CASE REPORT

Epigenetics of Solid Pseudopapillary Neoplasm of the Pancreas

Vera Lucia Antunes Chagas[1], Bruna dos Santos Paiva Ribeiro[1], Marcelo Soares da Mota e Silva[1], Danielle Nunes Forny[2], Fernando Colonna Rosman[1], Maria da Gloria da Costa Carvalho[1]

[1]Department of Pathology, Federal University of Rio de Janeiro, Rio de Janeiro, RJ, Brazil

[2]Physician of the Pediatric Surgery Services of Martagão Gesteira Institute of Pediatrics and Child Care, Federal University of Rio de Janeiro, Rio de Janeiro, RJ, Brazil

ABSTRACT

Background The solid pseudopapillary neoplasm of pancreas is a rare neoplasm with low-grade malignancy and uncertain origin that constitutes about 1-2% of all exocrine pancreatic neoplasms. Epigenetic events such as deoxyribonucleic acid methylation lead to altered gene expression, resulting in altered control of cell proliferation. Based on the fact that epigenetic changes are reversible, the importance of epigenetic studies lies in the better understanding of tumor progression as well as target therapy. **Objective** This study had the objective to evaluate the intratumor heterogeneity of solid pseudopapillary neoplasm of pancreas in three macroscopically distinct areas, comparing gene polymorphism and gene methylation. **Material and Methods** The samples were collected from three distinct tumor areas from one female patient diagnosed with Solid pseudopapillary neoplasm of pancreas and submitted to a pancreatectomy. Deoxyribonucleic acid was extracted from the fresh tissues by using proteinase K digestion and phenol-chloroform isoamyl alcohol followed by ethanol precipitation. The functionality of deoxyribonucleic acid was investigated for *GSTT1* (glutathione S-transferase theta 1) and *GSTM1* (glutathione S-transferase mu 1) genes by multiplex polymerase chain reaction. Methylation-specific polymerase chain reaction analysis was used to determine the methylation status of: *p16*, *RB1*, *E-cadherin*, *TIMP2* and *DAPK* genes promoter by bisulfite modification. **Results and Conclusion** The results show a null genotype for *GSTT1* in tumor areas 1 and 3 when comparing with area 2, showing that there is heterogeneity in the tumor. Tumor fragment 1 was not detected for any investigated genes in methylation, probably due to deoxyribonucleic acid degradation in this region. *TIMP-2* (tissue metallopeptidase inhibitor 2) and *p16* were methylated in areas 2 and 3, and *E-cadherin* presented a small methylation only in fragment 3*; DAPK* and *RB1* (retinoblastoma 1) were unmethylated. As far as we know, this is the first work to show deoxyribonucleic acid methylation in solid pseudopapillary neoplasm of pancreas.

INTRODUCTION

The solid pseudopapillary neoplasm of pancreas (SPTP) is a rare neoplasm with low-grade malignancy. It constitutes about 1-2% of all exocrine pancreatic neoplasms and occurs mainly in young women, having a prolonged, indolent clinical course [1, 2, 3]. This tumor received different denominations, including "Frantz tumor", "cystic solid tumor", "papillary cystic tumor", "papillary epithelial neoplasia", among others [2]. It was also considered as an uncommon carcinoma or non-functioning carcinoma of the pancreatic islets [3]. Through Frantz's description, in 1959, it was recognized as a specific entity [3] and in 1996 it was defined by the World Health Organization (WHO) as "solid pseudopapillary tumor" [2]. Diagnosis of these tumors is sometimes difficult, since their histomorphology and immunophenotype may suggest other exocrine and endocrine pancreatic tumors [4].

Despite the diverse studies with electron microscopy and immunohistochemistry, the cellular origin of this tumor remains uncertain, favoring for many researchers the hypothesis of its origin of a multipotential primitive cell [1, 3]. The extrapancreatic origin has been suggested by some authors [5, 6]. Because of their rarity, clinical data on these tumors are mostly limited to case reports or small series mainly performed among Asian populations [1].

Heterogeneity within the tumor has been described for several types of cancer. Chromosomal abnormalities and unbalanced chromosomal translocation have been reported by many authors for SPTP [7, 8, 9, 10, 11].

