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Avaliação de Biomarcadores Prognósticos em Câncer de Esôfago.

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Tese submetida ao Programa de Pós-Graduação em Patologia da Universidade Federal de Ciências da Saúde de Porto Alegre como requisito para a obtenção do grau de Doutor

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Resumo da Tese

Introdução: A elevada incidência mundial de câncer de esôfago, e a manutenção das baixas porcentagens de sobrevida global dos pacientes, estimulam estudos sobre potenciais biomarcadores prognósticos. Pesquisas têm sido realizadas sobre o papel das enzimas antioxidantes no processo carcinogênico, como a Superóxido Dismutase 2 (SOD2) e em especial o polimorfismo Val16Ala de SOD2, mas seu impacto prognóstico no câncer de esôfago não foi estabelecido. Estudos também avaliaram a associação entre a infecção por Papilomavírus Humano (HPV), principalmente HPV16, e a expressão da proteína supressora de tumor p16, com correlações divergentes destes com a progressão da doença. **Objetivos:** Investigar SOD2, HPV16, e p16 como potenciais biomarcadores prognósticos em câncer de esôfago. **Metodologia:** Artigo I - revisão sistemática sobre o polimorfismo Val16Ala de SOD2 e a associação com o prognóstico de pacientes com câncer. Artigo II - Análise dos genótipos do polimorfismo Val16Ala e a expressão imunohistoquímica de SOD2 no câncer de esôfago, e sua associação com a sobrevida global. Artigo III - Associação entre infecção por HPV16 e a expressão imunohistoquímica de p16 no câncer de esôfago, e sua correlação com a sobrevida. **Resultados:** Artigo I - Foram identificadas evidências na literatura da associação entre o polimorfismo Val16Ala (alelo Ala), e o prognóstico de pacientes com câncer. Artigo II - Polimorfismo Val16Ala foi associado ao risco de câncer de esôfago (RR 2.18, 95%CI 1.23-3.86), mas não a sobrevida dos pacientes (P=0.53). A baixa expressão de SOD2 foi associada a menor sobrevida (HR, 0.41; 95% CI, 0.22-0.79). Artigo III - Não houve associação de HPV16 e p16 com a sobrevida dos pacientes com câncer de esôfago (P=0.56). **Conclusão:** Na investigação de biomarcadores para câncer de esôfago, destacamos o polimorfismo Val16Ala-SOD2 (modelo AA vs AV+VV) associado ao maior risco dessa neoplasia, e os resultados promissores da expressão imunohistoquímica de SOD2 como biomarcador prognóstico.

Palavras-chave: Câncer de esôfago, Val16Ala-SOD2, HPV, p16, biomarcadores.

Abstract

Introduction: A large number of esophageal cancer worldwide, and the maintenance of low percentages of overall patient survival, encourage studies on potential prognostic biomarkers. Research has been carried out on the role of antioxidant enzymes in the carcinogenic process, such as Superoxide Dismutase 2 (SOD2) and in particular the SOD2 Val16Ala polymorphism, but their prognostic impact on esophageal cancer has not been established. Studies have also evaluated an association between an infection by Human Papillomavirus (HPV), mainly HPV16, and an expression of the tumor suppressor protein p16, with divergent correlations between these and the progress of the disease. **Aim of study:** To investigate SOD2, HPV16, and p16 as potential prognostic biomarkers in esophageal cancer. **Materials and methods:** Article I - systematic review of evidence on the Val16Ala SOD2 polymorphism and its association with the prognosis of cancer patients. Article II - Analysis of Val16Ala polymorphism genotypes and SOD2 immunohistochemical expression in esophageal cancer and its association with overall survival. Article III - Analysis of the association between HPV16 infection and immunohistochemical expression of p16 in esophageal cancer and its correlation with survival. **Results:** Article I - Evidence from literatura of an association between the Val16Ala polymorphism (Ala allele) and the prognosis of cancer patients was identified. Article II - Val16Ala polymorphism was associated with esophageal cancer risk (RR 2.18, 95%CI 1.23-3.86), but not patient survival (P=0.53). Weak SOD2 expression was associated with shorter survival (HR, 0.41; 95% CI, 0.22-0.79). Article III - There was no association of HPV16 and p16 with patient survival (P=0.56). **Conclusion:** In the investigation of esophageal cancer biomarkers, we highlight the Val16Ala-SOD2 polymorphism (AA vs AV VV model) associated with a higher risk of this neoplasia, and the promising results of the immunohistochemical expression of SOD2 as a prognostic biomarker.

Keywords: Esophageal cancer, Val16Ala-SOD2, HPV, p16, biomarkers.

Lista de abreviaturas

AC: Adenocarcinoma

AJCC: *American Joint Committee on Cancer*

BAK: *BCL2-antagonist/killer*

CAT: Catalase

CCE: Carcinoma epidermóide de Esôfago

CDK: Quinase dependentes de ciclina

cTNM: Estadiamento clínico, sistema TNM

DNA: Ácido Desoxirribonucleico

E1 à E7: Oncoproteínas virais do HPV

E2F: Fatores de Transcrição E2F

EGFR: Receptor de fator de crescimento epidérmico

EROs: Espécies Reativas de Oxigênio

G1: Fase de Crescimento

S: Fase de síntese

GPx: Glutationa peroxidase

HER2: *Human Epidermal Growth Factor Receptor-type 2*

H2O: Água

H_2O_2: Peróxido de hidrogênio

HO_2: Hidroperoxila

HPV: Papilomavírus Humano

HPV16: HPV tipo oncogênico 16

HPV18: HPV tipo oncogênico 18

IHC: *immunohistochemistry*

INCA: Instituto Nacional do Câncer

JEG: junção gastroesofágica

L1 e L2: Codificam proteínas estruturais do capsídio

LCR: Long control region

MTS: *Mitocondrial Target Sequence*

RNAm: Ácido Ribonucleico mensageiro

O_2: Oxigênio

O_{2-}: Superóxido

OH: Hidroxila

p21 e p27: Proteínas reguladoras da transição, inibidores de CDK - p21 e p27

p16: Proteína supressora tumoral celular $p16^{INK4a}$

PD-1:do inglês: *programmed cell death 1*

PD-L1: ligante 1de PD-1

pRb: Proteína do retinoblastoma

p53: Proteína supressora de tumor

pTNM: Estadiamento patológico

SNP: Polimorfismo de Nucleotídeo Simples

SOD: Superóxido Dismutase

SOD1: Superóxido Dismutase 1, associada a cobre e zinco (Cu/ZnSOD)

SOD2: Superóxido Dismutase 2, associada a manganês (MnSOD)

SOD3: Superóxido Dismutase 3, associada a cobre e zinco com localização extracelular (ecSOD)

URR: *Upstream Regulation Region*

VEGF: Fator de crescimento endotelial vascular

ypTNM: Estadiamento pós-neoadjuvante

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SUMÁRIO

1. REFERENCIAL TEÓRICO

O presente estudo aborda o câncer de esôfago, tumor do sistema gastrointestinal, e possíveis biomarcadores prognósticos a ele associados. O referencial teórico a seguir embasa a construção dos objetivos da tese, e justifica os três estudos científicos realizados (Anexos). No primeiro item, revisamos o estado atual do conhecimento sobre o câncer de esôfago e suas características, epidemiologia, fatores de risco, métodos de diagnóstico, tratamento e estadiamento, bem como o contexto prognóstico desfavorável da doença. O segundo item aborda a importância dos biomarcadores com destaque para biomarcadores prognósticos em câncer de esôfago, que podem ser identificados a partir de estudos sobre fatores associados à progressão da carcinogênese. Esse item introduz os principais temas abordados nesta tese, que serão posteriormente apresentados e contextualizados nos itens: Estresse Oxidativo e Sistema Antioxidante [subitens: Superóxido Dismutase 2 (SOD2) e câncer; e Polimorfismo Val16Ala de SOD2 em câncer de esôfago]; Infecção viral por Papilomavírus Humano (HPV) e câncer de esôfago; e P16 e câncer de esôfago. Assim, neste referencial teórico é detalhada a importância de SOD2, da infecção por HPV especialmente HPV16, e de p16 em câncer de esôfago, e justificamos a realização de estudos para investigá-los como biomarcadores prognósticos.

1.1 Câncer de esôfago

O câncer de esôfago é uma neoplasia maligna do sistema digestivo, que se inicia a partir do revestimento interno do esôfago (mucosa) e invade outras camadas à medida que progride **(Figura 1).** Os dois tipos histológicos de câncer de esôfago mais comuns são o Carcinoma epidermóide de esôfago (CCE) e o Adenocarcinoma esofágico (ou AC)[1].

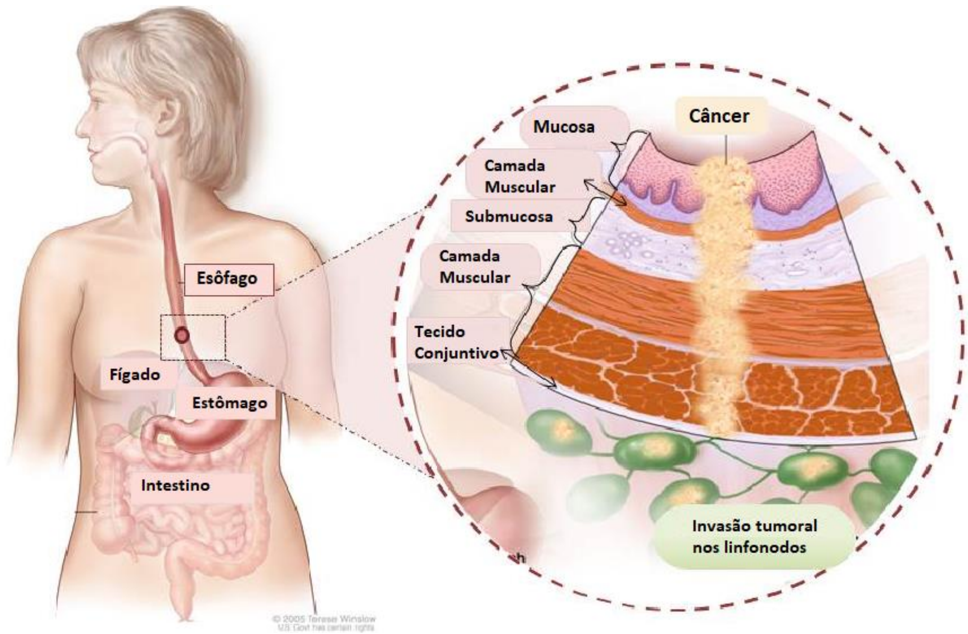

Figura 1 Ilustração do comprometimento neoplásico a parir da camada muscular da parede do esôfago, atingindo os linfonodos.
Fonte: Adaptado de PDQ Adult Treatment Editorial Board. NCI, 2021.

