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Mestrado Integrado em Medicina

Expression of ENPP1 in testicular germ cell tumors: exploring its role in the pathobiology of distinct histotypes and in prognosis

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Expression of ENPP1 in testicular germ cell tumors: exploring pathobiology of distinct histotypes and in prognosis

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Reconhecendo que as palavras são sempre insuficientes para traduzir plenamente a gratidão, deixo aqui o meu mais sincero agradecimento a todos aqueles que me estenderam a mão e me guiaram num percurso em direção a uma meta que, em tempos, me parecia inalcançável. Expresso uma menção especial ao Doutor João Lobo e ao Professor Doutor Rui Henrique, cuja generosidade, empenho e constante apoio foram pilares fundamentais para a concretização deste trabalho. Recordo, a propósito, as palavras do médico William Osler: *"A gratidão é uma das maiores virtudes do espírito humano e talvez a mais esquecida."* Com esta consciência, agradeço ainda, de forma sentida, a todos os que me acompanharam, mesmo em silêncio, com gestos ou palavras que deixaram marca - mesmo sem referência, todos têm lugar neste agradecimento.

Resumo

Introdução: Os tumores de células germinativas do testículo (TGCTs), as neoplasias sólidas mais comuns entre os homens jovens, têm cada vez maior incidência. Apesar da boa resposta à quimioterapia à base de cisplatina, os efeitos secundários a curto e longo prazos afetam negativamente a qualidade de vida do doente. O desenvolvimento recente de inibidores da ectonucleótido pirofosfatase/fosfodiesterase 1 (ENPP1) abre oportunidade para uma terapia seletiva dirigida. No entanto, a expressão de ENPP1 nos TGCTs não foi descrita até à data.

Objetivos: O nosso objetivo foi avaliar a imunoexpressão da ENPP1 numa coorte de TGCTs primários e metastáticos, representativa do ponto de vista clínico e anatomopatológico, bem como no parênquima testicular normal, investigando padrões de imunoexpressão diferenciais entre subtipos e estabelecendo correlações clinicopatológicas.

Materiais e métodos: No total, foram avaliados 90 componentes tumorais individuais de TGCT de 63 doentes diagnosticados e tratados num centro compressivo de cancro, tendo sido utilizada imunohistoquímica para determinar a expressão do biomarcador (pontuação combinada da percentagem de células tumorais marcadas e intensidade da marcação). Foram realizadas análises *in silico* dos dados do The Cancer Genome Atlas (TCGA) e do Human Protein Atlas (HPA) para validação adicional. A significância estatística foi estabelecida em $p<0,05$.

Resultados: Foram encontradas diferenças significativas na expressão de ENPP1 nos diversos subtipos de TGCT. Os seminomas e carcinomas embrionários apresentaram uma expressão de ENPP1 significativamente superior em comparação com outros subtipos, sendo os níveis mais elevados encontrados no carcinoma embrionário, o que foi confirmado pela análise *in silico* do TCGA e do HPA. A expressão nas amostras metastáticas foi, em geral, menor em comparação com os TGCTs primários. A ENPP1 não foi expressa em células germinativas de parênquima testicular saudável.

Discussão e conclusão: Demonstramos que a ENPP1 é expressa nos TGCTs, principalmente no carcinoma embrionário (um subtipo de tumor frequentemente agressivo), seguido pelo seminoma (o subtipo mais comum de TGCT), sugerindo um potencial de resposta aos novos inibidores da ENPP1. A ausência de expressão em células germinativas de parênquima testicular saudável sugere especificidade para as células tumorais e baixa probabilidade de toxicidade.

Palavras-chave: ENPP1, tumores de células germinativas do testículo, biomarcadores, terapias dirigidas.

Abstract

Background: Testicular germ cell tumors (TGCTs), the most common solid malignancies among young men, are increasingly incident. Despite good response to cisplatin-based chemotherapy, short- and long-term side effects negatively affect patient quality of life. Recent development of ecto-nucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) inhibitors opens opportunity for selective targeted therapy, but ENPP1 expression in TGCTs has not been characterized, thus far.

Objectives: We aimed to assess ENPP1 immunoexpression in a clinically and pathologically well-characterized cohort of primary and metastatic TGCTs, and in normal testicular parenchyma, looking into differential immunoexpression patterns among subtypes and clinicopathological correlations.

Materials and methods: Overall, 90 TGCT individual tumor components from 63 patients diagnosed and treated at a comprehensive cancer center were assessed, using immunohistochemistry to ascertain biomarker expression (combined score of stained tumor cells percentage and staining intensity). *In silico* analysis of The Cancer Genome Atlas (TCGA) and Human Protein Atlas (HPA) datasets were performed for additional validation. Statistical significance was set at $p<0.05$.

Results: Significant differences in ENPP1 expression across TGCT subtypes were disclosed. Seminomas and embryonal carcinomas showed significantly higher ENPP1 expression compared to other subtypes, the highest levels being found in embryonal carcinoma, which was confirmed with *in silico* analysis of TCGA and HPA. Expression in metastatic samples was overall lower compared to primary TGCTs. ENPP1 was not expressed in germ cells of healthy testicular parenchyma.

Discussion and Conclusion: We demonstrate that ENPP1 is expressed in TGCTs, mostly embryonal carcinoma (a frequently aggressive tumor subtype), followed by seminoma (the most common TGCT subtype), suggesting potential responsiveness to novel ENPP1 inhibitors. Absent expression in germ cells of healthy testicular parenchyma suggest cancer cells specificity and low probability of fertility-related toxicity.

Keywords: ENPP1, testicular germ cell tumors, biomarkers, targeted therapies.