Glutathione S-Transferases (GSTs) are enzymes of detoxification of phase II that block the formation of electrophilic products [12, 13]. People from different origins have different patterns of silencing of these genes, varying from region to region [13].

Epigenetics studies the hereditary changes in gene activity and expression that occur without alteration in the DNA sequence [14]. Epigenetic events are a characteristic of

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Abbreviations DAPK death-associated protein kinase; DNA Deoxyribonucleic acid; MSP methylation-specific polymerase chain reaction; SPTP solid pseudopapillary neoplasm of pancreas;
Correspondence Maria da Gloria da Costa Carvalho
Department of Pathology
Universisdade Federal do Rio de Janeiro
Rua Professor Rodolpho Rocco, 255, Rio de Janeiro, RJ, Brazil
Phone +55 21 39382112
Fax +55 21 39382112
E-mail gloria@gcarvalho.org

human cancer. Epigenetic events such as DNA methylation lead to altered gene expression, resulting in altered control of cell proliferation [15]. DNA methylation is an inherited epigenetic labeling that involves the transfer of a methyl group to carbon 5 from cytosine in DNA [16]. Methylation is one of the most studied epigenetic modifications in mammals. It is usually removed during zygote formation and then reestablished around the implantation stage [16, 17]. These changes are reversible; however they are very stable and have a great impact on the regulation of gene expression [18]. DNA methyltransferase is responsible for the methylation pattern. It is known that inactivation of some suppressor and tumor genes occur by hypermethylation of the promoter regions. These hypermethylations have the same effect of a mutation in the promoter region of the genes and some studies call them mimicry [17, 18]. Based on the fact that epigenetic changes are reversible, the importance of epigenetic studies lies both in the better understanding of tumor progression and target therapy.

This study aims to evaluate the intratumor heterogeneity of solid pseudopapillary neoplasm of pancreas in three macroscopically distinct areas, comparing gene methylation in three different tumor areas.

MATERIAL AND METHODS

Samples

A 12-year-old female patient was admitted to the surgical division of the Hospital Universitário Clementino Fraga Filho (HUCFF) of Universidade Federal do Rio de Janeiro (UFRJ), complaining of pain in the right hypochondrium and nausea that had started one month prior to hospital admission. Physical examination revealed a palpable mass in the right hypochondrium, with a well-defined contour, painful to palpation. Computed tomography of the abdomen showed expansive, heterogenous and rounded formation located in the head of the pancreas. The lesion was predominantly solid, nonhomogeneous, suggestive of a solid pseudopapillary neoplasm of the pancreas and a mean of 6.7 (T) × 6.3 (AP) × 6.4 (L) cm **(Figure 1)**. The patient was submitted to Whipple's surgery and the surgical specimen was referred to the Pathology Anatomy Service of the hospital. At macroscopic examination, the tumor was located in the head of the pancreas, measuring 6.0 cm in diameter, surrounded by a fibrous capsule. At cut, clear borders and whitish surface, with solid, granular and hemorrhagic areas were detected. The solid areas were mainly located in the periphery of the tumor, while the granulosa and hemorrhagic areas were mainly located in the intermediate and central areas, respectively. Samples 1, 2 and 3 for the study of molecular biology were collected from the fresh material of the three distinct macroscopic areas of the tumor: the peripheral solid-appearing area, the intermediate area of granular appearance and the more central and hemorrhagic area respectively **(Figure 2)**.

Histopathological examination of hematoxylin and eosin stained slides revealed neoplastic cells with eosinophilic or clear cytoplasm, with ovoid or rounded nuclei, sometimes grooved, with slight anisokaryosis, uniform chromatin, and about 6 mitoses in 10 HPF. The cells had a polyhedral aspect, without cohesion, constituting cellular masses permeated by a delicate connective-vascular stroma, or even arranged perpendicularly around this axis, forming pseudopapillae, with nuclei located at the apical cellular border.

