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Original Article

EVALUATION OF THE OVARIANTAG BIOMARKER PANEL'S APPLICABILITY IN DETECTING KRUKENBERG TUMORS

AVALIAÇÃO DA APLICABILIDADE DO PAINEL DE BIOMARCADORES OVARIANTAG NA DETECÇÃO DE TUMORES DE KRUKENBERG

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RESUMO

O tumor de Krukenberg é um adenocarcinoma metastático de ovário, geralmente originado do trato gastrointestinal, especialmente do câncer gástrico. Ele representa uma proporção significativa dos tumores ovarianos, sendo mais comum em populações asiáticas. Esses tumores

normalmente afetam mulheres mais jovens e apresentam um prognóstico desfavorável. O diagnóstico é desafiador devido às semelhanças clínicas com outros tipos de câncer de ovário, exigindo uma abordagem multimodal, incluindo exames de imagem, análise histopatológica e marcadores bioquímicos. O CA-125 pode ser útil no diagnóstico, mas apresenta baixa especificidade. A identificação de biomarcadores moleculares específicos para os tumores de Krukenberg poderia melhorar o diagnóstico e o tratamento. Este estudo retrospectivo investigou o uso do painel de biomarcadores OvarianTag®, desenvolvido para avaliar o prognóstico do câncer epitelial de ovário (CEO), com o objetivo de diferenciar entre cânceres primários de ovário e tumores metastáticos de outros sítios, como aqueles de origem gastrointestinal. A pesquisa analisou amostras de tumores de Krukenberg e amostras de câncer primário de ovário, utilizando RNA extraído dos tecidos tumorais e análise de expressão gênica por RT-qPCR. Apesar da análise genética, o algoritmo OvarianTag® não conseguiu distinguir com precisão os tumores de Krukenberg dos cânceres primários de ovário. A expressão dos genes TNFSRF10B, TNFSRF10C e CASP8 não mostrou diferenças significativas entre os grupos, e o algoritmo falhou em classificar corretamente os tumores de Krukenberg. Esses resultados sugerem que o OvarianTag® não é eficaz para essa aplicação específica, ressaltando a necessidade de novos biomarcadores para melhorar o diagnóstico de tumores ovarianos metastáticos.

Palavras-chave: Tumor de Krukenberg, Câncer de ovário, Biomarcadores, Estudo retrospectivo, OvarianTag®, Aprendizado de máquina.

ABSTRACT

Krukenberg tumor is a metastatic ovarian adenocarcinoma, typically originating from the gastrointestinal tract, especially gastric cancer. It accounts for a significant proportion of ovarian tumors, being more common in Asian populations. These tumors usually affect younger women and have a poor prognosis. Diagnosis is challenging due to clinical similarities with other ovarian cancers, requiring a multimodal approach, including imaging tests, histopathological analysis, and biochemical markers. CA-125 may be helpful in diagnosis, but it lacks specificity. Identifying molecular biomarkers specific to Krukenberg tumors could improve diagnosis and treatment. This retrospective study investigated the use of the OvarianTag® biomarker panel, developed to assess the prognosis of epithelial ovarian cancer (EOC), to differentiate between primary ovarian cancers and metastatic tumors from other sites, such as those of gastrointestinal origin. The research analyzed Krukenberg tumor samples and primary ovarian cancer samples, utilizing RNA extracted from tumor tissues and gene expression analysis by RT-qPCR. Despite genetic analysis, the OvarianTag® algorithm could not accurately distinguish Krukenberg

tumors from primary ovarian cancers. The expression of genes TNFSRF10B, TNFSRF10C, and CASP8 showed no significant differences between the groups, and the algorithm failed to classify Krukenberg tumors correctly. These results suggest that OvarianTag® is not effective for this specific application, highlighting the need for new biomarkers to improve the diagnosis of metastatic ovarian tumors

Keyword: *Krukenberg tumor, Ovarian cancer, Biomarkers, Retrospective study. OvarianTag®, Machine learning.*

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1. INTRODUCTION

The Krukenberg tumor is a metastatic adenocarcinoma of the ovary, most commonly originating from the gastrointestinal tract, particularly gastric cancer. It accounts for a significant proportion (1%-21%) of ovarian tumors, with a higher frequency in Asian populations due to the increased prevalence of gastric cancer. These tumors typically present as bilateral lesions and manifest in solid, cystic, or mixed forms. Unlike primary ovarian cancers, Krukenberg tumors often affect younger women and are associated with a poor prognosis. Surgical resection, while palliative, may provide some survival benefits [(1)].