Lista de abreviaturas e siglas

ABCG2: ATP-Binding cassette sub-family G member 2 gene

ADP: adenosine diphosphate

AFP: alpha-fetoprotein

AJCC: American Joint Committee on Cancer

AMP: adenosine monophosphate

A-NED: alive with no evidence of disease

ATP: adenosine triphosphate

AWD: alive with disease

CD: cluster of differentiation

cGAMP: cyclic guanosine monophosphate-adenosine monophosphate

CH: choriocarcinoma

CSC: cancer stem-cell

CTCs: circulating tumor cells

DFD: died from disease

DNA: deoxyribonucleic acid

EC: embryonal carcinoma

EMT: epithelial-mesenchymal transition

ENPP1: ecto-nucleotide pyrophosphatase/phosphodiesterase 1

GCNIS: germ cell neoplasia in situ

GCT: germ cell tumor

GSC: glioma stem-cell

HER-2: human epidermal growth factor receptor 2

HGSOC: High-Grade Serous Ovarian Cancer

IB: immunoblotting

IF: immunofluorescence

IGCCCG: International Germ Cell Cancer Collaborative Group

IHC: immunohistochemistry

IL: interleukin

IPO Porto: Instituto Português de Oncologia do Porto

IQR: interquartile range

MDM2: murine double minute 2

mRNA: messenger RNA

NAD: nicotinamide adenine dinucleotide

NK: natural killer

NS: nonseminoma

OSCC: Oral Squamous Cell Carcinoma

PARP: poly-ADP ribose polymerase

PC1: plasma cell alloantigen 1

PCR: polymerase chain reaction

PD-1: programmed cell death protein 1

PD-L1: programmed death-ligand 1

Pi: phosphate

PPi: inorganic pyrophosphate

qRT-PCR: quantitative real time polymerase chain reaction

RNA: ribonucleic acid

RT: radiotherapy

SE: seminoma

STING: stimulator of interferon genes

TCGA: The Cancer Genome Atlas

TE: teratoma

TGCT: testicular germ cell tumor

TMA: tissue microarray

TNF: tumor necrosis factor

TPM: transcript per million

WB: Western Blot

WHO: World Health Organization

YST: yolk sac tumor

***β*-HCG -** Human chorionic gonadotropin *β*

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Introduction

Germ cell tumors (GCTs) comprise a diverse category of neoplasms connected to developmental biology. They can develop in different anatomic regions, including the testes, the ovaries and extragonadal sites, such as the mediastinum, central nervous system and retroperitoneum. Extragonadal GTCs only account for 4% of the GCTs.[2]

Testicular GCTs (TGCTs) make up 95% of all testicular neoplasms and are the most common solid malignancies in young men aged 15 to 40 years, who have a long-life expectancy.[2,3] The most common TGCTs are the so-called type II TGCTs. These originate from the precursor lesion germ cell neoplasia *in situ* (GCNIS) and enclose the seminomas (SEs) and nonseminomas (NSs). The latter include several subtypes, including embryonal carcinoma (EC), postpubertal-type yolk-sac tumor (YST), choriocarcinoma (CH) and postpubertal-type teratoma (TE), as well as mixed tumors.[3,4]

TGCTs represent one of the most curable solid neoplasms, with 5-year survival rates over 90% in the cisplatin era.[5] However, long-term toxicity from cisplatin treatment in young TGCT survivors, as well as emergence of cisplatin-resistant disease, are two major concerns in the field. Therefore, it is important to uncover novel less toxic targeted therapies, as well as predictive biomarkers.[1,3,6-8] Molecular biomarkers are increasingly assuming a critical role in the management of cancer patients.[5] Considering the overall rarity of certain types of testicular neoplasms, progress in biomarker research has been relatively slow.[8,9] Despite notable progress, including the discovery of liquid biopsy biomarkers such as microRNAs of the 371~373 cluster, there is a lack of studies on biomarkers predicting response to targeted therapies.[1,10]

Recently, ecto-nucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) inhibitors were developed. These inhibitors have revealed immunostimulatory effect, and their combination with other therapies, such as immune-checkpoint blockade, STING activation, DNA damage response inhibitors and radiotherapy (RT), represents a promising path to enhance antitumor immune responses and improve clinical outcomes in several tumors.[11,12] ENPP1 expression has been assessed in some tumor models, including lung, ovarian, colorectal, gallbladder and breast carcinomas [11,13-15], but not in TGCTs. Several forms of TGCTs are characterized by dense infiltration of immune cell populations, namely SEs. An example of the role of the immune system includes the “regressed germ cell tumor” phenotype, also called “burned out tumor”. However, clinical trials using immune checkpoint inhibitors (modulating the PD-1/PD-L1 axis) or PARP inhibitors in monotherapy have showed low efficacy.[12] Therefore, novel ways of modulating

the immune microenvironment or DNA damage response for targeting TGCT cells are much desired, and may be key for better understanding how to unlock an anti-tumor immune response.

Because ENPP1 expression has not been investigated in TGCTs, we sought to assess ENPP1 immunoexpression in a clinically and histopathologically well-characterized cohort of primary and metastatic TGCTs, as well as in normal testicular parenchyma, which could suggest potential response to ENPP1 inhibitors. Furthermore, we aim to explore the differential ENPP1 immunoexpression patterns across TGCT subtypes and to establish clinicopathological correlations, assessing a putative prognostic role.

Material and Methods

In silico analysis

In silico analysis of TCGA (Testicular Germ Cell Cancer-TCGA, PanCancer Atlas) database using the publicly available cBioPortal tool (https://www.cbioportal.org/), was performed as previously described [6]. The TCGA cohort, including 124 TGCT patients, was selected for evaluation of ENPP1 mRNA expression (obtained via RNA-sequencing). Clinicopathological features analyzed included "Cancer Type," "Cancer Type Detailed," "American Joint Committee on Cancer Tumor Stage Code," "Neoplasm Disease Lymph Node Stage American Joint Committee on Cancer Code," "American Joint Committee on Cancer Metastasis Stage Code," and "Neoplasm Disease Stage American Joint Committee on Cancer Code."

Moreover, *in silico* analysis of protein expression was performed, using the publicly available Human Protein Atlas database (https://www.proteinatlas.org/), including tissue microarray (TMA) data from 11 TGCT cases, as well as distribution of protein expression among various tumor sites in the human body.

Patients and sample collection

The study includes retrospective TGCT samples from patients diagnosed and treated between 2005 and 2018 at the Portuguese Oncology Institute (IPO) of Porto. The study cohort full details are reported elsewhere.[2] Exclusion criteria were consultation-based cases, incomplete histological material and a history of extra-gonadal GCTs. All patients underwent surgery,

treatment, and follow-up at our institution. This study is part of a larger project on TGCTs, previously approved by the IPO Porto Ethics Committee (CES-IPO-12-018).