These tissue patterns were observed in the three tumor areas, with predominance of solid areas with cells with eosinophilic cytoplasm and with clear cytoplasm in the region of fragment 1. In the region of fragments 2 and 3 besides the solid areas, a greater number of pseudopapillae and cells with cytoplasmic degeneration of foamy aspect (vacuolated) were noted, associated with

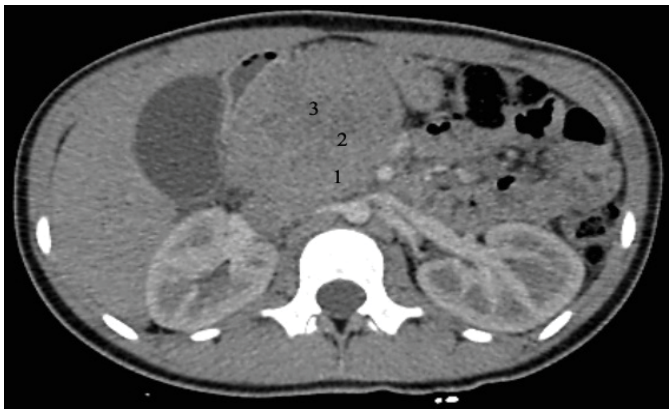

Figure 1. Computed Tomography Image of the abdomen, with arterial contrast, showing a transverse section of the head of the pancreas with a rounded and heterogeneous mass, predominantly solid, measuring 6.7 cm (Transverse) × 6.3 cm (Anteroposterior) × 6.4 cm (Longitudinal), with discrete and heterogeneous contrast. Areas 1, 2 and 3 are the topographical areas where fresh tissue samples were collected for the epigenetic analysis. Compare with Figure 2.

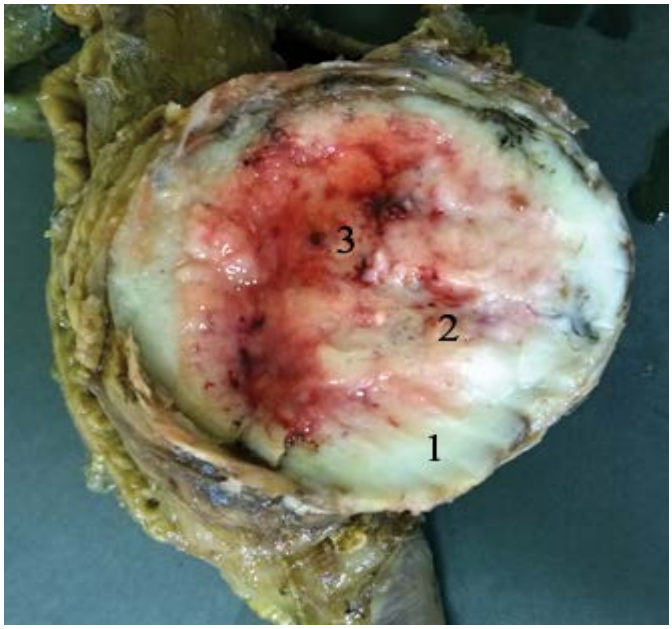

Figure 2. Transverse section of a fresh solid pseudopapillary neoplasm in the head of the pancreas showing rounded and heterogeneous aspects, predominantly solid and whitish, associated with red hemorrhagic area. On the left side, observe the duodenum. Regions 1, 2 and 3 are the regions where fresh tissue samples were collected for the epigenetic analysis. Compare with Figure 1.

intra- and extracytoplasmic eosinophilic granules negative for staining by the Periodic Acid-Schiff method (PAS) and hemorrhagic areas, mainly in the region of fragment 3.

This study was approved by the Research Ethics Committee of the HUCFF of UFRJ (#64915717.0.0000.5257).

DNA Extraction

DNA was extracted from the fresh tissues by using proteinase K digestion and phenol-chloroform isoamyl alcohol followed by ethanol precipitation. DNA concentration was done on Thermo Nanodrop® device.

DNA Functionality Test

The functionality of DNA extracted from three distinct areas was investigated for *GSTT1* and *GSTM1* genes by multiplex PCR following the methodology described by Joseph and collaborators [19] with some modifications [20]. For the *GSTM1* and *GSTT1* genes, the polymorphism investigated is of the deletion type, where the absence of the gene corresponding band (220 base pairs for *GSTM1* and 450 bp for the *GSTT1* gene) represents its deletion.

