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Research Article
Genetic Polymorphisms and DNA Methylation Evaluation in a Rare Pediatric Case Carrying a Solid Pseudo Papillary Neoplasm of the Pancreas

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Abstract

Background and Objectives: Heterogeneity within the tumour has been described for several types of cancer, including Solid Pseudo Papillary Neoplasm of the Pancreas (SPNP). Tumour heterogeneity may provide distinct molecular signatures that can significantly affect treatment response. This study aimed to investigate the molecular heterogeneity of three different tumour areas (peripheral, intermediate and central) from a pediatric case with SPNP submitted to Whipple's surgery in the Clementino Fraga Filho University Hospital, Rio de Janeiro, Brazil. **Materials and Methods:** Polymorphisms of the detoxification genes *GSTP1*, *CYPA1*m1 and *CYPA1*m2 were investigated by PCR-restriction fragment length polymorphism (PCR-RFLP) technique and the promoter Methylation profile of the genes $p14^{ARF}$, *GSTP1*, *hTERT* and *MGMT* were assessed by Methylation-Specific PCR (MSP). **Results:** The *CYPA1*m1 and *CYPA1*m2 showed wild-type genotype and *GSTP1* showed heterozygote genotype. Regarding the methylation status, *MGMT* showed no detectable methylation in any of the 3 tumour regions; in contrast, both $p14^{ARF}$ and *hTERT* showed detectable methylation in all three regions, whereas, *GSTP1* showed methylation in peripheral and intermediate areas. **Conclusion:** Epigenetic inactivation of critical genes and reduced detoxification activity revealed the SPNP tumour biology heterogeneity, which could represent new molecular targets for SPNP treatment.

Key words: Solid pseudopapillary neoplasm of pancreas, tumour heterogeneity, pediatric patient, polymorphism, DNA methylation

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Solid Pseudo Papillary Neoplasia of the Pancreas (SPNP) is characterized as a rare neoplasm of the low potential of malignancy, comprising about 1-2% of all exocrine pancreatic neoplasms. The young women are the most affected by the disease, with a prolonged and indolent clinical course[1]. The SPNP symptoms, when present, are not specific and may include abdominal pain, nausea and vomiting[2]. Surgical resection is the standard treatment and generally leads to a good prognosis. Also, some patients may experience an aggressive clinical course and resistance to anticancer therapy[3]. Cancer is a heterogeneous disease revealing distinct molecular signatures within the same tumour. Tumour heterogeneity can be described into two types, intertumoral and intratumoral and molecular heterogeneity can significantly influence treatment response[4]. This influence on therapeutic response is observed in pancreatic ductal adenocarcinoma, in which the fast drug resistance is related to tumour heterogeneity[5]. Since it is a rare tumour, the molecular events involved in SPNP heterogeneity need to be better explored.

Cells exhibit several mechanisms of response to protect their functioning against toxic substances. Detoxification enzymes are part of this process and can be represented by Cytochrome P450 (CYP) and Glutathione S-Transferase (GSTs) enzymes[6]. The CYPs enzymes are phase I detoxification enzymes responsible for the oxidation of xenobiotics, while GSTs are phase II detoxification enzymes that protect cellular macromolecules from attack by reactive electrophiles[7]. Therefore, genetic polymorphisms in these enzymes can influence their activity, contributing to increasing cell instability in the face of toxic and/or mutagenic agents or even affecting the response of therapeutic drugs. The *GSTP1* (rs1695) polymorphism involves A→G transition (c.313A>G) that results in an isoleucine to valine exchange, showing clinical relevance[7]. The most common *CYP1A1* variants are represented by *CYP1A1*m1 (rs4646903, T>C) and *CYP1A1*m2 (rs1048943, A>G) polymorphisms and may contribute to cancer risk and treatment efficacy[8-10].

Epigenetic events, such as DNA methylation, can lead to gene expression changes able to induce silencing of crucial genes, thereby promoting altered control of cell proliferation[11,12]. It is well known that the inactivation of some tumour suppressor genes, such as $p14^{ARF}$, occurs by hypermethylation of the promoter region[11,13]. The $p14^{ARF}$ protein is involved in the cell cycle control by its antiproliferative role. Therefore, $p14^{ARF}$ inactivation may contribute to cancer development and progression. Another important epigenetic event in cancer is observed in *hTERT* promoter hypermethylation. The *hTERT* gene encodes the catalytic subunit of telomerase, which may be reactivated and upregulated in numerous tumour cells by hypermethylation, thus leading to tumour formation and progression[14]. The O6-methylguanine-DNA methyltransferase (MGMT) is a DNA repair enzyme able to remove adducts at the O^6 position of guanine induced by alkylating agents, thus avoiding mutagenesis. Since this mechanism promotes resistance to alkylating drugs, the investigation of MGMT activity by gene promoter hypermethylation in tumours is useful to predict treatment response[15,16].