Patients' clinical files and pathology reports were reviewed, and all histological slides were classified according to the latest (2022) WHO Classification of Tumors of the Urinary System and Male Genital Organs. Follow-up data (progression and/or relapse events) was updated until November 2019. Tissue samples were obtained via radical inguinal orchiectomy, formalin-fixed, and paraffin-embedded (FFPE) for routine histopathological assessment.

Immunohistochemistry

Immunohistochemistry was performed on 4µm-thick sections. The protocol was performed with the monoclonal antibody anti-ENPP1/PC1 [EPR22262-22] in automated stainers used for diagnostic purposes at IPO Porto. Appropriate controls (liver, pancreas and placenta tissues) were included in each run.

Immunoexpression was assessed by an experienced uropathologist, considering both intensity and percentage of stained cells, in a combined score. Importantly, each histological subtype among mixed tumors was independently considered for immunoexpression analysis.

Statistical analysis

For statistical analysis, the highest expressed component in mixed tumors was considered. Statistical significance was set at a two-tailed *p-value* < 0.05. The Mann–Whitney U test and Kruskal–Wallis tests were used to compare continuous variables between two or more independent groups, with Bonferroni's correction applied for pairwise comparisons.

Results

***In silico* study - cBioportal data**

In silico analysis using the TCGA database showed no statistically significant differences in ENPP1 expression levels between SEs and NSs (p=0.3873, Figure 1A). However, when comparing all

tumor subtypes, ECs disclosed higher expression levels compared to SEs and to mixed tumors (p=0.0278 and p=0.0399, respectively, Figure 1B).

Concerning tumor stage, ENPP1 expression was significantly higher in stage II/III tumors compared to stage I (p=0.0149, Figure 1C). No statistically significant difference in ENPP1 expression was observed between pT1 and pT2/3 stages (p=0.0617, Figure 1D).

***In silico* study - Human Protein Atlas data**

Considering the Human Protein Atlas *in silico* analysis (immunohistochemistry performed on TMAs), ECs showed a higher proportion of cases with moderate intensity staining and with 25-75% stained cells when compared to SEs. Staining in SEs was overall lower and more heterogenous, including positive but also negative cases (Figure 2A-B).

Regarding ENPP1 expression across tumor models represented on Human Protein Atlas (Figure 2C), TGCTs were the 4th most highly expressing tumor, following breast, thyroid and hepatocellular carcinomas. Expression in other tumor types was overall low.

Cohort characterization

This study included a total of 63 TGCT patients. The clinicopathological characteristics of the cohort are summarized in Table 1. The median age at diagnosis was 29.5 years (interquartile range = 25-37 years).

Among the 63 patients, 42.8% had pure SE, while mixed tumors, pure EC, and pure TE accounted for 38.1%, 15.9%, and 3.2% of cases, respectively. CH and YST were only identified as components of mixed tumors. When considering each tumor component of mixed tumors as an independent sample, a total of 90 tumor samples were analyzed, consisting of 34.4% SE, 25.5% EC, 17.8% YST, 5.6% CH and 16.7% TE components. Most patients presented with stage I disease (44.4%).

Additionally, 17 metastatic TGCT cases were assessed. These included eight metastases in retroperitoneal lymph nodes classified as mature teratoma, five metastatic ECs (three to lymph nodes and two to the lungs), one metastatic SE to a lymph node, one metastatic CH to the brain, and two metastatic YST to the brain. Most metastases (15/17, 88%) emerged after treatment with cisplatin-based chemotherapy.

Finally, a series of normal testicular parenchyma was also included in the study, consisting of blocks representative of healthy testicular parenchyma (without evidence of TGCT or GCNIS) from patients undergoing orchiectomy for reasons other than a TGCT (median age of 55 years).

ENPP1 immunoexpression in primary tumors according to histologic subtype

Overall, there was no significant difference in ENPP1 expression between SE and NS patients (p=0.8849, Figure 3A). However, EC patients showed significantly higher levels of ENPP1 expression compared to TE patients (p=0.0455, Figure 3B).

Because ENPP1 expression was overall heterogeneous in mixed tumors (Figure 3B), we investigated ENPP1 expression per tumor subtype, considering each component within mixed tumors individually. In this "subtype-specific" analysis, SE components expressed significantly higher ENPP1 levels compared to TE (p=0.0009) and YST (p=0.0438) components. Also, EC components showed the highest levels of ENPP1 expression, being significantly higher than in CH (p=0.0027), YST (p<0.0001) and TE (p<0.0001). Also, YST components showed significantly higher expression levels compared to TE components (p=0.0031) (Figure 3C). Illustrative examples of ENPP1 expression in primary tumors are depicted in Figure 4.

ENPP1 immunoexpression and clinicopathological correlates

No significant differences in ENPP1 expression were found between stages I and II/III (p=0.4350, Supplementary Figure 1A). Notwithstanding overall higher levels in pT2/3 disease, the difference towards pT1 tumors did not reach statistical significance (p=0.4002, Supplementary Figure 1B).

Also, despite overall higher levels in patients which experienced disease recurrence, the difference did not reach statistical significance (p=0.3643, Supplementary Figure 1C).

ENPP1 immunoexpression in metastatic TGCTs and in healthy testicular parenchyma

ENPP1 expression was significantly higher in primary TGCTs compared to metastatic TGCTs (p<0.0001, Figure 5A). The highest expression (strong staining in 75-100% of cells) was found in a metastatic SE, without prior chemotherapy, followed by a YST metastatic to the brain (strong

staining in 1-25% of tumor cells) and an EC metastatic to a mediastinal lymph node (strong staining in 25-50% of tumor cells), corresponding to cisplatin-resistant disease.

Of note, we found no ENPP1 expression in germ cells of healthy testicular parenchyma, with only Leydig cells disclosing intense staining. Illustrative examples of ENPP1 expression in metastatic tumors and healthy testicular parenchyma are depicted in Figure 5B-E.