Methylation

Methylation-specific PCR (MSP) analysis was used to determine the methylation status as previously described: *p16* [21], *RB1* [22], *E-cadherin* [23], *TIMP-2* [24] and *DAPK* [21] genes promoter by bisulfite modification.

For all the analyses, the amplified products were detected by 10% electrophoresis polyacrylamide gel, using 100 base pair molecular weight marker (Pharmacia Biotech, USA).

RESULTS

A multiplex PCR was performed for *GSTT1* and *GSTM1* genes using samples from the three distinct tumor areas. The results show a null genotype for *GSTT1* in tumor areas 1 and 3 when comparing with area 2. These results indicate a probable specific loss of genomic integrity in response to tumour DNA degradation due to cellular degeneration. The null genotype for *GSTT1* in areas 1 and 3 of the tumor indicates the tumor heterogeneity **(Figure 3)**.

In relation to the methylation status of investigated genes, fragment 1 of the tumor was only detected for small unmethylation for *RB1*, probably by DNA degradation in this region. *TIMP-2* and *p16* were methylated in areas 2 and 3*; DAPK*, *RB1* and *E-cadherin* were unmethylated in fragments 2 and 3; 1 and 2; 2 and 3, respectively **(Figure 4)**.

DISCUSSION

The results of the Polymorphism analysis show that there is a molecular heterogeneity between the tumor fragments. The difference found is probably because of DNA damage in areas 1 and 3 of the tumor. Fragment 3 was collected from an area with high levels of degeneration and

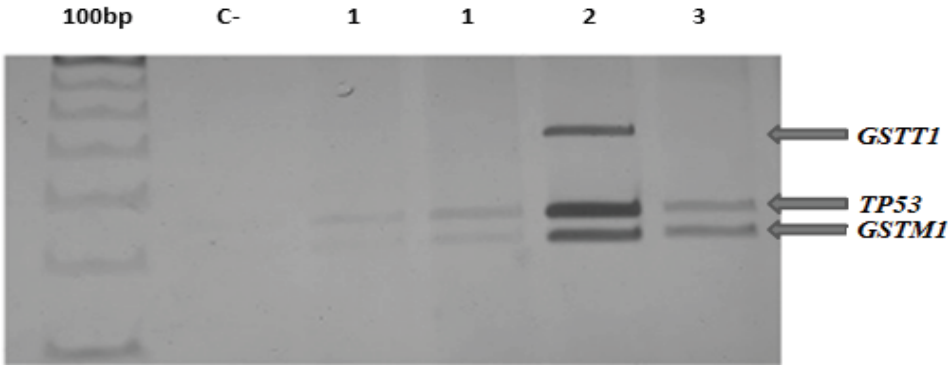

Figure 3. Multiplex PCR products analyzed by 10% polyacrylamide gel electrophoresis. *GSTT1* deletion polymorphism was observed in fragments 1 and 3. Tumor fragment 2 shows amplification for *GSTT1* 450 bp. *GSTM1* 220 bp and also to *TP53* 274 bp. Lane 1: 100 bp ladder, Lane 2: negative control and Lanes 3 to 6: tumor fragments. For tumor fragment 1, 20 and 50 ng of DNA were used, and for fragments 2 and 3, 20 ng of DNA in the Multiplex PCR reaction. The arrows point to the corresponding band of each gene.

