore, there is a comparable level of snoRNA64 present in IPMA specimens in comparison to IPMC specimens (Fig. 1).
An EMT-like pattern is observed in a snoRNA64 knockdown pancreatic cancer cell line.

As compared to a scramble control cell line, PK-8 cell lines knocked down for snoRNA64 showed a significant reduction in snoRNA64 expression (Fig. 4A). The gene-modified cell line has demonstrated significantly reduced expression of epithelial markers E-cad, and CK-8 when compared to the control (Fig. 4B, 4C). On the other hand, this cell line significantly increased the expression of mesenchymal markers Vim, CK-19, and MMP-3 when compared to scramble cell line. Furthermore, a noticeable increase in MMP-2 expression is also observed as compared to the control group (Fig. 4D).

Analysis of stage-by-stage pancreatic carcinogenesis has shown snoRNA64 is highly expressed in the normal pancreatic duct as compared to the primary IPMA and IPMC and the invasive stage originating from IPMA. Data is retrieved from GEO profiles database (GDS3836).

Figure 1. snoRNA64 is significantly more abundant in normal pancreatic ducts than in tumors.

Figure 2. A variable expression level of snoRNA64 in several pancreatic cancer cell lines with different characteristics. Cell lines PK-1 and PK-8 are significantly expressed high levels of snoRNA64 compared to PK-4 and Mia PaCa-2 cell lines.

Figure 3. Pancreatic cancer cell lines show significant variation in their expression of epithelial and mesenchymal markers. (A, B) PK-1 and PK-8 express significantly higher levels of E-cad and CK-8 than PK-4 and Mia PaCa-2. (C) PK-1 significantly express low Vim than Mia PaCa-2. In addition, PK-1 and PK-8 express significant lower mRNA of mesenchymal markers CK-19, MMP-2, and MMP-3 than PK-4 and Mia PaCa-2.
Figure 4. PK-8 with snoRNA64 knockdown exhibited EMT. (A) Analysis of snoRNA64 expression levels using siRNA to knockdown PK-8 compared to scramble controls. (B) A significant reduction in E-cad in the knockdown cell line compared to the scramble control. (C) A significant reduction in CK-8 in the knockdown cell line compared to the scramble control. (D) A significant increase in Vim, CK-19, and MMP-3 in the knockdown cell line compared to the scramble control.

Discussion

Pancreatic cancer is considered a disease with a poor prognosis due to the lack of early detection markers, genetic heterogeneity, and a high risk of metastasis. In most cases, patients with PDAC are diagnosed with incurable, localized, or metastatic disease with 5 years survival rate on average. Detecting PDAC in its early stages is crucial due to the complex microenvironment of the disease, which encourages the growth of the tumor and can lead to resistance to treatment. For treatment and/or surgical resection, early diagnosis by highly specific, sensitive, cost-effective, and minimally invasive means is necessary.

Generally, serum carbohydrate antigen19-9 (CA 19-9) is used as a marker for PDAC, however, it is not specific. It may be possible to determine PDAC by combining several protein biomarkers, including C4b-binding protein chain (C4BPA), IGFBP2 and IGFBP3, along with CA 19-9. Additionally, metabolomic biomarkers may be useful for the early detection of PDAC, such as M2-pyruvate kinase (M2-PK), phosphatidylcholine, glucitol, inositol, histidine, proline, xylitol, sphingomyelin, isocitrate, palmitic acid, and ceramide tested as a set of several biomarkers in combination with CA19-9. Researchers are currently investigating ways in which snoRNAs can be manipulated in pancreatic cancer. A possible diagnostic value has been identified for sets of snoRNAs found in the plasma or serum of PDAC patients. According to a study, miR-1290, a novel prognostic biomarker, demonstrated superior diagnostic performance over CA19-9 in distinguishing early PDAC from healthy individuals.