The clinical management of Krukenberg tumors poses considerable challenges due to diagnostic uncertainty, reduced responsiveness to systemic therapies compared to other metastatic cancers, and the often debilitating symptoms. Timely and accurate diagnosis is essential for optimizing treatment and improving outcomes[(2)]. Diagnosis involves a multimodal approach integrating imaging techniques, histopathological analysis, and biochemical markers. Ultrasound is typically the first step in identifying ovarian masses, followed by computed tomography for a more detailed anatomical assessment. Histopathological confirmation is obtained via biopsy, revealing characteristic mucin-producing signet ring cells and ovarian stromal involvement. Elevated serum CA-125 levels can further support the diagnosis and assist in monitoring treatment efficacy. If a Krukenberg tumor is found, endoscopic evaluation is recommended to locate the primary gastrointestinal malignancy [(3)].

One of the main challenges in diagnosing Krukenberg tumors is their nonspecific clinical presentation, which often resembles primary ovarian cancers or benign conditions, leading to diagnostic delays. The aggressive nature of these tumors and their tendency to present at advanced stages complicates early detection. Imaging can show bilateral ovarian involvement, but distinguishing Krukenberg tumors from

primary ovarian cancers requires histopathological confirmation. Furthermore, although CA-125 is often elevated, it is not specific to Krukenberg tumors, as many ovarian malignancies share similar findings. The need to locate the primary malignancy in the gastrointestinal tract adds complexity to the diagnosis, necessitating thorough endoscopic and radiological assessments [4].

Therefore, the research and validation of novel molecular biomarkers are crucial to enhancing the diagnosis and treatment of Krukenberg tumors. The identification of specific biomarkers would not only enable more accurate and timely diagnosis and allow for better differentiation between Krukenberg tumors and other ovarian malignancies [5].

OvarianTag® is a biomarker panel based on gene expression developed to assess the prognosis of patients with epithelial ovarian cancer (EOC). The OvarianTag® panel is designed to aid in prognostic discrimination by analyzing the aggressiveness of the disease and the causes of relapse in EOC patients. The test is based on RNA extraction from ovarian tissues, such as normal tissues, serous cystadenoma, and EOC tumors, followed by quantitative PCR (qRT-PCR) gene expression analysis. Using machine learning algorithms, the OvarianTag® panel classifies patient data and identifies prognostic molecular markers [6]. The present study aims to investigate whether the OvarianTag® panel could differentiate primary ovarian cancers from tumors originating in other sites that metastasize to the ovary. This is a significant clinical challenge for determining the appropriate treatment of Krukenberg tumors.

2. METHODOLOGY

This retrospective cohort study analyzed paraffin-embedded ovarian tumor samples obtained from the biobank of the Instituto Mário Penna. Clinical and pathological data were collected, followed by histological evaluation, RNA extraction and quantification, and gene expression analysis using RT-qPCR. The OvarianTag® algorithm was applied to classify tumors based on molecular profiles. The institutional ethics committee approved all procedures. Detailed descriptions of each methodological step are provided in the following sections.

2.1 Study Cohort and Sample Characteristics

The samples used in this study were sourced from the biobank of the Instituto Mário Penna. Two paraffin blocks were selected from patients diagnosed with Krukenberg tumors, based on specific clinical and pathological criteria, focusing on

metastatic cases confirmed by immunohistochemistry. The patients, aged 36 and 73 years, presented with a variety of symptoms, including abdominal pain, bloating, changes in bowel habits, nausea, weight loss, and ascites. Imaging studies, such as computed tomography and ultrasound, revealed large, often bilateral ovarian masses, characteristic of metastatic tumors, with some showing solid-cystic features and associated peritoneal and lymph node metastases. Elevated levels of tumor markers, such as CA-125 and CEA, further supported the diagnosis of ovarian metastasis. Given the advanced stage of the disease, most patients were indicated for palliative treatment, with a median survival of less than two years, consistent with the aggressive nature of Krukenberg tumors.