O CCE tem origem a partir de células epiteliais escamosas, mais frequentemente na parte superior e média do esôfago. Enquanto que, o adenocarcinoma surge a partir de células glandulares, próximas ao esfíncter esofágico inferior ou Junção Esofagogástrica (JEG)[2, 3].

As histórias naturais dos tipos histológicos também diferem sobre a qual modelos de transição para CCE descreveram o epitélio escamoso sofrendo alterações inflamatórias que progridem para displasia e alteração maligna in situ. Enquanto que adenocarcinomas iniciariam a partir do epitélio metaplásico colunar do tipo intestinal, comumente conhecido como esôfago de Barrett, que substitui o epitélio escamoso durante a esofagite de refluxo e pode progredir para displasia[4]. Assim, ocorre uma série de mecânicos ou celulares que favorecem o processo carcinogênico em CEC e adenocarcinomas[5].

Por apresentar características histológicas diferentes e ser uma doença com vários fatores que aumentam o risco do desenvolvimento neoplásico, uma causa comum para todos os cânceres de esôfago é desconhecida[2,6]. Porém, estudos têm demonstrado que alterações genéticas e epigenéticas parecem ser necessárias para a promoção do câncer de esôfago, e que fatores ambientais, imunológicos e infecciosos podem favorecer esse processo[7].

1.1.1 Epidemiologia

A incidência mundial do câncer de esôfago aumentou rapidamente nos últimos anos, e é atualmente o sétimo tipo de câncer mais incidente (604.000 novos casos) e o sexto em mortalidade geral (544.000 óbitos). O câncer de esôfago é uma e uma das principais causas de morte relacionada ao câncer em todo o mundo[8, 9], sendo responsável por um a cada 18 óbitos por câncer em 2020[2].

Em relação às regiões geográficas, quatro em cada cinco casos de câncer de esôfago ocorrem em nações não industrializadas, com taxas de incidência mais altas na Ásia e na África, sendo a Ásia o continente com maior taxa de mortalidade em 2020[2] **(Figura 2).** Além disso, Aproximadamente 70% dos casos ocorrem em homens, com uma diferença de 2 a 3 vezes nas taxas de incidência e mortalidade entre os sexos[10].

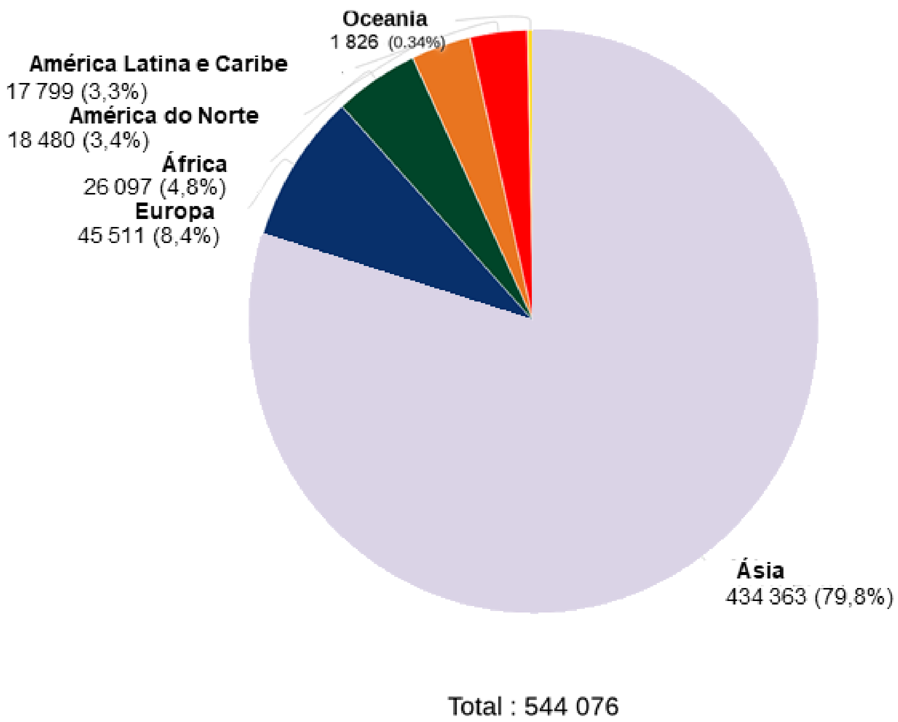

Figura 2 Estimativa de óbitos em 2020 por câncer de esôfago no mundo.
Fonte: Adaptado de IARC - GLOBOCAN 2020.

No Brasil, a taxa de incidência de câncer de esôfago estimada, para cada ano do triênio de 2020-2022, é de 8,32 novos casos a cada 100 mil homens e 2,49 novos casos a cada 100 mil mulheres[(10)]. Apesar da redução do número de óbitos por câncer de esôfago no Brasil, relacionado ao maior desenvolvimento socioeconômico e melhores indicadores de saúde, em 2019 foram registrados 8.617 óbitos por essa neoplasia[(11)].

A região Sul é a localidade brasileira com maior incidência da doença e segunda maior taxa de mortalidade, situação que se mantém há décadas[(11, 12)]. O câncer de esôfago é o quinto tipo de câncer mais frequente em homens (14,48/100 mil) e o décimo terceiro mais frequente em mulheres (4,52/100 mil)[(10)], sendo a causa de 1.998 óbitos por câncer em 2019[(11)].

A variação geográfica na incidência de câncer de esôfago também difere substancialmente de acordo com o tipo histológico e fatores de risco, sendo relacionados e discutidos a seguir [13].

1.1.2 Fatores de risco

Atualmente, os carcinomas esofágicos compreendem mais de 90% de todos os casos de câncer de esôfago, sendo o etilismo e o tabagismo excessivo, assim como seus efeitos sinérgicos, os principais fatores de risco, especialmente em populações ocidentais[2].

Entretanto, em países de baixa renda, incluindo partes da Ásia, América do Sul e África Subsaariana, os principais fatores de risco para CCE são componentes dietéticos, como: a deficiência de micronutrientes (betacaroteno, folato, vitamina C, vitamina E, entre outros), o consumo de vegetais em conserva, carnes vermelhas ou processadas, e o hábito de ingestão de bebidas quentes (65ºC ou mais - como chimarrão, chá e café)[10, 13].

Em relação ao chimarrão, é consenso na literatura que a ingestão cumulativa dessa bebida é um importante fator de risco, especialmente na região Sul do Brasil, onde a bebida é comumente consumida[10, 14]. Porém, alguns estudos verificaram que contaminantes mutagênicos relacionados à prática de processamento da erva-mate (Ex: Hidrocarbonetos Aromáticos Policíclicos) também poderiam ser fatores de risco para CCE[15, 16].

Ressalta-se que a incidência de CCE em certas áreas de alto risco (por exemplo, China) está em declínio e pode estar associada a ganhos econômicos e melhorias dietéticas, enquanto que em vários países de alta renda (por exemplo, Estados Unidos) as reduções estão associadas ao declínio no consumo de cigarros[2].

Em relação aos adenocarcinomas, a obesidade, a doença do refluxo gastroesofágico e esôfago de Barrett, no contexto de inflamação crônica secundária à exposição ao ácido e bile, são considerados os principais fatores de risco. Apesar de serem observados principalmente em países de alta renda, vêm apresentando um aumento significativo em diversas regiões, devido a mudanças nos hábitos dietéticos locais[2, 10].

Além dos supracitados, podemos incluir a idade superior a 55 anos, histórico de carcinomas associados ao fumo (como câncer oral ou pulmonar), distúrbio de motilidade esofágica congênito (acalasia), tilose hereditária, síndrome de Plummer-Vinson, infecção pelo Papilomavírus Humano (HPV), mutações e alterações epigenéticas [3, 7, 10, 17].

1.1.3 Diagnóstico, Tratamento e Estadiamento

Clinicamente, pacientes com câncer de esôfago são geralmente assintomáticos em estágios iniciais da doença. Porém, o crescimento da massa tumoral durante o processo de carcinogênese pode levar a obstrução e estreitamento da luz do tubo esofágico, refletindo em disfagia progressiva e odinofagia, além de dor torácica, dor retroesternal, refluxo, e consequentemente perda de peso não intencional identificados em casos mais avançados da doença [18, 19].

A sintomatologia tardia dificulta o diagnóstico precoce, uma vez que não há recomendações para rastreamento dessa neoplasia na população em geral[18]. A detecção do câncer de esôfago pode ser feita por meio da investigação médica, com exames clínicos, laboratoriais e de imagem. Para avaliação diagnóstica inicial é realizado o exame de endoscopia digestiva, seguido de biópsia em casos de lesão esofágica[1].

As opções de tratamento para câncer de esôfago, em comparação com outros tipos de neoplasia, são restritas principalmente em nações não industrializadas. Assim, a tríade processo cirúrgico, radioterapia e quimioterapia, aplicadas em associação ou singularmente, se estabeleceram ao longo dos anos como os tratamentos mais utilizados. Entretanto, novas alternativas de tratamento estão sendo amplamente investigadas, como a imunoterapia e a terapia direcionada [9, 20, 21].

O esquema de tratamento a ser aplicado dependerá do subtipo histológico do tumor, localização anatômica no esôfago (cervical, torácico ou distal), estágio em que a doença se encontra e as condições clínicas do paciente, sendo fundamental para tanto, o estadiamento do câncer [1, 22].

Os tumores esofágicos são classificados nesse processo de estadiamento conforme o Sistema TNM, sendo detalhadamente apresentado no quadro abaixo **(Quadro 1).**

Quadro 1 Estadiamento do câncer de esôfago - Sistema TNM (AJCC)

T - Grau de invasão do tumor	
Tx	O tumor não pode ser avaliado
T0	Não há evidência de tumor primário
Tis	Tumor "*in situ*"
T1	Tumor invade a lâmina própria, muscular da mucosa ou submucosa.
T1a	Tumor invade a lâmina própria ou muscular da mucosa.
T1b	Tumor invade a submucosa
T2	Tumor invade a muscular própria
T3	Tumor invade a adventícia
T4	Tumor invade as estruturas adjacentes
T4a	Tumor invade a pleura, pericárdio, ázigos, diafragma ou peritônio.
T4b	Tumor invade outras estruturas adjacentes, como aorta,

	vértebras ou vias aéreas.
N - Envolvimento linfonodal	
Nx	Linfonodos regionais não podem ser avaliados
N0	Sem metástase de linfonodos regionais
N1	Metástase em um ou dois linfonodos regionais
N2	Metástase em três a seis linfonodos regionais
N3	Metástase em sete ou mais linfonodos regionais
M - Metástase à distância	
M0	Não há metástases à distância
M1	Metástases à distância

Fonte: Adaptado de *American Joint Committee on Cancer* – AJCC 2017.