Discussion

ENPP1 is a type II transmembrane glycoprotein which has a role in the hydrolysis of different purine nucleotides in various different physiologic processes.[12] For instance, ENPP1 is highly expressed in the osteochondral compartment, where it displays homeostatic functions in regulating physiologic mineralization by regulating the balance between phosphate (Pi), a substrate of mineral deposition, and inorganic pyrophosphate (PPi), an inhibitor of mineralization by hydrolyzing cGAMP (cyclic (di-)nucleotides).[16] However, and of relevance for this work, ENPP1 also tilts the balance of adenosine triphosphate (ATP) / adenosine in conjunction with other components (CD38, CD39 and CD73), overall promoting an immunosuppressive tumor microenvironment [17]. This feature makes it an interesting subject of study in cancer, considering that immune surveillance evasion is a well-known hallmark of cancer.[18] Importantly, ENPP1 was frequently found to be overexpressed in local relapses and metastases in several tumor models, allowing tumors to escape from anti-tumor immune responses through STING activation, promoting fast oxidation of exported cGAMP.[11,12] Therefore, targeting ENPP1 shows promise as an anti-cancer strategy. Since no studies have assessed the expression of ENPP1 in TGCTs, thus far, we aimed to characterize its expression in a well-annotated cohort of primary and metastatic TGCT samples.

Extracellular ATP is hydrolyzed into adenosine through two distinct yet overlapping pathways within the tumor microenvironment. The canonical pathway involves the sequential enzymatic activity of CD39 and CD73, converting ATP to ADP, then to AMP, and finally to adenosine.[12,14] In contrast, the noncanonical pathway is mediated by CD38, a cell-surface ectoenzyme expressed in T cells, neutrophils, lymphocytes and monocytes, particularly under inflammatory conditions. CD38 catalyzes the hydrolysis of nicotinamide adenine dinucleotide (NAD^+) into ADP-ribose, which is further converted into adenosine monophosphate by ENPP1. CD73 then dephosphorylates AMP into adenosine and PPi.[12,13,15] This accumulation of extracellular

adenosine facilitated by ENPP1, in cooperation with CD39 and CD73, plays a crucial role in immune modulation, which makes ENPP1 an interesting target to investigate in a cancer setting. Specifically, adenosine exerts its effects through A2A receptors expressed on various immune cell populations.[12,19,20] Tumor cells with high ENPP1 expression induce significant tumor–immune remodeling by promoting polymorphonuclear myeloid-derived suppressor cell (PMN-MDSC) chemotaxis, reducing dendritic cell infiltration, increasing M2 macrophage polarization and enhancing regulatory $CD4^+$ T cell (Treg) activity.[12,21] Simultaneously, elevated adenosine levels suppress cytotoxic responses from natural killer (NK) cells and $CD8^+$ T cells, creating an immunosuppressive environment that hinders effective antitumor immunity [12,22].

ENPP1 expression has been assessed in other tumor models, although the number of studies looking into expression of this biomarker with potential for therapeutic targeting is limited (Supplementary Table 1) [16,23-31]. Studies suggest that measuring ENPP1 levels in primary breast cancer tissues could influence response to chemotherapy[32] and its overexpression could be also associated with bone metastases [16]. Moreover, ENPP1 was also shown to be highly expressed in gallbladder tumors, glioblastoma and lung cancer compared to normal tissues [25,31,33], and its expression was positively correlated with higher tumor grade, rate of progression and poorer prognosis, in line with its immunosuppressive role [23,26,34]. This is also in line with our findings of higher expression in EC, which is a proliferative tumor subtype with features of aggressiveness when compared to most SEs, or even to TE (which despite being resistant to chemotherapy, has a more quiescent biology). Li et al., in a study comprising 165 hepatocellular carcinomas, verified that exosome-associated ENPP1 could be a potential hepatocellular carcinoma biomarker, by affecting immune infiltration and therefore influencing prognosis [26].

In our cohort of 63 patients, ECs exhibited the highest ENPP1 protein immunoexpression, which is a consonant finding with the ENPP1 mRNA expression in the TCGA TGCT database. This was followed by SEs, which are commonly populated by a rich inflammatory background. Klein et al. analyzed inflammatory infiltrates associated with SEs [35], showing that a unique and complex immune microenvironment, comprising a mixture of T cells, B cells, pro-inflammatory cytokines, such as IL-1β, IL-6, IL17a and tumor necrosis factor (TNF) α and macrophages, contributes to tumorigenic growth [7,35-37]. Our results with ENPP1 expression suggest that ENPP1 could be an additional player in mediating the immune microenvironment in these tumors, also promoting tumor growth. The heterogeneity in expression among SEs may be explained, however, by the two subtypes of SEs proposed by Medvedev et al, one showing differences regarding immune response, metabolism and DNA damage response [38].

Furthermore, studies have showed that ENPP1 is overexpressed in glioma stem-cells (GSC) [24,33], being important for maintaining their undifferentiated state and proliferation. Bageritz et al. discovered that suppressing ENPP1 in cultured GSCs not only leads to a broad reduction in the expression of genes associated with stemness, but also promotes differentiation towards an astrocytic lineage, disrupts the formation of neurospheres, increases cell death, causes cells to accumulate in the G1/G0 phase of the cell cycle and enhances sensitivity to chemotherapy.[24] In this study, comprehensive gene expression profiling and analysis of nucleoside and nucleotide levels showed that ENPP1 knockdown interferes with purine and pyrimidine metabolism, indicating a connection between ENPP1 activity and the regulation of nucleoside–nucleotide balance in GSCs. The observed phenotypic changes in ENPP1-deficient GSCs are believed to be the result from reduced transcriptional activity of transcription factor E2F1. Overall, these findings demonstrated that ENPP1 functions upstream of E2F1 and is essential for sustaining the undifferentiated, proliferative state of GSCs in vitro.[24]

Also, high levels of ENPP1 in lung cancer were associated with favoring cancer stem cell traits, promoting a more aggressive behavior [25]. Min Hu et al demonstrated that ENPP1 plays a key role in both initiating and sustaining epithelial–mesenchymal transition (EMT) characteristics and cancer stem cell (CSC) traits in lung cancer.[25] According to this study, ENPP1-knockdown in HCC827 and A549 lung cancer cell lines culminates in suppressed colonogenic formation, anchorage-independent growth in vitro and tumorigenicity in vivo. They also found that ENPP1-knockdown results in lowered expression of CSC markers such as ABCG2, SOX2, NANOG and CD44. It also reverses EMT features induced by TGFβ, including reduced cell migration, restoration of E-cadherin expression and decreased vimentin levels.[25]

In our work, the highest expression of ENPP1 was found in EC and SE, which may be related to their pluripotent potential [39], as opposed to the lowest expression in TEs, the most differentiated and somatically-prone TGCT subtype. This is also in line with the concept of EC representing the neoplastic counterparts of embryonic stem cells. Indeed, Human Protein Atlas expression data across several tumor types shows that ENPP1 has a low expression in most models, with TGCTs however constituting an exception, being on the high end of expression. Therefore, ENPP1 expression may accompany pluripotent-like features, pointing that their role in reprogramming may be worth exploring in future works, especially considering previous studies which disclosed the impact of immune microenvironment specific populations on these processes [40].