Additionally, 18 samples were selected from patients diagnosed with primary ovarian câncer. The age at diagnosis ranged from ≤50 years in 7 patients (38.9%) to >50 years in 11 patients (61.1%), with a mean age of 60 years. The histological subtypes included high-grade serous carcinoma in 10 patients (55.5%), high-grade serous papillary carcinoma in 4 patients (22.2%), high-grade serous papillary cystadenocarcinoma in 1 patient (5.6%), and high-grade serous adenocarcinoma in 3 patients (16.7%). Clinical staging, according to the FIGO classification, showed that six patients (33.3%) were diagnosed at early stages (I-II), while 12 patients (66.7%) presented with advanced-stage disease (III-IV).

Control samples were obtained from healthy ovaries of patients over 18 years of age, who underwent ovarian removal as part of surgery for non-neoplastic conditions. Histopathological examination confirmed the absence of neoplasms.

All sample donors voluntarily participated in the study, providing informed consent for using their biological material in research. The study was approved by the Ethics Committee of the Instituto Mário Penna (CAAE: 70737223.0.0000.5121).

2.2 Tissue Sectioning and Staining

The surgically excised tumor specimens were carefully fragmented, fixed in formalin (10% neutral buffered formalin) for 24 hours, and then embedded in paraffin using standard tissue processing techniques. The paraffin blocks were sectioned into 3 µm-thick slices using a rotary microtome (HM 340E, UK). The resulting tissue sections were mounted onto glass slides and stained using the Hematoxylin and Eosin (H&E) staining protocol. Briefly, the sections were deparaffinized in xylene and rehydrated through graded alcohols before being stained with Hematoxylin (NEON, USA) for 5 minutes, followed by eosin (Sigma-Aldrich, USA) for 2 minutes. The slides were then dehydrated and mounted under a light microscope for histopathological evaluation.

2.3 RNA extraction

For RNA extraction, paraffin-embedded tumor samples were sectioned into 10 µm-thick slices using a microtome (Microtome HM 340E, UK). Excess paraffin was carefully removed using a fine brush. RNA extraction was performed using the EZ2 Connect System (Qiagen, Germany), utilizing the EZ2 AllPrep FFPE Kit (Qiagen, Germany). According to the manufacturer's protocol, tissue samples were incubated with a lysis buffer and processed through the automated EZ2 Connect platform. The system employs magnetic beads to selectively bind and purify RNA, ensuring high yield and purity by removing contaminants such as paraffin and protein. The extracted RNA was eluted in RNase-free water and stored at -80°C until further analysis.

2.4 RNA quantification

RNA concentration and quality were assessed using the Qubit™ 4 Fluorometer (Thermo Fisher Scientific, USA), combined with the Qubit RNA HS Assay Kit (Thermo Fisher Scientific, USA), following the manufacturer's protocol. A working solution was prepared by combining 199 µL of buffer and 1 µL of reagent provided by the kit, which was vortexed to ensure homogeneity. 198 µL of the working solution was mixed with 2 µL of RNA extract for each sample. The fluorescence emitted by the dye bound to the RNA was measured at an excitation of 485 nm and emission of 528 nm, allowing precise quantification of RNA concentration.

2.5 RT-qPCR Execution and Analysis

The molecular analysis of the extracted RNA was performed through reverse transcription followed by quantitative PCR (RT-qPCR). TaqMan probes specific to the *TNFSRF10B* (TRAIL R2), *TNFSRF10C* (TRAIL R3), and *CASP8* pathways were used, with *TBP* (TATA-box binding protein) as the endogenous reference gene for normalization. RNA samples were first treated with DNase to remove any contaminating genomic DNA. The reverse transcription was carried out using the RNA as template. Subsequently, qPCR amplification was performed using the TaqPath™ qPCR Master Mix (Thermo Fisher Scientific, USA), according to the manufacturer's instructions.