Proposto pela *American Joint Committee on Cancer* - AJCC (2017), o sistema TNM resulta em 5 estágios principais da doença (0,I, II, III, IV), mas que podem ser subdivididos em mais grupos (a e b) conforme o subtipo histológico do tumor (carcinoma ou adenocarcinoma) e o estadiamento (clínico ou patológico)[22]. O estadiamento clínico (cTNM) é realizado com base em estudos de imagem e informações histológicas mínimas, sendo utilizado para definir o tratamento primário. Já o estadiamento patológico (pTNM), é realizado pós-ressecção cirúrgica do tumor, possibilitando incluir informações de neoadjuvância (ypTNM), e sendo amplamente utilizado para análise de resposta terapêutica completa[22].

Em estágios clínicos iniciais a ressecção endoscópica e a cirurgia são os principais tratamentos para câncer de esôfago[19, 23, 24]. Em estágios II a III, opta-se pela terapia neoadjuvante com quimioterapia ou quimiorradiação, tendo por finalidade reduzir o tamanho do tumor para posterior remoção cirúrgica[19, 23-25].

Entre as técnicas cirúrgicas, destaca-se a esofagectomia, fundamental no tratamento curativo, principalmente em tumores localmente avançados. Porém, por ser altamente invasiva está associada à perda de qualidade de vida e mortalidade no período pós-operatório [19]. Quando a cirurgia se torna inviável, o tratamento único com quimioterapia e/ou radioterapia pode ser realizado. No estágio IV, mais avançado da doença, quando já existe metástase recomenda-se o tratamento paliativo[20, 23, 25-27].

A imunoterapia e terapia direcionada podem ser alternativas de tratamento menos invasivas para estágios iniciais, e nos casos avançados em que a cirurgia por si só não pode alcançar a cura[28]. O tratamento com imunoterapia para inibidores *programmed cell death 1* (PD-1) junto com seu ligante (PD-L1) foi aprovado recentemente no Brasil pela Anvisa (anticorpo anti-PD-1 nivolumabe), para pacientes com CCE irressecável e avançado ou metastático após quimioterapia prévia[29, 30].

Nos casos de pacientes positivos para o receptor do fator de crescimento epidérmico humano tipo 2 (HER2 - *Human Epidermal Growth Factor Receptor-type 2*), também está aprovada a terapia com anti-HER2 transtusumabe, desde que o câncer seja localmente avançado e recorrente ou metastático mas com expressão de PD-L1 positiva e que tenham recebido uma ou mais linhas anteriores de terapia sistêmica[31].

As opções imunoterapêuticas estão sendo amplamente pesquisadas em ensaios clínicos, indicando a necessidade de estratégias multidisciplinares para o tratamento de pacientes com câncer de esôfago[24, 28, 32].

1.1.4 Prognóstico

Na maioria dos casos, o câncer de esôfago é uma doença tratável, mas raramente tem cura[1]. O prognóstico para essa neoplasia varia entre as áreas geográficas, mas estudos de base populacional mostraram uma melhora na sobrevida geral de cinco anos, de menos de 5% na década de 1960 para cerca de 20% na última década em alguns países europeus, os EUA, e China[19].

Apesar da melhora nas taxas de sobrevida devido ao avanço das estratégias de tratamento nos últimos anos (quimioradioterapia, imunoterapia e terapia direcionada) e mesmo nos casos de ressecção completa, a sobrevivência dos pacientes continua substancialmente baixa[33, 34].

A principal causa é o estágio avançado da doença quando no momento do diagnóstico. Pacientes com doença em estágio inicial têm 46,4% de chance de sobrevivência em cinco anos. Porém, somente 17,5% dos pacientes são diagnosticados nesse estágio da doença[1]. Nos casos de câncer de esôfago localmente avançado a recorrência precoce da doença pode chegar a 43,3% em seis meses após o tratamento trimodal[21].

Nesse contexto, o prognóstico da doença depende de vários fatores, associados principalmente à invasão local da doença, bem como a disseminação da neoplasia para estruturas regionais e distantes[13]. Assim, durante a avaliação prognóstica, serão verificadas informações acerca do tipo histológico tumoral, localização anatômica, indiferenciação celular (grau histológico 3), aumento da profundidade de invasão do tumor, invasão patológica venosa, ressecção microscopicamente ou macroscopicamente incompleta (R1-R2), proporção aumentada de linfonodos positivos ressecados, envolvimento de linfonodos extracapsulares, e superexpressão de HER2 nos casos de adenocarcinoma[21, 22, 25].

Além disso, estudos verificaram associações prognósticas com idade, etnia, sexo biológico, histórico familiar oncológico, nutrição, perda de peso e comorbidades pré-existentes, complicações pós-operatórias e estado imunocomprometimento pós-operatório[13, 19, 21, 22].

Destaca-se que outros fatores ainda não totalmente compreendidos, podem influenciar na gênese e progressão da doença, e estarem associados ao prognóstico de pacientes com câncer de esôfago, sendo investigados, por exemplo, o estresse oxidativo e a infecção persistente por HPV, os quais serão amplamente discutidos neste trabalho[35-37].

Na busca por individualização do tratamento oncológico, existem esforços na identificação de fatores preditivos e prognósticos para câncer de esôfago, que apresentem relevância clínica[38]. Nesse contexto, além da utilização de HER-2 e ~~EGFR~~, novos biomarcadores podem ajudar a prever a resposta ao tratamento, otimizando as ferramentas terapêuticas existentes, ou ainda, auxiliar na vigilância rigorosa pós-tratamento a fim de melhorar ainda mais a sobrevida de paciente com câncer de esôfago[19].

1.2 Biomarcadores

Os biomarcadores são parâmetros biológicos mensuráveis em células cancerígenas ou através de células normais como resposta à presença do câncer[39]. Eles auxiliam na distinção dos processos normais dos patológicos com aplicações no diagnóstico e tratamento das neoplasias[40].

Esses parâmetros são obtidos a partir de uma variedade de amostras biológicas, os diferentes métodos de análise definirão a presença de uma macromolécula alvo, das alterações moleculares na mesma, ou ainda mensurará as variações de nível do marcador em questão[39].

A partir dos avanços científicos sobre a influência do câncer nos processos biológicos e da necessidade de um microambiente tumoral propício para o desenvolvimento e progressão da doença, uma ampla gama de biomarcadores para câncer tem sido investigada[41].

Esses biomarcadores são classificados geralmente conforme sua função biológica, e sua relação com "*Hallmarks* do câncer", ou seja, características comuns entre os tipos de câncer e que definem o processo neoplásico. As categorias definidas por Hanahan e Weinberg (2011) [41] são apresentadas na **Figura 3**.

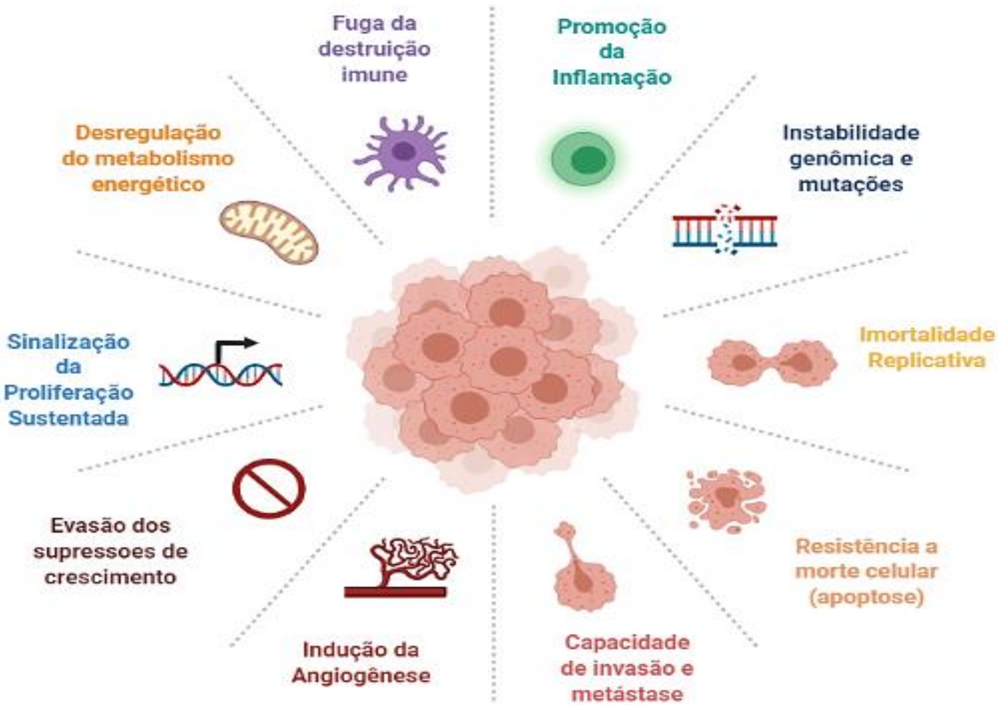

Figura 3 Ilustração dos *Hallmarks* do Câncer.
Fonte: Adaptado de Hanahan e Weinberg, 2011. Created with BioRender.com

Os biomarcadores para câncer de mama, câncer de pulmão e tumores cerebrais estão bem estabelecidos na prática clínica, diferentemente do câncer de esôfago, para o qual não há um painel de marcadores padrão que seja aceito em consenso [17, 19, 36, 40, 42, 43].

Além disso, há variabilidade quanto ao subtipo tumoral, estágio da doença, técnicas de detecção e objetivos da investigação[44, 45]. Enquanto os biomarcadores preditivos são úteis para determinar quais pacientes são adequados para cada tipo de tratamento, os biomarcadores prognósticos estão associados à sobrevida, história natural da doença e desfechos clínicos[46].

Para câncer de esôfago, é crescente a necessidade de biomarcadores que auxiliem na avaliação de prognóstico e vigilância terapêutica[19, 45, 47]. Assim, estudos que investiguem possíveis fatores associados à progressão da carcinogênese, como por exemplo, o estresse oxidativo e processos infecciosos, podem identificar novos marcadores prognósticos para câncer de esôfago[39, 48-51].