The genetic and epigenetic changes described above are some of the molecular events that could contribute to increased tumour heterogeneity. In this context, the study aimed to investigate the molecular heterogeneity within the tumour of an SPNP pediatric case that was reported in previous studies[17,18]. In the present study, the molecular analyzes were evaluated by assessing the polymorphisms of *CYP1A1*m1 (rs4646903), *CYP1A1*m2 (rs1048943) and *GSTP1* (rs1695) and the methylation status of the $p14^{ARF}$, *GSTP1*, *hTERT* and *MGMT*.

MATERIALS AND METHODS

Study area: The patient investigated in this study received the clinical diagnosis of solid pseudopapillary neoplasm of the pancreas in 2016. The molecular investigation reported in this study started in 2018 and ended in 2020.

Patient presentation: This study investigated a 12 years-old female patient admitted at the surgical division of the Clementino Fraga Filho University Hospital, Rio de Janeiro, Brazil, as previously described[17,18]. The patient presented a palpable mass in the right hypochondrium and a lesion found on the head of the pancreas was suggestive of a solid pseudopapillary neoplasm of the pancreas. The patient was submitted to Whipple's surgery and continues to be regularly monitored at the hospital, using Pancreatin (Creon®) four times a day. This study was approved by the Research Ethics Committee of the Hospital (# 64915717.0.0000.5257).

Samples collection: Three distinct macroscopic areas of the tumour were obtained for investigations: (1) The peripheral solid-appearing area, (2) The intermediate area of granular appearance and (3) the more central and hemorrhagic area.

Table 1: Primers sequences, annealing temperatures, fragment sizes and restriction enzyme of polymorphism and methylation analysis

Genes	Primer sequence	Annealing (°C)	Fragment size	Enzyme
*CYP1A1*m1	F: CAGTGAAGAGGTGTAGCCGCT	64	200 and 140 bp homozygous	MspI
	R: TAGGAGTCTTGTCTCATGCCT		340, 140 and 200 bp heterozygous	
*CYP1A1*m2	F: GAAAGGCTGGGTCCACCCTCT	64	232 bp wild type and 263 bp variant	NcoI
	R: CCAGGAAGAGAAAGACCTCCCAGCGGGCCA			
GSTP1	F: TCCTTCCACGCACATCCTCT	68 (first 20 cycles)	294 pb, 234 pb e	BsmAI
	R: AGCCCCTTTCTTTGTTCAGC	51 (10 final cycles)	60 pb	
GSTP1 M	F: TTCGGGGTGTAGCGGTCGTC	55	91	NA
	R: GCCCCAATACTAAATCACGACG			
GSTP1 U	F: GATGTTTGGGGTGTAGTGGTTGTT	55	97	NA
	R: CCACCCCAATACTAAATCACAACA			
hTERT M	F: GAGGTATTTCGGGAGGTTTCGC	62	121	NA
	R: ACTCCGAACACCACGAATACCG			
hTERT U	F: GGGAGGTATTTTGGGAGGTTTTGT	62	126	NA
	R: CAAACTCCAAACACCACAAATACCA			
P14^{ARF} M	F: TTTTTGGTGTTAAAGGGTGGTGTAGT	60	122	NA
	R: CACAAAAACCCTCACTCACAACAA			
P14^{ARF} U	F: GTGTTAAAGGGCGGCGTAGC	60	132	NA
	R: AAAACCCTCACTCGCGACGA			
MGMT M	F: TTCGACGTTCGTAGGTTTTCGC	59	80	NA
	R: GCACTCTTCCGAAAACGAAACG			
MGMT U	F: TTTGTGTTTTGATGTTTGTAGGTTTTTGT	59	94	NA
	R: AACTCCACACTCTTCCAAAAACAAAACA			

NA: Not applied, M: Methylated, U: Unmethylated

DNA extraction: Genomic DNA was extracted from the fresh tissues of the three distinct tumour areas by the phenol-chloroform method, according to standard protocols[19]. DNA concentration was measured by Nanodrop 2000® (Thermo Fisher Scientific) and stored at -20°C until further analysis.