By studying genes, sequences, and other data obtained from sequencing and other studies, scientists can predict a specific drug’s efficacy based on important information such as gene expression in specific diseases. In Slico analysis is a method to analyze and represent the data on GEO profiles in order to determine disease progression, gene implications, and possible biomarkers. In our study, the statistical analysis of the online data reveals differences among the different grades of tumor which suggests an important role for snoRNA64 in the initiation and development of pancreatic cancer. Compared to IPMA, IPMC has more indications of malignancy due to the presence of mural nodules and a larger pancreatic duct. Such indications are critically important for the surgical procedure. When combined with other biomarkers,
snoRNA64 may provide insights into a tumor's grade, providing physicians with the information they need to make an informed decision regarding treatment. In our study, different levels of snoRNA64 are expressed in experimental pancreatic cancer cell lines. The PK-1 and PK-8 cell lines exhibit a significantly higher level of snoRNA64 than the PK-4 and Mia PaCa-2 cell lines. On the other hand, PK-1 and PK-8 exhibit significantly lower levels of mesenchymal markers Vim, CK-19, MMP-2, and MMP-3 than PK-4 and Mia PaCa-2.

The epithelial tissue integrity is maintained by E-cad, a constituent of the adherens junction and its main structure. A reduction in the expression of Cadherin protein results in cancer progression and the dissemination of some cancer cells.\(^{24,25}\) CK-8 is an intermediate filament-forming protein that is found within the cytoplasm of simple epithelial cells. In conjunction with other keratin proteins, they create a stabilizing framework that is essential to determining a cell's shape and allowing it to handle mechanical stress.\(^{25}\)

In terms of malignancies, CK-8 is an excellent marker since it allows for early detection as well as the identification of potentially spread tumor cells. Based on our results, snoRNA64 expression is positively correlated with the expression of genes responsible for epithelial characteristics.

EMT is referred to as a morphologic cellular phenomenon, described as the phenotypic transition of the cell from an epithelial to a mesenchymal state, where the mesenchymal state is considered to be meta-stable, and the epithelial phenotype is considered to be stable.\(^{27}\) The increased enrichment of mesenchymal markers in tumor cells is evidence of their ability to detach themselves and migrate via blood to other sites. Tumor cells can reach distant organs, such as the lung, liver, brain after escaping to the bloodstream via extravasation.\(^{12,27}\)

CK-19 is a member of the keratin family and is an important component of the cytoskeleton. CK-19 is highly expressed in a variety of cancerous cell types and may play a vital role in their development. CK-19 is an expression molecule elevated even in the circulating tumor cells and its high expression correlates with metastatic behavior in hepatocellular carcinoma.\(^{28,29}\) Therefore, CK-19 is a reliable stem cell marker for the detection of tumors in late, metastatic, and/or recurrent stages.\(^{29}\)

The vimentin protein forms intermediate filaments in both mesenchymal and non-mesenchymal cell structures. During embryonic development, vim is involved in the migration of cells and the construction of neurons.\(^{30}\) It is co-expressed with keratins and forms close associations with intermediate filaments, suggesting some degree of overlap among these proteins.\(^{31}\) Vim reflects a phenotypic characteristic that contributes to the migration and aggressiveness of epithelial tumor cells.\(^{30,32}\) Knockdown of vim expression hampered colony growth and mutations in its sequence significantly reduced migration of the tumor cells.\(^{31}\)

Metalloproteinases (MMPs) are a large family of enzymes that can degrade extracellular matrix (ECM) proteins. It is likely that abnormal expression of MMPs in the tumor microenvironment would contribute to loss of homeostasis of the extracellular matrix, thereby accelerating the progression of PDAC.\(^{33,34}\) Therefore, MMPs are used as indicative markers for tumor aggression in several types of cancer or treatment responses.\(^{35}\) In the modern world, the decrease of MMP levels is considered to be the best indicator of how effective any potential anti-tumorigenic drug might be. A possible therapeutic approach that could reduce tumor invasion and metastasis involves MMP-2 and MMP-3 inhibition. According to studies, increased MMP-2 and MMP-3 levels have been associated with clinical characteristics including survival, metastasis, and tumor stage.\(^{36,37}\) Treatment with MMP-2 and MMP-3 selective synthetic inhibitors significantly limited tumor growth, angiogenesis, and metastasis in several cancer types.\(^{33,38,39}\)