1.3 Estresse oxidativo e Sistema Antioxidante

Ao longo dos anos, o estresse oxidativo tem sido amplamente estudado nos processos de modulação bioenergética e fisiopatológicos. É caracterizado por um desequilíbrio nos sistemas oxidantes e antioxidantes celulares, em que há produção elevada e déficit na redução de radicais livres[52, 53].

Os radicais livres são moléculas que contêm um elétron desemparelhado, resultando em instabilidade e reatividade molecular [53]. A formação dessas moléculas ocorre principalmente no metabolismo do oxigênio e a nível mitocondrial, através da redução de oxigênio (O_2) em água (H_2O), promovendo a formação de produtos intermediários reativos, como superóxido (O_2-), hidroperoxila (HO_2), hidroxila (OH) e peróxido de hidrogênio (H_2O_2)[53, 54].

Também é um promotor desses metabólito a redução incompleta do O_2, devido a fatores externos como a exposição à radiação ionizante e xenobióticos(53-55).

Observa-se que nem todos os intermediários reativos apresentam elétrons desemparelhados em sua última camada (definição de radical livre). Assim, no decorrer deste texto e conforme a literatura(53, 56) utilizaremos o termo "Espécies Reativas" para referirmo-nos a eles.

Ressalta-se que outras moléculas além do oxigênio, como o enxofre, o carbono e o nitrogênio também podem produzir espécies reativas, no entanto, as espécies reativas de oxigênio recebem maior atenção, dada a importância do oxigênio nos processos metabólicos celulares (53, 57).

As Espécies Reativas de Oxigênio (ERO) estão envolvidas em processos fisiológicos normais, como resposta imune, crescimento e diferenciação celular, apresentando um efeito benéfico na sua produção(53).

Entretanto, quando em excesso (estresse oxidativo), os EROs estão associados a diversas alterações patológicas no organismo. Podem causar alteração e destruição de membranas biológicas, inativação de proteínas, danos ao DNA, indução de mutação celular e carcinogênese. Além disso, podem induzir a ativação de fatores de transcrição associados à ativação de cascatas de sinalização que culminam na liberação de citocinas e inflamação(53, 55, 58, 59).

Esses efeitos prejudiciais dos EROs podem ser bloqueados pela ação do sistema antioxidante. A ativação antioxidante ocorre quando as células tentam neutralizar os efeitos oxidantes e restaurar o equilíbrio redox (reações de oxidação-redução) pela ativação ou silenciamento de genes que codificam enzimas defensivas, fatores de transcrição e proteínas estruturais. Além de defesa, esse sistema pode realizar a prevenção e reparo físico-químico dos danos oxidativos nos diversos sistemas orgânicos(55).

A **Figura 4** ilustra a relação entre a produção de espécies reativas, estresse oxidativo, desenvolvimento de doenças e o papel dos antioxidantes e da variação genética.

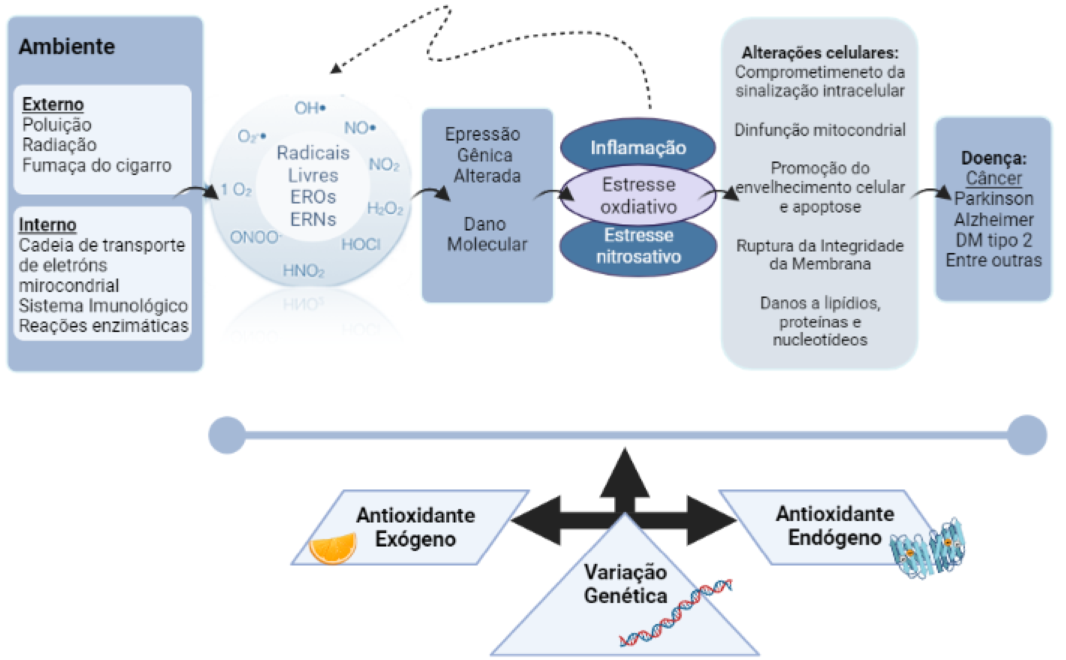

Figura 4 Estresse Oxidativo na promoção de doenças.
Acúmulo de EROs e espécies reativas de nitrogênio (ERNs) a partir de estímulos ambientais podem causar danos moleculares e resultar em estresse oxidativo e nitrosativo. Também podem alterar a expressão gênica, levando à liberação de citocinas e inflamação, a qual resulta em mais produção de espécies reativas. Esse excesso de espécies reativas pode causar diversas alterações celulares que podem resultar no desenvolvimento de doenças como o câncer. Por isso os antioxidantes são importantes, pois buscam reduzir o acúmulo de espécies reativas e o dano celular. Porém, sua função pode ser modificada pela variação genética individual. DM = Diabetes Mellitus.
Fonte: Adaptado de Da Costa *et al*. 2012.Created with BioRender.com

Os antioxidantes podem ser classificados de diversas formas, mas principalmente pelos mecanismos de ação. Os antioxidantes não enzimáticos ou mecanismos exógenos, como vitaminas, ácido úrico, flavonoides, entre outros, interrompem as reações em cadeia dos radicais livres, sendo antioxidantes encontrados em diversos alimentos. Já os antioxidantes enzimáticos, ou mecanismos endógenos, especialmente as enzimas superóxido dismutase - SOD, catalase – CAT, e glutationa peroxidase – GPx, funcionam reduzindo e removendo os EROs [57, 60].

Entre essas enzimas, destacamos a SOD, uma família de enzimas antioxidantes de ação primária, que são capazes de reduzir com eficiência o ânion superóxido em peróxido de hidrogênio (H2O2), posteriormente transformado em água (H2O) e oxigênio (O2) pelas enzimas CAT e GPx. Em humanos, existem três isoformas enzimáticas principais, a SOD1 (associada a cobre-zinco Cu/ZnSOD com localização citosólica), a SOD2 (associada a manganês MnSOD e localização mitocondrial), e a SOD3 (associada a cobre-zinco mas com localização extracelular ecSOD).Devido a elevada produção de EROs e especialmente de superóxido intracelular na mitocôndria, a SOD2 é considerada uma das enzimas mais importantes do sistema antioxidante e um potencial biomarcador para câncer [53].

1.3.1 Superóxido Dismutase 2 (SOD2) e câncer

A SOD2 é uma molécula homotetramétrica de 23 kDa, codificada pelo gene nuclear de mesmo nome localizado no cromossomo 6 região q25.3. É sintetizada inicialmente na forma inativa com uma pequena sequência de direcionamento mitocondrial denominada *mitocondrial target sequence, MTS*, que é posteriormente traduzida no citoplasma e transportada para a mitocôndria[61].

Ao passar pelos poros da membrana mitocondrial interna, o segmento peptídico *MTS* é clivado pelos lisossomos, tornando a proteína um ativo homotetrâmero e uma enzima funcional, agindo na detoxificação do ânion radical superóxido em peróxido de hidrogênio **(Figura 5)** [61].

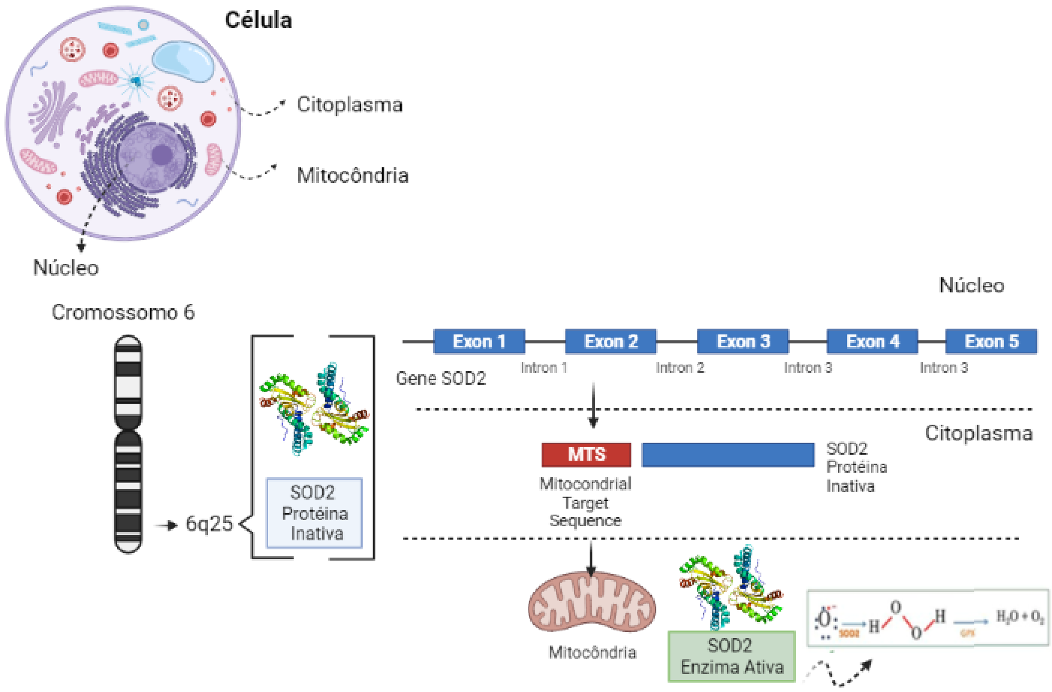

Figura 5 Codificação e ativação da enzima SOD2.
Fonte: Adaptada de Barbisan, 2017. Created with BioRender.com

A SOD2 é considerada essencial para a sobrevivência aeróbia, com papel chave na regulação de eventos de morte celular[53, 60]. Assim, em processos carcinogênicos a expressão dessa enzima pode estar alterada, apresentando um papel dicotômico no câncer (alternando entre supressor e promotor tumoral)[62].