Polymorphism of selected genes: The *GSTP1* (rs1695), *CYP1A1*m1 (rs4646903) and *CYP1A1*m2 (rs1048943) polymorphisms were investigated by Polymerase Chain Reaction Restriction Fragment Length Polymorphism (PCR-RFLP) technique, as described by Joseph *et al.*[9], with some modifications. Briefly, PCR products were digested with specific restriction endonuclease enzyme in Table 1 and the corresponding fragments were visualized on non-denaturing 10% polyacrylamide gels.

DNA methylation analysis: Methylation-specific PCR (MSP) analysis was used to determine the methylation status in the promoter regions of *p14*^{ARF}[13], *hTERT*[14], *MGMT*[15] and *GSTP*[20]. Genomic DNA was bisulfate-treated using the EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA), according to the manufacturer's instructions. After MSP reactions using specific primer sequences expressed in Table 1, methylated and unmethylated products were visualized on nondenaturing 10% polyacrylamide gels.

RESULTS

For the present study, the tumour heterogeneity in an SPNP pediatric case was investigated by assessing the polymorphic and the methylation status of critical genes for genome stability and treatment response. PCR-RFLP analysis revealed *CYP1A1*m1 and *CYP1A1*m2 with a non-digestion fragment, representing the wild-type (WT) genotype for all three tumour areas in Fig. 1a and Table 2. In contrast, all three tumour areas showed *GSPT1* heterozygote genotype, being represented by fragments of digested PCR products of 294/234/60 bp in Fig. 1b and Table 2.

Regarding the methylation status, detectable methylation was observed in all three tumour areas for the *hTERT* and *p14*^{ARF} genes in Fig. 2 and Table 2. It is important to note that *p14*^{ARF} methylation showed a weak signal in the intermediate tumour area (area 2) compared with the hypermethylation observed in peripheral (area 1) and central (area 3) regions. This variation observed in the *p14*^{ARF} methylation signal could represent a distinct molecular signature of tumour heterogeneity. For *GSTP1* methylation, the peripheral and the intermediate areas showed detectable methylation and the central area showed undetectable methylation (unmethylated), representing a tumour heterogeneity profile for *GSTP1* methylation rates in Fig. 2 and Table 2). In contrast, *MGMT* showed undetectable methylation for all three distinct tumour areas (data not shown).

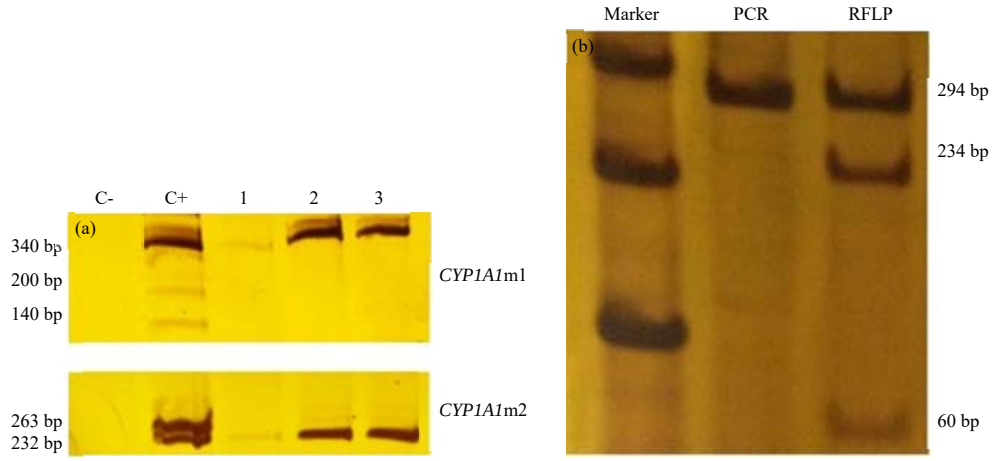

Fig. 1(a-b): PCR-RFLP analyzes for (a) *CYP1A1*m1, *CYP1A1*m2 and (b) *GSTP1* in representative non-denaturing 10% polyacrylamide gels