Controls were included in each reaction to ensure the specificity and reliability of the results. Negative controls without reverse transcriptase (No RT) were used to verify the absence of genomic DNA contamination. Additionally, for each gene of interest (*TNFSRF10B, TNFSRF10C*, and *CASP8*), a negative control reaction (NTC)

was also set up. This reaction contained all reagents but excluded the RNA sample, confirming that no non-specific amplification occurred. All reactions were performed in triplicate to ensure reproducibility and accuracy.

The RT-qPCR reactions were conducted under the following conditions: an initial incubation at 50°C for 2 minutes, followed by 20 seconds at 95°C to activate the enzyme, and 45 amplification cycles consisting of 3 seconds at 95°C (denaturation) and 30 seconds at 60°C (annealing and extension). These conditions allowed for the efficient amplification of cDNA, facilitating accurate quantification of gene expression levels. The cycle threshold (CT) values were recorded, and relative expression was calculated using the ΔΔCt method, normalizing against the TBP reference gene.

2.6 Comparative analysis using the OvarianTag® Algorithm

The comparative analysis was performed using the OvarianTag® algorithm, a computational tool developed by the Oncotag group that evaluated gene expression data associated with ovarian cancer. This algorithm employs advanced predictive models based on gene expression profiles, enabling the accurate classification of tumor samples and providing a robust approach to tumor prognosis [6].

The CT values, obtained through gene analysis using the RT-qPCR technique, were input into OvarianTag® to stratify the samples into primary or metastatic ovarian tumors.

3. RESULTS

The results described below encompass histopathological findings, gene expression levels, and the performance of the classification algorithm applied to the tumor samples.

3.1 Histopathological analysis of tumor samples

The histological analysis of the tumor samples, after sectioning and staining with H&E, revealed the presence of signet-ring cells. These cells were identified under optical microscopy, where the nucleus was pushed to the cell's periphery due to the presence of cytoplasmic mucin. The distinct morphological features of signet-ring cells, characteristic of Krukenberg tumors, were observed.

Microscopic images of these findings are shown in Figure 1, illustrating the typical appearance of ovarian tumors with signet-ring cells.

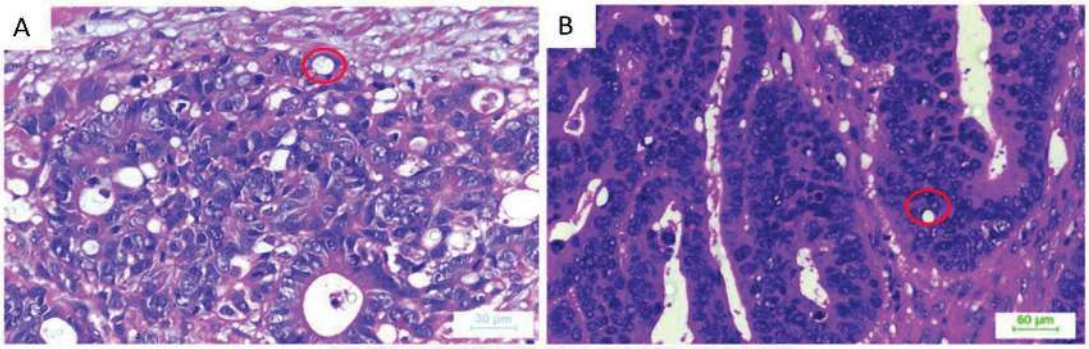


Figure 1: Optical microscope images of ovarian tumor sections stained with Hematoxylin and Eosin (H&E). Panels A and B show the tissue samples from patients with Krukenberg tumors 1 and 2 at 40x magnification. In all panels, the red circle highlights cells suggestive of signet-ring cell morphology, characterized by a peripheral nucleus and a mucin-filled cytoplasm, which appears white. No characteristic structures of healthy ovarian tissue, such as the capsule, germinal epithelium, cortex, tunica albuginea, or follicles, are identified, as the samples consist of tumor tissue.

3.2. Comparison of TNFSRF10B, TNFSRF10C, and CASP8 gene expression levels in Krukenberg tumors and primary ovarian tumors

A comparative analysis of the relative expression levels between the groups of patients with Krukenberg tumors and those with primary ovarian tumors did not reveal statistically significant differences for any of the three genes evaluated: *TNFSRF10B*, *TNFSRF10C*, and *CASP8* (Figure 2).