As alterações na expressão e atividade de SOD2 também são dependentes do tipo de câncer[62, 63]. De acordo com a revisão de Kinulla e Kappo *et al*. (2004), a expressão dessa enzima foi considerada elevada em alguns casos de câncer de esôfago[63]. Em relação ao prognóstico, identificamos poucos estudos com câncer de esôfago, que observaram a alta expressão da enzima associada ao pior prognóstico para CCE[64] e a baixa expressão de SOD2 associada à progressão de Esôfago de Barret para Adenocarcinomas esofágicos[65].

Outros estudos em CCE verificaram associações divergentes in vivo e in vitro. In vivo, a baixa expressão de RNAm (Ácido Ribonucleico mensageiro) e da proteína SOD2 foi associada com a invasividade no câncer de esôfago[66-68]. Porém, em experimentos in vitro, a superexpressão da enzima foi associada à radioresistência[69, 70], crescimento celular e resistência a apoptose, enquanto que a expressão moderada diminui as taxas de crescimento e aumentou a apoptose[69].

A atividade antioxidante da SOD2 pode ser modificada por diversos fatores, relacionados ao indivíduo e/ou ambientais, como fatores genéticos, dieta nutricional, tabagismo, consumo de álcool, atividade física, entre outros[53]. No caso de pacientes com câncer, estudos também indicam a influência do tratamento oncológico sobre a atividade antioxidante.

Em relação aos fatores genéticos que influenciam na atividade de SOD2, alteração genética mais estudada é o polimorfismo de nucleotídeo (SNP), que ocorre quando bases únicas de genes são alteradas ou deletadas, resultando na modificação de aminoácidos em posições específicas e, portanto, em fenótipos alterados. Mais de 190 SNPs foram descritos para o gene que codifica a SOD2, sendo um dos mais estudados o polimorfismo Val16Ala (rs4880), especialmente na gênese e progressão do câncer[53, 61].

1.3.2 Polimorfismo Val16Ala de SOD2 em câncer de esôfago

O polimorfismo Val16Ala de SOD2 é caracterizado por uma mutação estrutural, devido a substituição de uma timina (T) por uma citosina (C) no exon 2. A substituição afeta o códon 16, traduzindo o aminoácido valina (G**T**T) em alanina (G**C**T). Isso resulta em uma variabilidade genética populacional, em que há o alelo Val (Valina) e o alelo Ala (Alanina) e, portanto, três genótipos possíveis: Val/Val (TT ou VV), Ala/Val (TC ou AV), ou Ala/Ala (CC ou AA) [53].

Essa substituição de aminoácidos também resulta em uma mudança conformacional da enzima SOD2 na região MTS (estrutura β-lâmina secundária para estrutura α hélice – fenotipicamente: Val com estrutura parcial de β-lâmina Ala com estrutura α-hélice, e variante Ala/Val apresentando estrutura helicoidal). Tal modificação poderia alterar a eficiência de transporte da enzima para a mitocôndria[53, 71].

Assim, a variante Ala é capaz de cruzar rapidamente ambas as membranas mitocondriais para alcançar a matriz mitocondrial, enquanto o precursor Val está parcialmente ligada à membrana interna da mitocôndria[53]. Além disso, de acordo com Sutton *et al.* (2003), o alelo Ala gera 30-40% a mais do homotetrâmero SOD2 processado, matricial e ativo em comparação com o alelo Val[72]. Nesse contexto, conforme os genótipos, uma disponibilidade diferente de SOD2 poderia ocorrer na mitocôndria **(Figura 6)** [53].

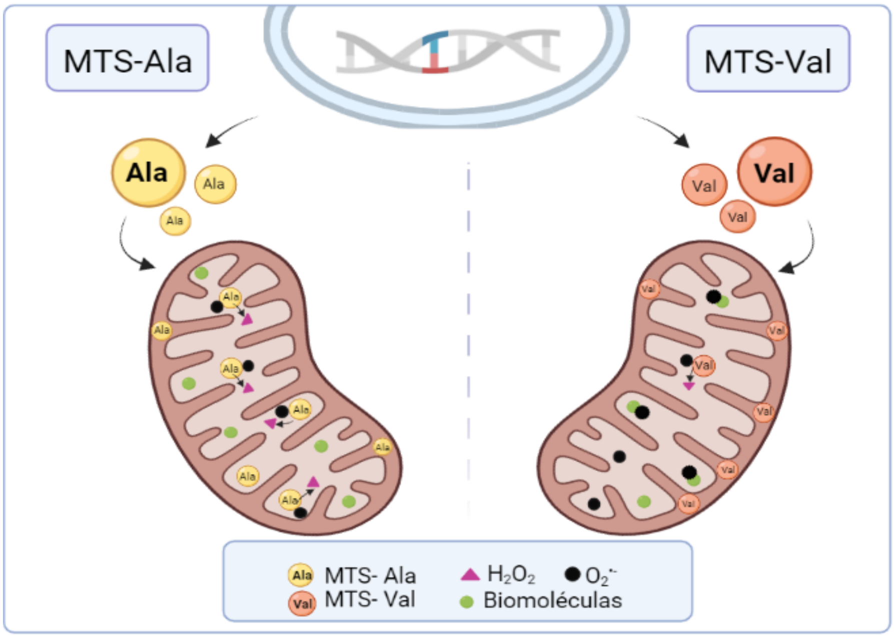

Figura 6 Disponibilidade de SOD2 na mitocôndria - Ala *vs* Val.
Fonte: Adaptado de Bresciani *et al.* 2015 Created with BioRender.com

Além das variações genéticas que influenciam no transporte mitocondrial, estudos têm observado que esse polimorfismo pode desregular a expressão e atividade enzimática, havendo diferenças nos níveis de mRNA, proteína e atividade de SOD2 entre os alelos Ala e Val[72-74].

Essas modificações na SOD2 são importantes uma vez que as mudanças na eficiência da enzima podem acarretar em um desequilíbrio do sistema antioxidante. Essa desregulação pode ser ainda maior quando avaliamos e comparamos células neoplásicas com células normais. A função mitocondrial tem um papel crítico durante a progressão do câncer, inclusive para a evasão tumoral e metástase[75-77].

Nesse contexto, extensos estudos foram realizados nas últimas duas décadas associando o polimorfismo Val16Ala de SOD2 com o desenvolvimento e progressão do câncer[78-80]. Embora, não haja consenso na literatura sobre essa associação. Os resultados diferem conforme o tipo de câncer, estadiamento tumoral, população estudada, etnia, fatores nutricionais e comportamentais [76, 79, 81-84].

Em relação ao câncer de esôfago, há poucos estudos avaliando o polimorfismo Val16Ala-SOD2 como um biomarcador, sendo todos com enfoque na suscetibilidade à doença[85-88]. A maioria dos estudos, incluindo um estudo na população brasileira[85], não identificaram uma associação significativa entre o risco para câncer de esôfago e o polimorfismo [86, 87, 89]. Porém, em uma metanálise realizada por Sun *et al.* (2013), foi identificado evidências de uma associação estatisticamente significativa do polimorfismo com risco para câncer de esôfago[84].

Em um estudo piloto de revisão sistemática (Protocolo Metodológico de Revisão disponível no Apêndice A), não identificamos trabalhos com análises conjuntas do polimorfismo Val16Ala de SOD2 e expressão tecidual da proteína em câncer de esôfago.

Devido à importância de SOD2 como antioxidante endógeno, no controle de espécies reativas e possíveis danos celulares associados, são necessários estudos que a avaliem como um possível biomarcador de prognóstico no câncer de esôfago. Nesse contexto, é fundamental investigar de forma conjunta a expressão de SOD2 (por exemplo, a expressão tecidual da proteína) e o polimorfismo Val16Ala associados aos desfechos clínicos da doença.

1.4 Infecção viral por Papilomavírus Humano e câncer de esôfago

Além do estresse oxidativo, outros fatores estão relacionados à gênese e progressão do câncer. Estima-se que 13% de todas as doenças malignas humanas estão associadas a agentes infecciosos[90, 91], sendo o

Papilomavírus humano (HPV, do inglês *Human Papiloma Vírus)* um dos quatro principais patógenos, responsável por 690 mil novos casos de câncer no mundo em 2018 [91].

O HPV pertence à família Papillomaviridae, grupo de pequenos vírus não envelopados, epiteliotrópicos, de simetria icosaédrica **(Figura 7)**. São vírus de DNA dupla fita circular, possuindo genes que se expressam precocemente após a infecção (E1, E2, E4, E5, E6, E7 - codificam proteínas da síntese de DNA viral) e outros tardiamente (L1 e L2 - codificam proteínas estruturais do capsídeo). Também há uma região reguladora *(URR – Upstream Regulation Region ou LCR – Long control region)* que não codifica proteína, mas mantém o controle da transcrição e replicação do vírus [90].