While in TCGA data we found a significant upregulation of ENPP1 in stage II/III cases compared to stage I disease, this could not be verified in our own patient cohort. Differences in

composition of both cohorts may help explain the discrepancy, and mostly differences in methodology, since TCGA used mRNA expression obtained by RNA sequencing, while we performed immunohistochemistry and further assessed a combined score in each specific component within mixed tumors. Immunohistochemistry is a reliable method for screening for biomarker expression in tumor sections, with rapid turnaround time, being ideal for assessing potential targets of therapy, such as PD-L1 of HER-2. Therefore, our results support that ability to score the expression of ENPP1 in TGCTs, possibly indicating patients which may benefit from ENPP1 inhibitors, in the future.

TGCTs are overall very sensitive to cisplatin-based chemotherapy.[2,41] However, systemic treatments have been associated with many serious adverse effects which impact patients' quality of life [35,37,41]. In our results, the monoclonal antibody Anti-ENPP1/PC1 antibody [EPR22262-22] had no expression in germ cells of normal testis, which argues in favor of specificity for neoplastic germ cells (TGCT cells). This finding, accompanied by higher expression levels (membranous expression) particularly in EC and SE, gives the first supportive data that targeting ENPP1 with already available ENPP1 inhibitors could result in anti-tumor effects, potentially with modest toxicity, supporting additional *in vitro* and *in vivo* studies. Therefore, it may further represent a therapeutic opportunity for targeted therapy in TGCTs. While expression in metastatic tumors was overall low, most consisted of post-chemotherapy residual TE masses, which are cured by resection. Strong expression in a SE metastasis (chemo-naïve), as well as in cisplatin-resistant cases (which have poor prognosis and lack additional effective therapies) may however indicate a putative role in this setting, requiring validation in additional tumor samples.

Limitations of our work include its retrospective nature and relatively limited number of cases included, which is expected given the overall low frequency of TGCTs. However, all cases were evaluated by the same pathologist with experience in TGCTs, histology and clinical charts were reviewed and detailed according to most recent classifications, and all patients were treated in the same institution by the same experienced multidisciplinary team. Moreover, results were further validated using publicly available databases (TCGA and Human Protein Atlas), strengthening our observations.

Conclusions

To conclude, our results highlight frequent and intense ENPP1 expression in ECs, followed by SEs, in comparison to lower levels in other more differentiated TGCTs. Also, ENPP1 expression was exclusively found in TGCT tissue, and not in healthy germ cells. At present, with the recent advances in ENPP1 inhibitors, we report the first expression data of ENPP1 in TGCT primary and metastatic tissues, supporting the investigation of ENPP1 inhibitors effectiveness in this tumor model.

A – Parecer da Comissão de Ética para a Saúde do IPO Porto

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DECLARAÇÃO

Júlio Manuel Ramos Maia de Oliveira, Presidente do Conselho de Administração, declara que Instituto Português de Oncologia do Porto Francisco Gentil, E.P.E., NIPC 506362299, autoriza e acolhe o trabalho académico "Expression of ENPP1 in testicular germ cell tumors: exploring its role in the pathobiology of distinct histotypes and in prognosis" do estudante **Miguel Bernardo Oliveira Alves**, da componente curricular "Dissertação/Projeto/Estágio", do Mestrado Integrado em Medicina, do Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto, cujo orientador é João Pedro da Silva Machado Lobo.

Ademais, afiança que serão o cumpridas as exigências legais em matérias de ética, de proteção de dados e de integridade científica, e que emitirá uma declaração similar após a conclusão do trabalho.

Dr. Júlio Oliveira
Presidente do Conselho de Administração
IPO Porto

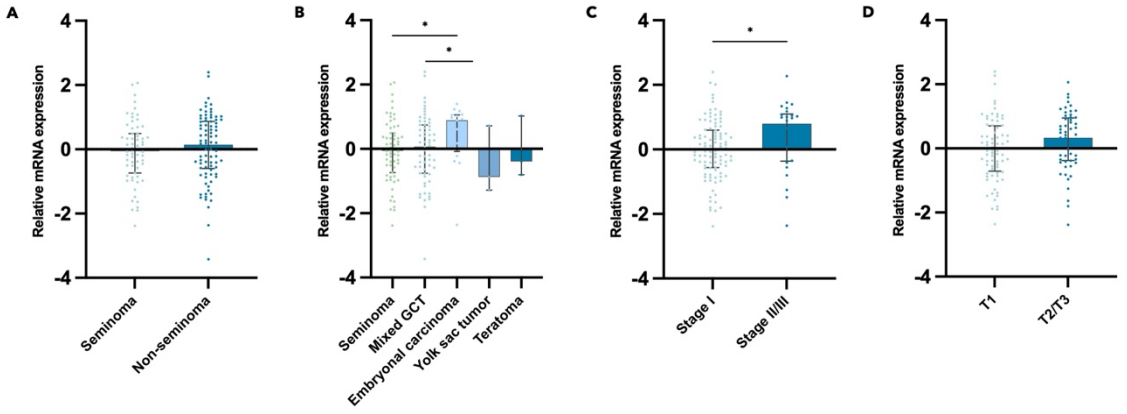


Figure 1. Scatter dot plots of relative ENPP1 mRNA expression from in silico analysis of TCGA database. Comparisons between **(A)** seminomas and non-seminomas, **(B)** different histological subtypes (seminoma, mixed germ cell tumor, embryonal carcinoma, yolk sac tumor, and teratoma), **(C)** tumor stage I and II/III, **(D)** pT1 stage and pT2/pT3, considering the median and interquartile range values. **p-value* (SE vs EC) = 0.0278; **p-value* (Mixed vs EC) = 0.0399; **p-value* (I vs II) = 0.0126. EC: embryonal Carcinoma; SE, Seminoma.