According to the results of this study, CK-19, Vim, MMP-2, and MMP-3 are expressed at high levels in the pancreatic cancer cell lines PK-4 and Mia PC-a, which both exhibit low expression of snoRNA64. In contrast, CK-19, Vim, MMP-2, and MMP-3 were expressed at low levels in cell lines PK-1 and PK-8 that express high levels of snoRNA64. Upon knockdown of snoRNA64 expression in PK-8 epithelial-like cells, mesenchymal makers were significantly increased compared to controls. Conversely, the expression of the epithelial markers E-cad and CK-8 decreased compared to the control group. This suggests a connection between snoRNA64 loss and the acquisition of features characteristic of mesenchymal tissue. Several studies showed that PDACs that have mesenchymal-like features are considered high grade tumors (poorly differentiated), whilst non-mesenchymal PDACs are considered low grade tumors (well differentiated).\(^{40,41}\)

**Conclusion:**

In this study, the results showed a correlation between the loss of snoRNA64 expression and the promotion of EMT in PDAC. In addition, the study confirms the possibility of using snoRNA64 as a diagnostic marker for both early and invasive stages.
of PDAC. Moreover, snoRNA64 may be considered as a barrier during tumor progression.

**Authors' declaration:**
- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.
- Authors sign on ethical consideration’s approval
- Ethical Clearance: The project was approved by the local ethical committee in Southern Technical University.
- Ethics approval: The manuscripts involve no human participants, human data, or human tissue. The *In Slicio* analysis have been obtained from GEO profile database and the cancer cell lines used in the experiments have been obtained commercially.

**References:**


snoRNA64 مؤشر حيوي جديد لتطور سرطان البنكرياس وانتشاره

مختار حيوي

قسم تقنيات صحة المجتمع، المعهد التقني البصرة، الجامعة التقنية الجنوبية

الخلاصة:

تشمل سرطان الاجيادات غدة البنكرياس (PDAC) أكثر من 90٪ من حالات سرطان غدة البنكرياس، كما أنه يمتاز بأعلى معدل للاندماج والانتشار مقارنة بورام البنكرياس الخبيثة الأخرى. صممت هذه الدراسة لتحديد تأثير snoRNA64 في نشوء وتطور PDAC. أظهرت النتائج الأولية أن snoRNA64 قد انخفض بشكل كبير في أورام البنكرياس الأولية والخبيثة مقارنة مع الأنسجة الطبيعية. باستخدام تقنية تفاعل البوليميراز للنسخ العكسي، عربت خطوط خلايا سرطان البنكرياس PK-1 وPK-8 وPK-4 وPK-1 Comparison مع مستوى مختلف من الجزء المودروف في خطوط الخلايا التي يغلب عليها الخصائص الطلائية PK-1. E. cadherin و Cytokeratin-8 و Vimentin و Cytokeratin-19 و MMP-2 و MMP-3 و MMP-8 كعلامات تعبير عن الخصائص الوسيطية. هذه التغيرات في التعبير الجيني جاءت نتيجة تثبيط التعبير الجيني لجزء snoRNA64 وبالتالي سيادة الخصائص الطلائية. تتكون وانتشار الورم الخبيث. بالإضافة إلى ذلك، نقترح اكتشاف إمكانية اعتبار snoRNAs جزء حيوي في تحول النسيج الطبيعي للورم الأولي، مما يظهر تدريجيا خلال تطور السرطان فقد يعمل مستوى تعبيره كحاجز لتحول النسيج الطبيعي للورم الأولي، مما يظهر تدريجيا خلال تطور السرطان. النواحي الفردود الصغير.