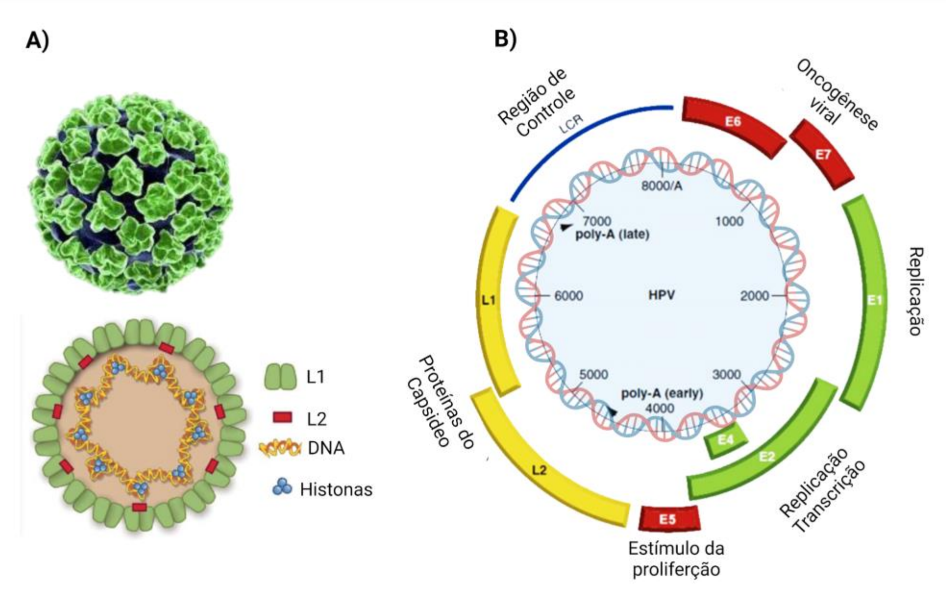

Figura 7 Estrutura viral e organização do genoma do HPV.
A) Modelo em 3D do vírus e diagrama do capsídeo, onde a proteína L1 é vista, bem como o empacotamento do genoma viral com histonas. Fonte: Adaptada de Mohamed *et al.* 2016[92]. B) Genoma circular de dupla-fita, mostrando a organização e localização dos genes. Fonte: Adaptada de Li e Kristensen 2008[93]. Created with BioRender.com

O envolvimento da infecção por HPV na etiologia do câncer esofágico foi sugerido pela primeira vez em 1982, a partir de observações histológicas de alterações celulares condilomatosas em CCE [94, 95]. Posteriormente diversos estudos foram realizados com o objetivo de verificar a associação do vírus com o desenvolvimento da neoplasia [96-101], e mais recentemente com o prognóstico[102], apresentando resultados divergentes.

A prevalência de HPV em CCE varia entre os estudos, de 0%[103] a 78%[104], sendo a prevalência média mundial de HPV nas revisões sistemáticas e metanálises de 11% a 38%[97-100, 102, 105, 106]. Na população brasileira, estudos identificaram uma frequência de HPV em CCE mais baixa, entre 0% a 16%[35, 107-111].

Em contrapartida, há um número limitado de estudos sobre a prevalência de HPV em adenocarcinomas esofágicos, sendo verificado na revisão sistemática de Li *et al*. (2017) uma prevalência mundial média de HPV de 35%, variando de 1% a 90%. Ressalta-se que na respectiva revisão nenhum estudo foi realizado na população brasileira[100].

Assim, as diferenças nas taxas de positividade de HPV entre os estudos podem estar relacionadas à variabilidade geográfica (diferenças regionais de prevalência de HPV e câncer de esôfago), assim como a diversidade de metodologias para detecção do vírus [98, 99].

Outra questão ainda não esclarecida é a transmissão do vírus para a mucosa do esôfago. De acordo com Liyange *et al.* (2013), como a mucosa esofágica é contínua com a mucosa orofaríngea, é possível um modo de transmissão de HPV semelhante, via prática de sexo oral e transmissão vertical (da mãe para o bebê durante o parto)[99].

Entretanto, para a carcinogênese além da aquisição viral é necessária a persistência da infecção e que o vírus seja capaz de promover a transformação maligna do fenótipo celular(91, 101, 112). Na maioria dos casos a infecção será assintomática, transitória e com regressão espontânea(102). Assim, vários cofatores têm sido propostos como envolvidos nesse processo, sejam eles ambientais, virais ou do hospedeiro **(Figura 8)** (113).

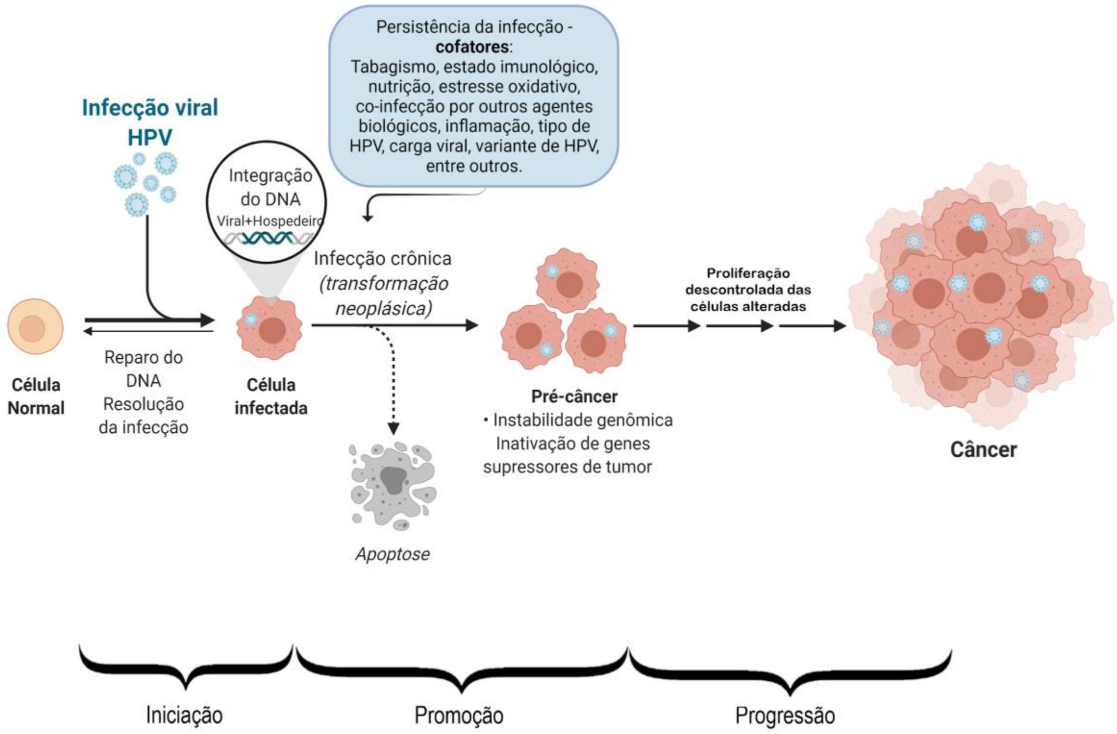

Figura 8 Processo de carcinogênese a partir da infecção pelo HPV.
Fonte: A autora. Created with BioRender.com

Atualmente, a transformação celular do HPV no câncer cervical é o modelo patogenético melhor definido(101). Os pontos chave seriam a perda da forma epissomal e integração do DNA do HPV ao genoma da célula hospedeira, bem como a expressão de oncoproteínas virais, como E6 e E7(90, 114).

A integração do HPV no genoma da célula poderia ser um fator crítico para a transformação celular, evento inicia e promove a carcinogênese [96, 115]. Durante a integração, o DNA viral é clivado na região onde se localiza os genes E1/E2, interferindo assim no controle transcricional exercido por E2 em E6 e E7. Essa interrupção resulta em aumento da expressão de E6 e E7, que desempenham um papel fundamental na imortalização e transformação neoplásica da célula hospedeira infectada [90, 99, 114].

A oncoproteína E6 interage com a proteína p53 (principal proteína supressora de tumor), o que resulta na degradação proteolítica de p53 e evasão da apoptose[96, 113, 116]. Além disso, E6 também induz a expressão do Fator de crescimento endotelial vascular (VEGF) e ativa a telomerase, uma etapa essencial na imortalização celular[117].

Alternativamente, a oncoproteína E7 induz a degradação de pRb (proteína do retinoblastoma), que é um regulador negativo do ciclo celular e associa-se com a família de fatores de transcrição E2F [96, 99, 114]. A ligação de E7 a pRb resulta no deslocamento de E2F, permitindo a produção de proteínas necessárias para a replicação do DNA[90, 114].

A interrupção do complexo pRB / E2F promovida por E7, permite por exemplo a expressão da ciclina E, um fator avançono do ciclo celular, da fase G1 para S[90, 113]. Destaca-se que ao avançar o ciclo celular nessa fase, há perda do ponto de checagem e reparo de danos no DNA, que antecede a replicação[90, 113].

E7 e E6, podem ainda ligarem-se a outras proteínas, contribuindo para o descontrole do ciclo celular em diferentes pontos de checagem (por exemplo, a ligação com inibidores de ciclina- dependente de kinases (CDKs) p21 e p27), e impedir a apoptose de células com danos no DNA irreversíveis (por exemplo, a ligação com BAK – do inglês: *BCL2 antagonist/killer*) **(Figura 9)**[90].

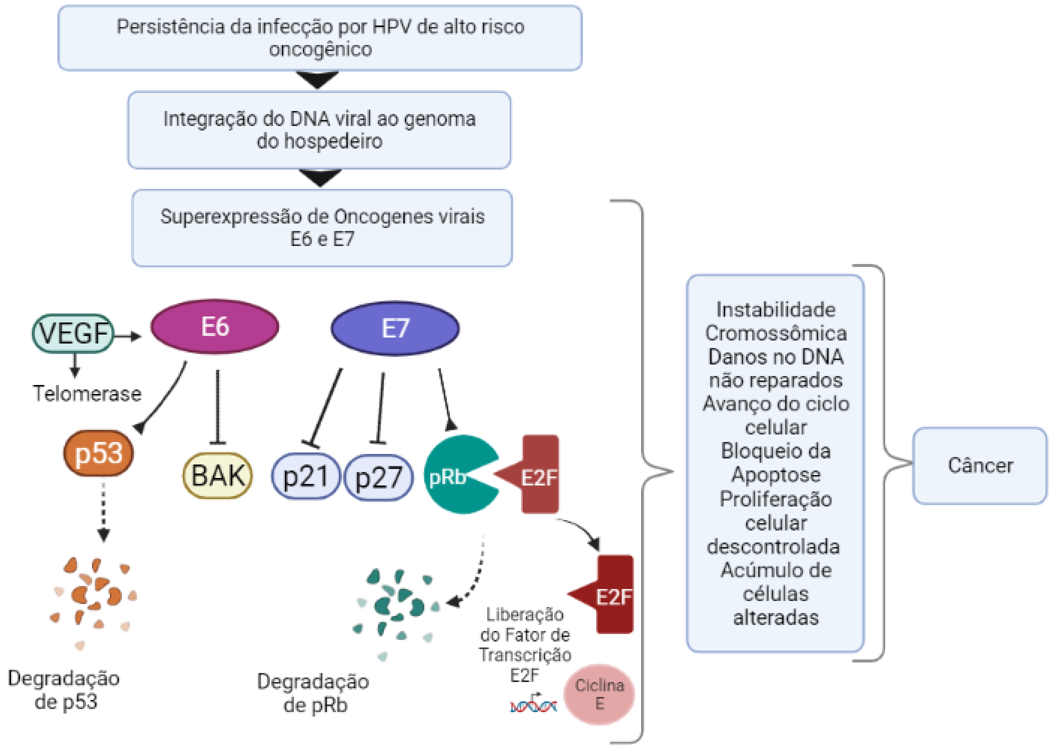

Figura 9 Oncoproteínas virais E6 e E7 e principais alterações celulares induzidas.
Fonte: A autora. Created with BioRender.com

Há mais de 200 tipos de HPV[118], com risco oncogênico diferindo entre baixo e alto, sendo relevantes para o processo neoplásico às infecções persistentes por tipos de alto risco oncogênico, pois teriam maior potencial de integração do DNA viral [119]. Entre os tipos de HPV, destaca-se o HPV-16 e o HPV-18, responsáveis por 72% de todos os casos de câncer associados à HPV[91].