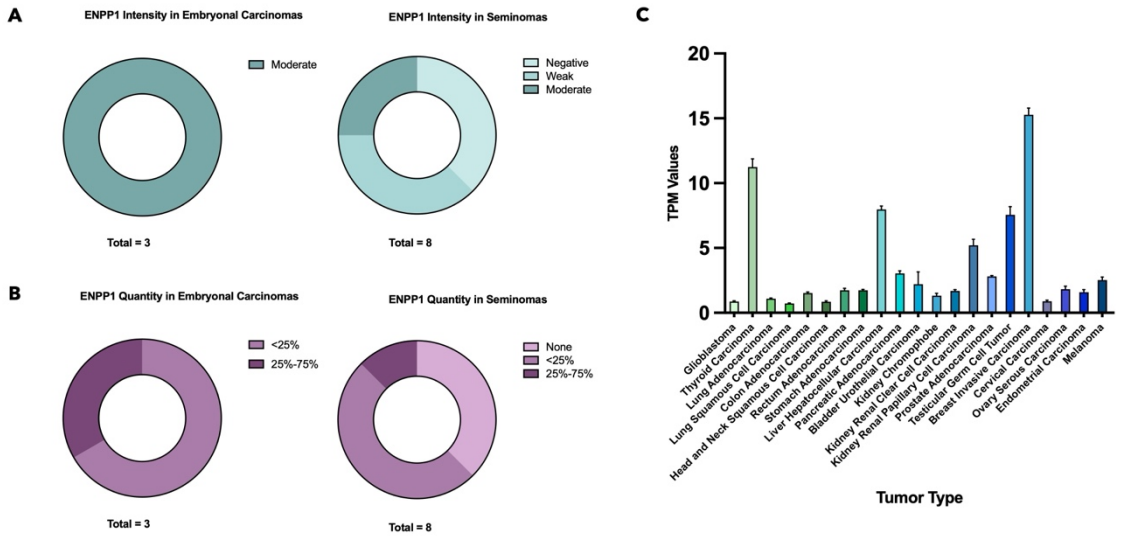


Figure 2. In silico immunohistochemistry analysis from Human Protein Atlas data. (A) ENPP1 intensity in TMAs from ECs and in SEs, **(B)** ENPP1 quantity in TMAs from ECs and in SEs. **(C)** ENPP1 gene expression data in several tumor models. ECs, embryonal carcinomas; SEs, seminomas; TMAs, tissue micro arrays.

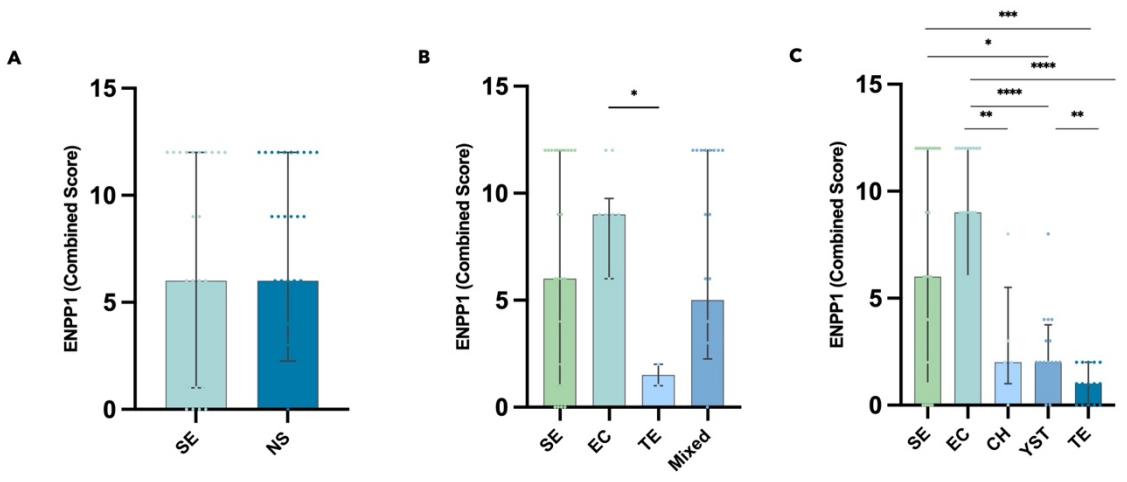


Figure 3. Scatter dot plots of ENPP1 immunoexpression combined score in IPO Porto cohort. Comparisons between **(A)** Seminomas and non-seminomas, **(B)** Different histological subtypes (SE, EC, TE and mixed) **(C)** Individual tumor components, considering the median and interquartile range values. CH, choriocarcinoma; EC, embryonal carcinoma; NS, non-seminoma; SE, seminoma; TE, teratoma; YST, yolk sac tumor. **p-value* (EC vs TE) = 0.0455, **p-value* (YST vs SE) = 0.0438, ***p-value* (CH vs EC) = 0.0027, ***p-value* (TE vs YST) = 0.0031, ****p-value* (SE vs TE) = 0.0009, *****p-value* (EC vs YST) < 0.0001, *****p-value* (EC vs TE) < 0.0001.