PCR-RFLP of *CYP1A1*m1 and *CYP1A1*m2 in the three tumour areas and a previously known sample used as a positive control (C+). The three areas show unique fragments representing a non-digestion site (wild-type genotype), *GSTP1* polymorphism representative of tumour area 2 showing heterozygote genotype (294/234/60 bp), bp: base pair

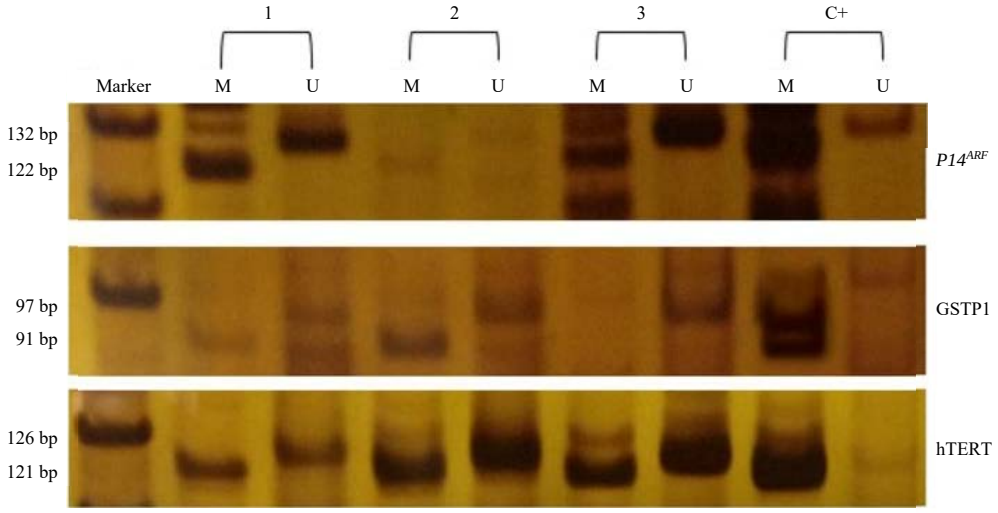

Fig. 2: Representative non-denaturing 10% polyacrylamide gels of MSP products for *p14*^{ARF}, *GSTP1* and *hTERT*

Methylated and unmethylated products and the corresponding band sizes are shown. Lanes: M, methylated, U: Unmethylated, C+: Positive control

Table 2: Polymorphism and methylation analysis of the three distinct tumour regions

	Polymorphism genotyping			Methylation status			
Tumor area	*CYP1A1*m1	*CYP1A1*m2	*GSTP1*	*p14*^{ARF}	*GSTP1*	*hTERT*	*MGMT*
1	WT	WT	Heterozygote	M	M	M	ND
2	WT	WT	Heterozygote	M	M	M	U
3	WT	WT	Heterozygote	M	U	M	U

WT: Wild type, M: Methylated, U: Unmethylated, ND: Not detected, Tumour areas 1: Peripheral, 2: Intermediate, 3: Central

DISCUSSION

Targeted therapy has significantly improved patient outcomes in a range of solid tumour types. However, the targeted therapies do not seem to benefit all selected patients, especially those with advanced disease. This clinical profile may be explained by tumour heterogeneity, in which the distinct molecular signatures found within the same

tumour have been reported to significantly affect therapeutic response and clinical outcome[4]. The SPNP is a rare tumour and thus the molecular events need to be better explored. In the present study, we assessed the genetic polymorphisms and the methylation status in an SPNP pediatric case to evaluate tumour heterogeneity in three distinct tumour areas.

Genetic polymorphisms in genes that encode detoxification enzymes may influence the elimination of carcinogenic compounds, contributing to increased genomic instability. CYPs enzymes are a superfamily of enzymes that play a role as detoxification enzymes. Polymorphisms in *CYP* genes, such as *CYP1A1*m1 and *CYP1A1*m2*,* show clinical relevance since may contribute to cancer risk or even affect treatment efficacy resulting in drug resistance[8-10]. However, some studies reported a lack of significant association between *CYP1A1*m1 (rs4646903) and *CYP1A1*m2 (rs1048943) polymorphisms and cancer susceptibility[21-23]. In the present work, both *CYP1A1*m1 and *CYP1A1*m2 exhibited wild-type genotype for the three regions investigated, revealing a similar tumour profile for *CYP* genes in this SPNP case. The GST family is another group of crucial metabolic enzymes involved in detoxification processes. The *GSTP1* polymorphism (rs1695, A>G) may affect the detoxification metabolism of chemical carcinogens such as benzene, being an important biomonitoring tool to investigate the health risks in individuals occupationally exposed to benzene, such as gas station attendants[24]. The *GSTP1* polymorphism can result in lower enzyme activity and reduced detoxification ability, thus affecting the prognosis and treatment of cancer patients[25]. Furthermore, the polymorphic *GSTP1* GG genotype was reported to be less stable than the wild-type genotype[25]. In the present study, all the three tumour regions showed *GSTP1* heterozygote genotype, which may contribute to increasing the molecular instability and facilitating the neoplastic development in this SPNP case.