Nesse contexto, o valor prognóstico do status do HPV foi investigado anteriormente em pacientes com câncer de esôfago, principalmente as infecções por HPV-16 e HPV-18. No entanto, os resultados são muito controversos em relação à CCE e escassos sobre os adenocarcinomas esofágicos[102, 109, 120-122].

Há estudos que indicaram a não associação da infecção viral com a interferência no prognóstico da neoplasia[82, 122], assim como estudos que indicaram a positividade para HPV associada a maior sobrevida global em cinco anos e sobrevida livre de progressão[120], bem como a melhor resposta à quimiorradioterapia[121].

O estudo de Guo *et al.* (2016) também indicou a possibilidade de que os pacientes HPV-16-positivos podem ter uma sobrevida significativamente favorável e que mais estudos são necessários para analisar a diferença de sobrevivência entre os diferentes genótipos de HPV[122].

Do mesmo modo como, é importante estudos com ajustes para fatores prognósticos potenciais ao comparar os resultados de sobrevida[122]. Nesse contexto, a utilização do status HPV como biomarcador prognóstico de câncer de esôfago deve ser ainda investigada, preferencialmente em conjunto com outros biomarcadores envolvidos no processo infeccioso e carcinogênico.

1.5 P16 e câncer de esofágico

A $p16^{INK4A}$ (referida como p16 ao longo do texto) é uma proteína de regulação essencial no ciclo celular e amplamente estudada como um biomarcador para diversos tipos de câncer. No processo de carcinogênese esofágica, a p16 é avaliada pelos estudos quanto a possíveis associações com alterações genéticas ou epigenéticas[123, 124] e com a infecção pelo

HPV(125-127), que causem perturbação da via da pRb de controle do ciclo celular.

A p16 é codificada pelo gene p16/*CDKN2A (inibidor de CDK, 2A)* no locus *CDKN2A* , localizado no cromossomo 9, na banda 9p21.Sua principal função é impedir a progressão do ciclo celular da fase G1 para S, ao se ligar a CDK 4/6 (quinase 4 e 6 dependente de ciclina)(124). Essa ligação inibe a formação do complexo ciclina D / CDK 4/6 quinase, que tem por função regular a fosforilação de proteínas relacionadas à progressão do ciclo celular **(Figura 10)** (124).

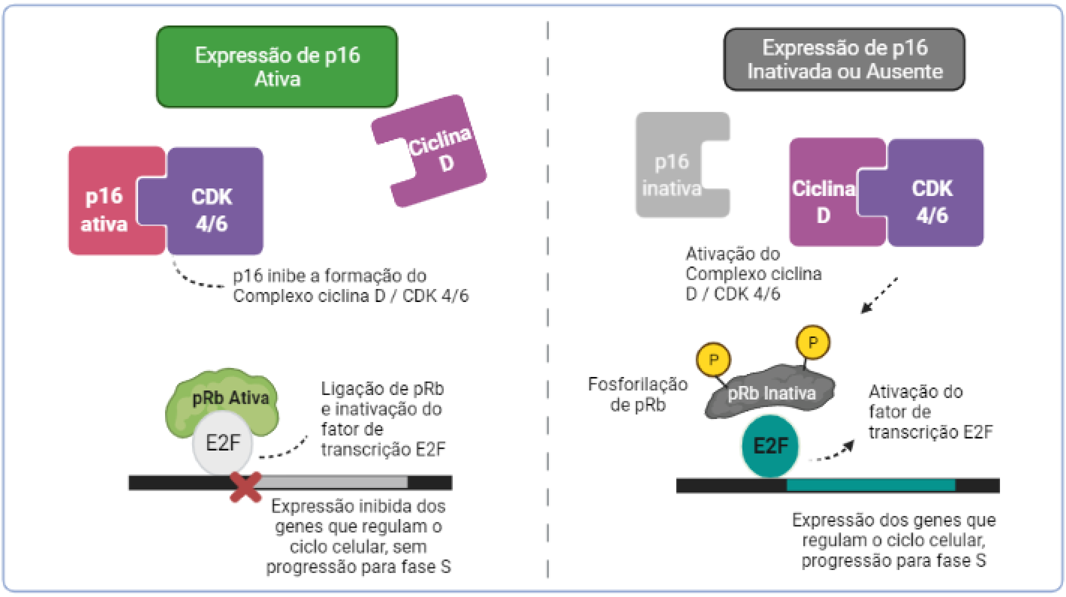

Figura 10 Esquema simplificado do papel de p16 no controle do ciclo celular.
Fonte: A autora. Created with BioRender.com

A ausência desse complexo evita a fosforilação de pRb que permanecerá na sua forma ativa (hipofosforilada) associada ao fator de transcrição E2F. Esse processo impede com que ocorra a transcrição de genes alvo de E2F, necessários para progressão do ciclo celular da fase G1 para a fase S (124, 125), mencionados anteriormente.

Essa via também parece colaborar com a cascata de sinalização mitogênica para a indução de EROs, que ativa a proteína quinase C delta, levando a uma parada irreversível do ciclo celular. Portanto, p16 participa não só da iniciação, mas também da manutenção da senescência celular. Através dos seus efeitos antiproliferativos, é considerada uma importante proteína supressora de tumor [123, 128].

Estudos têm demonstrado que a inativação de p16 é um ponto relevante no processo de carcinogênese, sendo relatados mecanismos moleculares como contribuintes para essa inativação[123]. No câncer de esôfago, a hipermetilação da região promotora do gene p16, por exemplo, causa a inativação de sua transcrição[82, 123, 124, 129, 130], favorecendo a fosforilação de pRb e liberação do fator E2F, e posteriormente, a proliferação celular. Assim, a inativação de p16 estaria associada ao maior risco de desenvolvimento neoplásico[124] e progressão da doença[82].

Inversamente, a superexpressão de p16 poderia ter um efeito benéfico em pacientes com câncer de esôfago[46, 126, 131]. Foi demonstrado que a alta expressão de p16 ou imuno-histoquímica positiva é um relevante preditor de resposta à (quimio)radioterapia em pacientes com câncer de esôfago[46, 126]. Além disso, a expressão isolada de p16 ou combinada com outros marcadores pode ser um fator de melhor prognóstico[131].

Ao longo dos anos a superexpressão de p16 em imunohistoquímica é investigada como um marcador substituto da infecção viral por HPV, indicando uma possível associação entre a infecção e a superexpressão[125-127].

De acordo com Zur Hauzen (2002), no modelo proposto com base nas observações em câncer cervical, a p16 poderia inibir a ação do oncogene viral E6. Isso ocorre, pois E6 ao ligar a p53 impede o estímulo da transcrição de p16 pelo gene supressor. Alternativamente, o oncogene viral E7 poderia controlar p16, regulando positivamente ou a inativando, e assim resgatando E6 da inibição por p16 [90].

Porém, há discrepância nos resultados dos estudos que avaliaram essa associação entre p16 e HPV para diferentes tipos de câncer, especialmente de câncer de esôfago[101, 120, 125, 132]. Esses achados aparentemente contraditórios da p16 levaram à confusão relacionada ao significado da funcionalidade da proteína na gênese e progressão do câncer.

Nesse contexto, Witkiewicz *et al.* (2011) propôs dois modelos para o significado da superexpressão de p16 em câncer, considerando uma série de estresses oncogênico distintos como causadores, incluindo danos ao DNA, envelhecimento fisiológico, e ação de oncoproteínas virais [133] **(Figura 11)**.

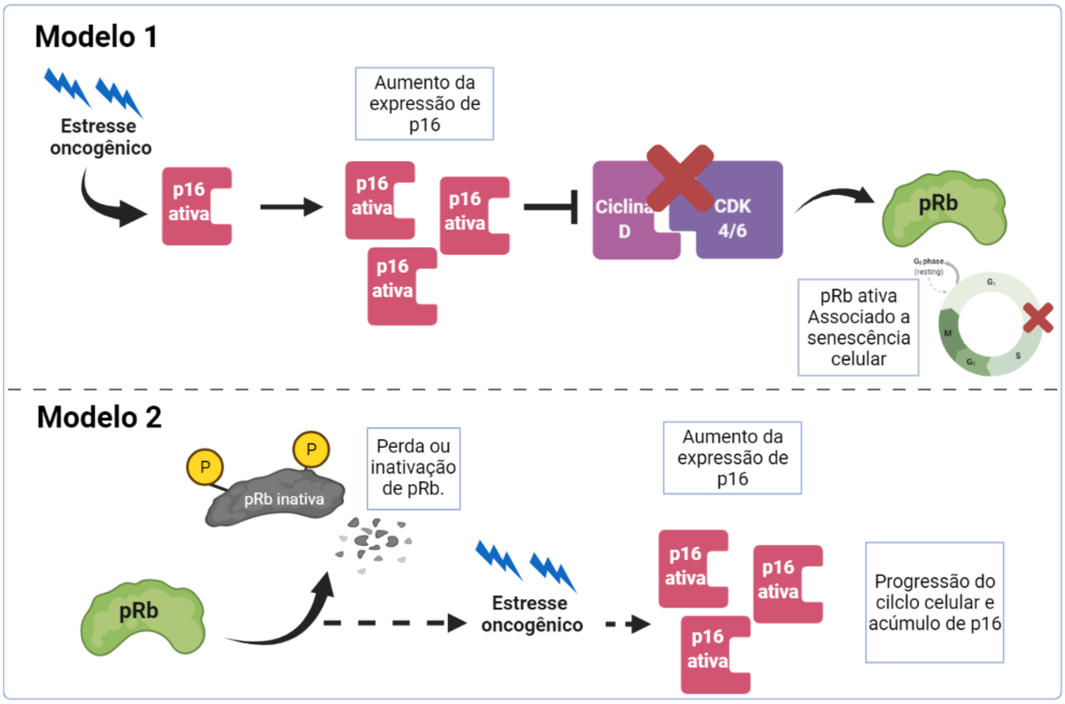

Figura 11 Vias oncogênicas para superexpressão de p16 (Modelos 1 e 2).
Fonte: Adaptado de Witkiewicz et al. 2011. Created with BioRender.com.