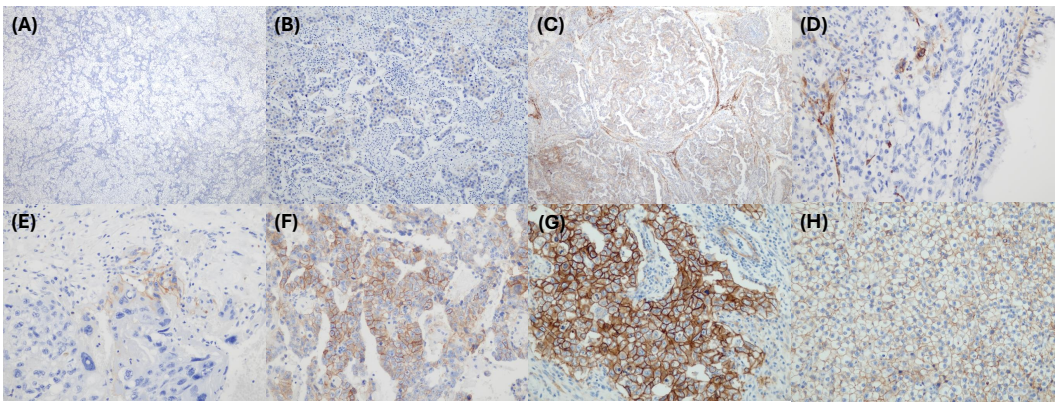


Figure 4. Illustrative examples of immunoexpression of ENPP1 in different histological subtypes. Null expression **(A)** and low expression **(B)** in seminomas. Strong and diffuse staining in embryonal carcinoma **(C)**. Immunoexpression observed in yolk sack tumor component of a mixed tumor, with adjacent negative teratoma component **(D)**. Focal positivity on the choriocarcinoma element of a mixed tumor **(E)** and intermediate expression in embryonal carcinoma **(F)**. Diffuse staining in an embryonal carcinoma component of a mixed tumor **(G)** and in a seminoma **(H)**, with different intensities.

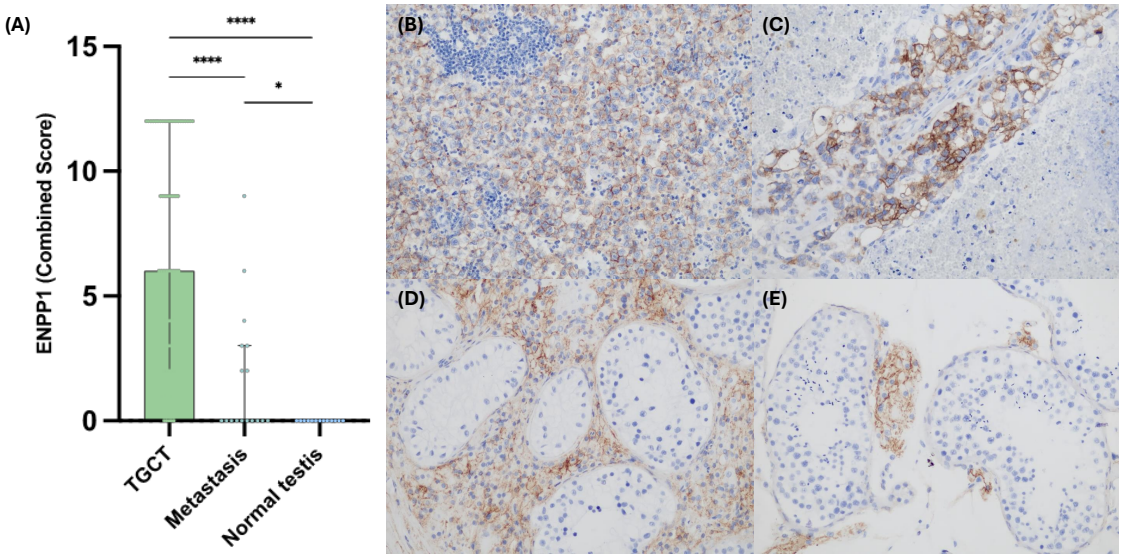
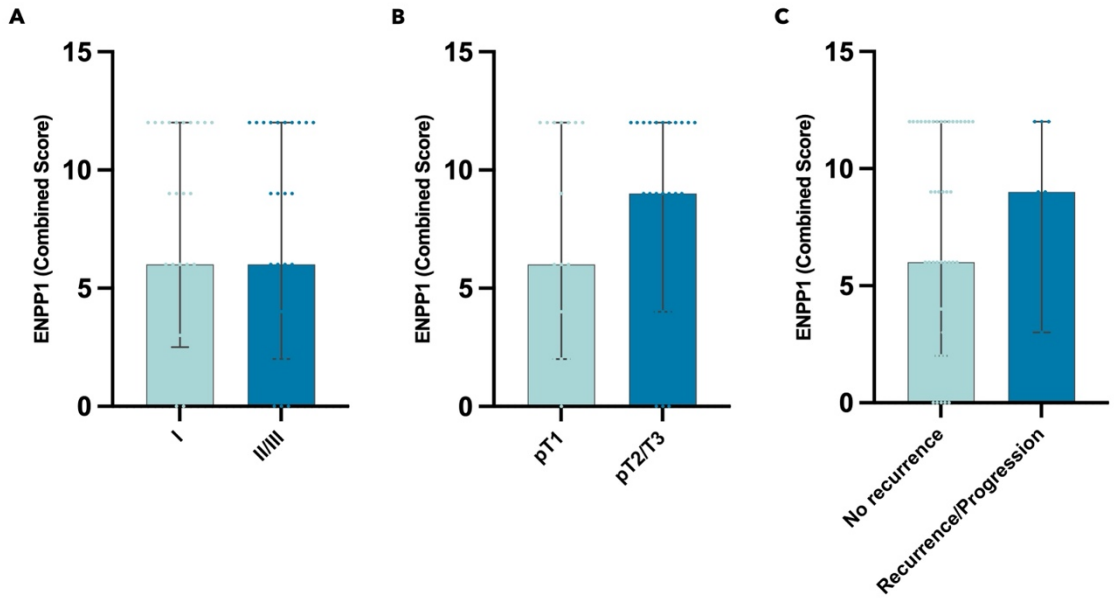


Figure 5. Immunoexpression of ENPP1 in primary TGCTs compared to metastases and testicular parenchyma. (A) Scatter dot plots of ENPP1 immunoexpression (combined score) in IPO Porto cohort for primary TGCTs, metastases and normal testis, considering median values and interquartile range values. TGCT, Testicular Germ Cell Tumor. **p-value* (TCGT vs metastasis) < 0.0001, **p-value* (TCGT vs normal testis) < 0.0001. Strong and diffuse ENPP1 expression in a seminoma lymph node metastasis **(B)** and strong but focal expression in a yolk sac tumor brain metastasis **(C)**. In normal testicular parenchyma, ENPP1 shows strong expression in the Leydig cells in between the seminiferous tubules, but complete lack of immunoexpression in germ cells (**D** and **E**).



Supplementary Figure 1: Scatter dot plots of ENPP1 immunoexpression combined score in IPO Porto cohort according to clinicopathological data. Comparisons between **(A)** tumor stage I and II/III, **(B)** pT1 stage and pT2/pT3, **(C)** tumor recurrence or non-recurrence, considering the median and interquartile range values.