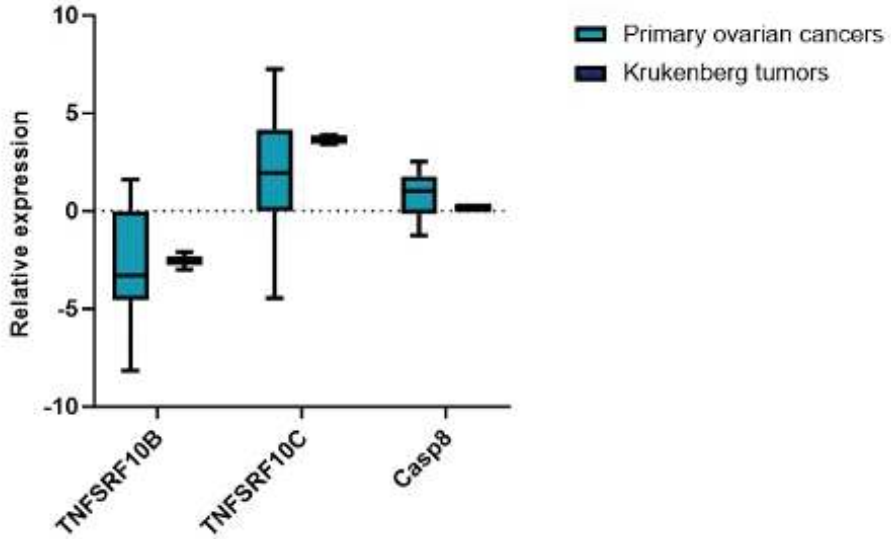


Figure 2: Relative expression of the *TNFSRF10B*, *TNFSRF10C*, and *CASP8* genes in patients with primary ovarian cancer and Krukenberg tumors. Gene quantification was performed using the 2-ΔΔCt method with *TBP* as the reference gene. Statistical analysis was conducted using the unpaired Student's t-test.

3.3. Comparison of *TNFSRF10B*, *TNFSRF10C*, and *CASP8* gene expression levels in Krukenberg tumors and primary ovarian tumors

Of the 20 samples analyzed, 18 were diagnosed with primary ovarian cancer, and two were identified as Krukenberg tumors — all were classified as primary ovarian cancer, as evidenced by the confusion matrix (Table 1), highlighting the algorithm's inability to correctly classify Krukenberg tumors.

Table 1: Confusion Matrix of the Classification of Primary Ovarian Cancer and Krukenberg Tumors Using the Ovariantag Algorithm

Actual / Predicted	Predicted Primary Ovarian Cancer	Predicted Krukenberg Tumor
Actual Primary Ovarian Cancer	18 (True Positive)	0 (False Negative)
Actual Krukenberg Tumor	2 (False Positive)	0 (True Negative)

4. DISCUSSION

The analysis of the results obtained in this study reveals important considerations about the applicability of the OvarianTag® biomarker panel in differentiating between primary ovarian tumors and metastatic tumors, specifically Krukenberg tumors. The accurate identification of this type of metastatic neoplasm, often originating from the gastrointestinal tract, is essential due to its aggressiveness, high lethality, and late diagnosis, in addition to the need for multiple complementary tests, such as CEA and CA-125 measurements, biopsies, and immunohistochemical analyses [7,1].

The histopathological analysis results evidenced signet-ring cells, a classic morphological feature of Krukenberg tumors, reinforcing the importance of microscopic evaluation for the identification of this neoplasm [1]. However, diagnostic confirmation requires the performance of immunohistochemical panels due to the need to differentiate metastases from primary ovarian tumors. Markers such as CK20, CDX2, and STAB2 are associated with colorectal and appendiceal neoplasms, while CK7, PAX8, ER, and PR are generally negative in this context, contributing to diagnostic accuracy [8,9].