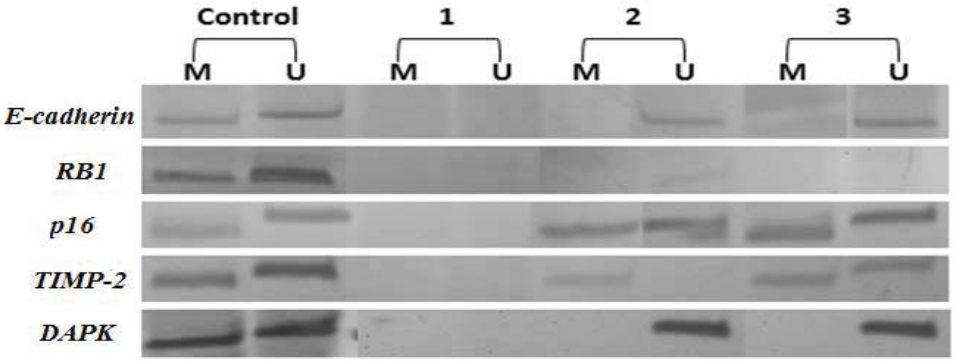

Figure 4. Methylation Specific PCR analyzed by 10% polyacrylamide gel electrophoresis. Methylation was observed for *p16* and *TIMP-2* in fragments 2 and 3. *E-cadherin*, *RB1* and *DAPK* had results for unmethylated genes in fragments 2 and 3; 1 and 2; and 2 and 3, respectively. A previously known sample of gastric cancer was used as positive control. The numbers correspond to the fragments. M is for Methylated and U for Unmethylated. The corresponding genes are listed on the left side of the image. For all samples, 50 ng of DNA were used.

hemorrhage, which is possibly the cause of the degradation in this region. Fragment 1 was collected from a solid and peripheral area. The methylation experiment also shows differences between the fragments, corroborating the GSTs analyses.

The methylation markers used in this study were chosen based on the literature, where the methylations of these genes are related with tumor progression and proliferation in pancreatic tumors [25]. Methylation of tumor suppressor genes has been reported before in papillary mucinous neoplasm of pancreas related to its malignant progression [26].

Silencing of *p16* through methylation is the most frequent early event in carcinogenesis. Study on pancreatic carcinoma suggests that the methylation of this gene could be associated with the tumorigenesis [27]. Alterations in this gene are common in pancreatic adenocarcinomas, through genetic and epigenetic modifications [28]. In the present study, it may indicate the malignant potential of the tumor. Another work shows a relation between tumor size and *p16* expression. Tumors were bigger when the expression was lower [29], which could possibly be another result of the silencing of the gene in this tumor, leading to the need of further studies to prove this hypothesis.

E-cadherin was not methylated. *E-cadherin* gene methylation could be involved in tumor formation, invasiveness or metastatic potential; its lower expression was more frequent on metastatic tissue of pancreatic adenocarcinoma [30]. The understanding of tumor biology could identify new molecular targets for the diagnosis of SPTP. Previous studies show that *E-cadherin* is hypermethylated in pancreas carcinomas [25]. Moreover, the methylation of E-cadherin was shown to be a worsened factor in pancreatic ductal tumor in patients with diabetics [31]; however, methylation was not found in the present work.

TIMP-2 showed differences between fragments 2 and 3. *TIMP-2* in fragment 2 showed only methylation, which is a result that can suggest the total methylation of the gene in this area. *TIMP-2* methylation can be related to the ability, though not accentuated, of neoplastic cells to permeate tissue adjacent to the tumor, such as the conjunctive capsule or, in some cases, the pancreatic parenchyma [32, 33].

The gene of protein *DAPK* methylation leads to transcription inactivation, and this gene probably relates to the cancer origin in the urinary bladder [34] and in the gastrointestinal tract [35]. However, regarding pancreatic tumor and especially SPPT, we did not find any reference about *DAPK* methylation. But, in the literature, the loss of DAP kinase expression has already been shown to be more frequent in the metastatic tissue than in the primary tumor in the pancreatic adenocarcinoma [30].

RB1 is the gene of the protein that is a cell cycle regulator and a tumor suppressor. Even though *RB1* was already found inactivated in pancreatic cancer [36], this gene did not have positive results for methylation in this study.

Curiously, in fragment 1, although it showed functional DNA **(Figure 1)**, only a discrete positivity for unmethylated *RB1* was observed.

CONCLUSION

This is the first study, as far as we know, to show gene methylation in different areas of a Solid Pseudopapillary Neoplasm of Pancreas. The silencing of genes tumor suppressors by methylation, indicates one of the mechanisms involved on the oncogenesis of this type of tumor, contributing for its malignancy.

This study shows the absence of *GSTT1* in both fragments 1 and 3, and, for the first time, a specific degradation of these genes.

Further studies are necessary to understand better the biology of this tumor.

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Conflict of Interest

The authors declare no conflict of interest.

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