The DNA methylation can promote the inactivation of key tumour suppressor genes and, therefore, shows association with tumorigenesis and tumour progression[11-13]. The present study, investigated the methylation status in the promoter region of the genes $p14^{ARF}$, *GSTP1*, *hTERT* and *MGMT*. Like the polymorphic genotype, the *GSTP1* promoter hypermethylation affects the enzyme activity and has been associated with cancer development and progression[26]. In this study, *GSTP1* showed detectable methylation in peripheral and intermediate SPNP areas, which corroborates the tumour heterogeneity observed in our previous studies using other molecular biomarkers for the same SPNP case[17,18]. Therefore, *GSTP1* epigenetic inactivation by DNA methylation may also have contributed to increase genomic instability and trigger neoplastic events in SPNP.

Telomeric DNA repeats maintain the chromosomal integrity and telomere shortening is a natural event leading to cell growth arrest after multiple cell divisions. However, cancer cells can reacquire longer telomeres and maintain unlimited cell division through telomerase activation[27]. The *hTERT*, catalytic subunit of telomerase, may be reactivated in the process of tumorigenesis and progression, in which *hTERT* is upregulated in several tumours via genetic and epigenetic mechanisms[14,27]. In the present study, *hTERT* showed detectable hypermethylation for all three SPNP areas, which may indicate that *hTERT* expression is being up-regulated in SPNP. Further investigations are needed to better establish the association between *hTERT* methylation and *hTERT* expression in SPNP.

The $p14^{ARF}$ encodes the $p14^{ARF}$ protein, which is a crucial tumour suppressor protein for cell cycle control. Therefore, $p14^{ARF}$ inactivation has been associated with several malignancies[11,13]. In this study, detectable methylation for $p14^{ARF}$ was observed for the three tumour areas. It is important to highlight, $p14^{ARF}$ methylation showed a weak signal in the intermediate tumour area (area 2) compared to peripheral (area 1) and central (area 3) regions. The $p14^{ARF}$ protein is encoded by the same *CDKN2A* locus as the $p16^{INK4A}$ protein. Our previous analysis revealed $p16^{INK4A}$ methylation in tumour fragments 2 and 3 (intermediate and central area, respectively)[17]. Although at the same locus, $p14^{ARF}$ and $p16^{INK4A}$ methylation can occur independently[13]. These findings revealed tumour heterogeneity in three distinct tumour areas of SPNP for the *CDKN2A* locus.

The *MGMT* is a DNA repair protein that avoids mutagenesis by removing adducts at the O^6 position of guanine induced by alkylating agents. The *MGMT* can be used as a target for chemotherapy to induce apoptosis[28]. In glioblastoma patients, *MGMT* methylation is associated with a better overall survival even when tumours are not suitable for resection[29]. In this study, no detectable methylation for *MGMT* was found in any of the three SPNP tumour regions, which may contribute to characterizing this neoplasm as a low-grade malignancy.

CONCLUSION

This study analyzed the molecular heterogeneity in three distinct macroscopic areas of a pediatric SPNP tumour. This study described the multiple molecular mechanisms that may be associated with SPNP to better characterize the molecular heterogeneity involved in this malignancy, showing relevant molecular targets for SPNP treatment.

SIGNIFICANCE STATEMENT

The molecular events involved in SPNP heterogeneity need to be better explored. This study described the molecular heterogeneity in three distinct macroscopic areas of tumour samples from a pediatric patient diagnosed with SPNP by assessing the genetic polymorphisms and methylation status of critical genes for genome stability. These findings may be useful to better characterize the molecular heterogeneity involved in this malignancy, showing relevant molecular targets for SPNP treatment response and clinical outcome.

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