Table 1. Clinicopathological data of the study cohort (primary tumors).

	Patient cohort (n=63) **Tumor samples (n=90)**
Age median [IQR], years	29.5 [25-37]
Laterality n, (%)	
Right	37/63 (58.73)
Left	26/63 (41.27)
Histologic subtypes n, (%)	
Pure SE	27/63 (42.86)
Pure EC	10/63 (15.87)
Pure TE	2/63 (3.17)
Mixed tumor	24/63 (38.10)
Tumor components n, (%)	
SE	31/90 (34.44)
EC	23/90 (25.55)
TE	15/90 (16.67)
YST	16/90 (17.78)
CH	5/90 (5.56)
Largest nodule size [cm (median, IQR)]	4.7 (2.8-7)

Rete testis invasion n, (%)	
Absent	27/61 (44.26)
Present	34/61 (55.74)
Vascular invasion n, (%)	
Absent	28/63 (44.44)
Present	35/63 (55.56)
Stage n, (%)	
I	28/63 (44.44)
II	17/63 (26.99)
III	18/63 (28.57)
Relapse/Progression n, (%)	
Absent	54/63 (85.71)
Present	9/63 (14.29)
Preoperative AFP n, (%)	
Elevated	25/62 (40.32)
Within normal range	37/62 (59.68)
Preoperative β-HCG n, (%)	
Elevated	38/62 (61.30)
Within normal range	24/62 (38.70)

Vital Status at last follow-up n, (%)	
A-NED	59/63 (93.65)
DFD	3/63 (4.76)
AWD	1/63 (1.59)
IGCCCG prognosis group n[a], (%)	
Good	24/35 (68.57)
Intermediate	4/35 (11.43)
Poor	7/35 (20.00)

Abbreviations: AFP, alpha-fetoprotein; A-NED, alive with no evidence of disease; AWD, alive with disease; CH, choriocarcinoma; DFD, died from disease; EC, embryonal carcinoma; IGCCCG, International Germ Cell Cancer Collaborative Group; IQR, interquartile range; SE, seminoma; TE, teratoma; YST, yolk sac tumor; *β*-HCG, beta subunit of human chorionic gonadotropin.

[a] For patients presenting with metastases at diagnosis.

Supplementary Table 1. Review of the literature regarding ENPP1 expression in different tumor models

Tumor model Sample size	**Methodology**	**Main findings**	**Reference**
Breast cancer 26 luminal-type cancer tissues + 9 normal tissues	qRT-PCR	Elevated ENPP1 expression in breast cancer patients. Value as biomarker for predicting malignant potential and responsiveness to chemotherapy	Takahashi, 2015 [32]
Breast cancer 2 triple-negative aggressive syngeneic breast cancer cell lines, ANV5 and 4T1	IHC	CTCs leverage the ENPP1/HP axis to drive local recurrence after surgery and radiation by suppressing myeloid suppressor cells, disrupting tumor engraftment and reducing immunosuppression, highlighting a promising therapeutic target	Córdoba, 2022 [28]
Gallbladder cancer Seeded cells in 24-well plates	IF staining WB Flow cytometry analysis Electrochemical measurements	ENPP1 integration with EpCAM enhanced cytosensor specificity for CTC detection and adapted to assess chemoresistance in gallbladder cancer	Zhang, 2023 [31]
Bile duct carcinoma 41 samples	WB IHC	ENPP1 and ENPP3 presented distinct localization patterns by IHC, apical cytoplasmic and apical plasma membrane, respectively. ENPP3 expression was significantly elevated in tumor tissues compared to surrounding tissues, while ENPP1 showed no such increase by WB	Yano, 2004 [30]
Astrocytic Brain Tumors 20 glioblastoma multiforme; 10 astrocytoma**;** 9 anaplastic astrocytoma; 2 pilocytic astrocytoma**;** 5 controls	IHC	ENPP1 protein is present in human astrocytic brain tumors, with its expression correlating with tumor grade, suggesting a potential role in tumor progression and classification	Aerts, 2011 [23]
Glioblastoma 12 samples	PCR WB Microscopic detection of apoptosis	ENPP1 is highly expressed in GSCs compared to normal brain and neural stem cells, possibly regulating purine and pyrimidine metabolism. This results from reduced E2F1 transcriptional activity, highlighting ENPP1 as essential for maintaining GSCs in an undifferentiated, proliferative state	Bageritz, 2014 [24]
Hepatocellular carcinoma 165 samples	IHC	Exosome-associated ENPP1 is downregulated in liver hepatocellular carcinoma and correlates with prognosis, and may influence progression by affecting immune infiltration	Li, 2022 [26]
Lung cancer (n=36 pairs)	WB IHC	ENPP1 was upregulated in most human lung tumor tissues compared to adjacent normal tissues. Dysregulated ENPP1 enhances lung cancer malignancy by promoting cancer stem-cells traits and EMT-like phenotypes	Hu, 2019 [25]
Oral Squamous Cell Carcinoma 96-well culture plates	IB IHC IF	ENPP1 downregulation inhibits OSCC growth, induces apoptosis, and triggers autophagic cell death	Ma, 2024 [27]

Ovarian carcinoma 21 normal ovarian epithelial tissues, 32 ovarian serous cystadenoma and 36 HGSOC	IHC WB qRT-PCR	Elevated ENPP1 expression may contribute to HGSOC development, indicating rapid progression and poor prognosis, with no correlation between ENPP1 expression and chemosensitivity	Wang, 2019 [34]
Breast carcinoma Representative cell lines	qRT-PCR IHC	ENPP1 overexpression enhanced bone tumor formation, confirmed by radiography and histology	Lau, 2013 [16]

Abbreviations: CTCs, Circulating Tumor Cells, EMT, Epithelial–Mesenchymal Transition; GSCs, Glioblastoma Stem-like Cells, HGSOC, High-Grade Serous Ovarian Cancer; IB, Immunoblotting; IF, Immunofluorescence; IHC, Immunohistochemistry; OSCC, Oral Squamous Cell Carcinoma; qRT-PCR, Quantitative Real-Time Polymerase Chain Reaction; WB, Western Blot.

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