In the molecular context, the analysis of the expression of the *CASP8*, *TNFSRF10B*, and *TNFSRF10C* genes, involved in cellular apoptosis pathways, did not reveal statistically significant differences between the studied groups, suggesting that these genes may not be suitable for distinguishing Krukenberg tumors from primary ovarian tumors. The absence of significant variation in gene expression indicates that, although these markers are relevant for characterizing apoptotic processes, their application in differentiating these tumor types is limited [10,11].

Additionally, the inability of the OvarianTag® algorithm to correctly classify Krukenberg tumor samples may be attributed to the small sample size of this group, compromising data representativeness and the effectiveness of the machine learning used by the predictive model. The underrepresentation of samples affects the algorithm's ability to generalize adequately, resulting in classification limitations [12].

Another relevant factor is the RNA extraction methodology used. While the initial validation of the panel was performed with fresh samples and a Trizol-based protocol, the present study used formalin-fixed and paraffin-embedded (FFPE) samples subjected to automated extraction. This difference can directly impact RNA integrity and purity, interfering with molecular analysis and contributing to the non-validation of the OvarianTag® panel in this context [13].

Furthermore, it is essential to consider that the OvarianTag® panel was initially developed to discriminate prognosis in epithelial ovarian cancer (EOC) patients, using fresh ovarian tissue, serous cystadenoma, and EOC tumors, with analysis based on qRT-PCR and machine learning. The high accuracy reported in the training phase (90%) and cross-validation (89%) was observed in a different context, with significant methodological and sample differences compared to the present study. In the later phase, with FFPE samples, the panel showed 100% specificity and 79% sensitivity to predict tumor recurrence and platinum resistance, reinforcing its efficacy for specific EOC subtypes but not necessarily for distinguishing Krukenberg tumors [6].

Finally, the literature suggests that other genes, such as *KRAS*, *SMAD4*, and *NTRK1*, are more frequently associated with ovarian metastases from colorectal carcinomas, indicating that expanding the biomarker panel could increase diagnostic accuracy for Krukenberg tumors [14]. Therefore, future investigations should consider including these markers and adopting standardized RNA extraction methods to improve the accuracy of molecular diagnosis.

5. CONCLUSION

In conclusion, this study highlights the limitations of the OvarianTag® panel in differentiating primary ovarian tumors from metastatic Krukenberg tumors, due to small sample size and variations in RNA extraction methods. Histopathological analysis emphasized the importance of microscopic evaluation and immunohistochemical panels for diagnosis. The molecular markers analyzed did not show significant differences, suggesting that alternative biomarkers may be more relevant for this differentiation. Future studies should focus on expanding the biomarker panel and standardizing RNA extraction methods to improve molecular diagnosis.

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AUTHORS' CONTRIBUTIONS

Ana Beatriz Ramos Do Nascimento and Jorge Gomes Goulart Ferreira contributed to the conception and drafting of the manuscript, data collection and analysis, discussion of results, and final review and approval of the article. Rafaela Lopes Figueiredo de Andrade, Eduardha Santos Temponi Barroso, Anna Carolina Almeida de Paula, and Pedro Henrique Villar Delfino contributed to data collection and analysis, discussion of results, and final review and approval of the article. Stephanie Braga Gonçalves da Silva, Thalía Rodrigues de Souza Zózimo, Aline Luiza Costa e Silva, Ramon De Alencar Pereira, and Paulo Guilherme de Oliveira Salles participated in the discussion of results and final approval of the article.

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AUTHOR DISCLOSURES

The authors declare no conflicts of interest.

ORIGIN OF THE WORK

This work is derived from the Undergraduate Thesis of the Biomedicine Bachelor's program at FUMEC University, located in Belo Horizonte, MG, conducted by the student Ana Beatriz Ramos do Nascimento, under the supervision of Dr. Jorge Gomes Goulart Ferreira.

ETHICS DECLARATION

This study was conducted following universal ethical principles and adhered to international ethical guidelines, including the Declaration of Helsinki and the International Ethical Guidelines for Biomedical Research Involving Human Subjects (CIOMS), as well as Brazilian regulations, such as CNS Resolution No. 196/96 and its complementary guidelines. The research project was reviewed and approved by the Research Ethics Committee of the Mário Penna Institute and is registered under CAAE: 70737223.0.0000.5121.

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