No primeiro modelo, os estresses oncogênicos induzem a expressão de p16 limitando a proliferação celular anormal. No entanto, este evento pode ser contornado por meio da perda de pRb, um evento secundário comum em fases avançadas facilitando a progressão da doença (133). Em um segundo modelo a perda de pRB produz um estresse oncogênico que induz a expressão de p16. Uma vez que o pRB já está comprometido, a indução de p16 não pode interromper a progressão do câncer e, portanto, os tumores se desenvolvem com altos níveis de p16 (133).

Assim, a expressão de p16 é heterogênea entre os tipos de câncer, e pode estar associada a prognósticos distintos que são modificados pelo tecido de origem, pela natureza do evento oncogênico originador e fatores associados [(133)]. Portanto, é fundamental a análise de p16 como potencial biomarcador prognóstico para câncer de esôfago, e sua real associação prognóstica com a infecção por HPV de alto risco oncogênico nesse tecido.

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3. OBJETIVOS

GERAL:

Investigar SOD2, HPV16, e p16 como potenciais biomarcadores prognósticos em câncer de esôfago.

ESPECÍFICOS:

a) Revisar na literatura as atuais evidências de associação entre o SNP Val16Ala de SOD2 e o prognóstico de pacientes com câncer (Artigo 1).
b) Identificar se o SNP Val16Ala de SOD2 e a expressão imunohistoquímica de SOD2 em câncer de esôfago estão associados à sobrevida geral (Artigo 2).
c) Avaliar a associação entre HPV16 e p16 no câncer de esôfago e sua correlação com a sobrevida geral (Artigo 3).

4. ARTIGO CIENTÍFICO REDIGIDO EM INGLÊS

4.1. ARTIGO I

"PROGNOSTIC IMPACT OF SOD2 VAL16ALA POLYMORPHISM (RS4880) IN PATIENTS WITH CANCER: A SYSTEMATIC REVIEW"

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PROGNOSTIC IMPACT OF SOD2 VAL16ALA POLYMORPHISM (RS4880) IN PATIENTS WITH CANCER: A SYSTEMATIC REVIEW

Short title: SOD2 VAL16ALA POLYMORPHISM AND CANCER PROGNOSIS

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ABSTRACT

Markers associated with oxidative stress have been widely investigated to evaluate the prognosis of patients with cancer. Superoxide dismutase 2 (SOD2), an antioxidant enzyme, plays an important role in defense against reactive species involved in inflammatory and carcinogenic processes. Previous studies have reported an association between SOD2 Val16Ala single nucleotide polymorphism (SNP) and the risk of cancer. However, the results regarding clinical and prognostic outcomes, as well as their potential as biomarkers, are divergent. This systematic review aimed to identify the prognostic impact of the Val16Ala SNP in cancer patients and the possible use of this polymorphism as a prognostic marker. We searched for original articles in electronic databases (PubMed-MEDLINE, Web of Science, and gray literature using Google Scholar). The article's search methodology and eligibility criteria are described in the review protocol PROSPERO (CRD42021254468). 1201 studies were identified, of which 12 were included in the final analysis. A total of 8 (66.6%) studies indicated an association between the Val16Ala, mainly for the Ala allele, and the prognosis of patients with cancer. Regarding the types of cancer, this association proved to be divergent. Owing to the heterogeneity of the studies, it was not possible to perform a meta-analysis on the data obtained. In conclusion, the association between the SOD2 Val16Ala SNP and oncological clinical outcomes indicated a possible impact of this polymorphism on the prognosis of patients with certain types of cancer. This current evidence encourages and directs further studies on the potential use of polymorphisms as prognostic tumor-related biomarkers.

KEYWORDS: superoxide dismutase 2, Mn-SOD, polymorphism, cancer, prognostic

1. BACKGROUND

1.1 Oxidative stress and antioxidants

It is known that some antioxidant enzymes may play a role in protecting cells against cellular oxidative stress generated by the accumulation of reactive oxygen species (ROS) in the cells. ROS induce genetic damage (oxidized bases, the formation of DNA adducts, and DNA strand breakage), which leads to an increase in the rate of mutation within cells and promotes oncogenic transformation (1).

The effects of high ROS can be counterbalanced by the action of non-enzymatic antioxidants when interrupting free radical chain reactions, or by antioxidant enzymes, which work by breaking down and removing free radicals (1, 2); low levels of ROS are crucial for maintaining normal cellular physiological functions. Along with these functions, ROS can act as signaling molecules in the cell cycle, proliferation, apoptosis, and senescence, as well as in inflammatory activation, contributing to the destruction of microorganisms and foreign bodies (2, 3).

1.2 Antioxidant enzymes, genes, and allelic variants

In aerobic organisms, the mitochondrial respiratory chain is a significant source of superoxide radical anions (O2 ●-). Inside the mitochondria, most superoxide anions are dismutated in hydrogen peroxide (H202) by the SOD manganese-dependent enzyme (MnSOD or SOD2). Soon after, the H202 formed is catalyzed in water by the enzymes catalase (CAT) and glutathione peroxidase (GPX) (1).

SOD2 is a member of the SOD family of antioxidant enzymes; however, it is the only enzyme in this family that is essential for the survival of life in aerobic environments under physiological conditions (4, 5). Experimental and epidemiological evidence supports the conflicting role of SOD2 in tumor biology (5, 6). The dual nature of the SOD2 enzyme in cancer is reflected in studies involving the widely studied Val16Ala polymorphism (rs4880) found in the gene of this enzyme (1, 4).

The SOD2 polymorphism Val16Ala is generated by swapping a single nucleotide (SNP T→C) at codon 16 in position 9, resulting in the replacement of valine (Val) with alanine (Ala) (Val16Ala) (7). Studies have shown that this SNP has functional consequences. The 16Ala variant with alpha-helical structure exhibits normal transport of the enzyme to the mitochondria, while the precursor 16Val, which has a beta leaf conformation, may have reduced enzymatic activity (7, 8).

According to Sutton et al. (2003), the Ala allele generates 30–40% more processed, matrixed, and active SOD2 homotetramers compared to the Val allele (8). In this context, depending on the genotype, different availability of SOD2 can occur in the mitochondria (4). Studies have also described a dysregulation in enzyme expression and activity, with differences in the levels of mRNA, protein, and SOD2 activity between the Ala and Val alleles (8-10).

In the global population, regarding the SOD2 Val16Ala polymorphism, the frequency of the Val allele is slightly higher than that of the Ala allele, i.e., 0.51, and 0.48, respectively. However, according to the National Library of Medicine - RefSNP report, there is a wide variation in the frequency of these alleles in published studies, from 0.26 to 0.88 for the Val allele and 0.11 to 0.74 for the Ala allele. These differences in the frequency distribution are related to the participants' ethnicity, demographics, and clinical characteristics (11).

1.3 SOD2 Val16Ala and prognosis.

Many studies have analyzed the Val16Ala-SOD2 and the risk of various types of cancer, with approximately 14 meta-analyses published on this topic. However, the prognostic impact of the Val16Ala-SOD2 polymorphism in patients with cancer is not entirely clear, indicating the importance of further reviews on this subject.

In the literature, there is a divergence between the association of each allele (Ala and Val) with risk of cancer, tumor aggressiveness, and survival. For example, while the meta-analysis performed by the Breast Cancer Association Consortium (2006) (12) described an association between the Ala-SOD2 allele and breast cancer risk, Ma et al. (2010) (13) and Cronin-Fenton et al. (2014) (14) found no significant association between SOD2 Val16Ala SNP and the risk of cancer or breast cancer recurrence.

A growing number of studies investigating the Val16Ala polymorphism in cancer patients may also indicate a possible use of this polymorphism as a tumor biomarker, and it is noteworthy that tumor markers have been widely investigated for clinical application and for the prognosis of cancer patients. Thus, studies are also needed to assess the potential of the Val16Ala-SOD2 polymorphism as a tumor prognostic marker.

1.4 Objective

In this context, the aim of this systematic review was to identify the impact of the SOD2 Val16Ala (rs4880) polymorphism in cancer patients and the possible use of this polymorphism as a prognostic marker.

2. MATERIALS AND METHODS

Observational studies evaluating the association between the SOD2 Val16Ala polymorphism and the prognosis of patients with cancer were reviewed. This review is based on recommendations and guidelines for HuGE analysis of gene-disease association studies (15), and the review protocol has been registered in the International Prospective Register of Systematic Reviews (PROSPERO) (http://www.crd.york.ac.uk/PROSPERO), registration number CRD42021254468.

2.1 Search strategy

The manuscript selection was performed using the methodology of the preferred reporting items for systematic reviews and meta-analyses (PRISMA) (16). The inclusion criteria were original manuscripts, full texts in any language, obtained from MEDLINE (PubMed), Web of Science, and gray literature by Google Scholar. Moreover, we scanned the reference lists of the identified publications for additional studies. To search for the manuscript, two main keyword descriptors were applied: “neoplasm” and “superoxide dismutase 2,” or their variations listed in **Supplementary Material 1**.

Considering that exhaustive reviews on the association between the SOD2 Val16Ala polymorphism and cancer were published until 2018, we focused on original research conducted between January 1st, 2010, and January 1st, 2020. Therefore, we analyzed the current knowledge on the subject and evaluated the clinical prognosis according to current treatments, reinforcing the importance of limited time since new treatments have been implemented in recent years for the respective neoplasms.

2.2 Selection of eligible studies

The manuscripts found in the initial search were independently reviewed by three reviewers (AVS, AJK, CMB), covering three stages: (1) title reading was performed, and we selected studies that presented all main keyword descriptors or their variations. Duplicates or publications outside the investigation period were excluded from the review. (2) Following which, abstracts of the articles selected in stage 1 were analyzed according to the eligibility criteria presented in **Table 1**. (3) Eligible articles were then approved for the full-text reading. Articles that did not assess the prognosis of cancer patients and their association with the Val16Ala-SOD2 polymorphism were excluded.

[Table 1 is here]

2.3 Data extraction and analysis

After the full-text review, the following data were extracted: first author, year of publication, study objective, country of study, ethnicity of the participants, sample number, study design (cohort or control case), selection of participants, type of cancer studied, follow-up time, results, and allele or genotype frequency distribution (according to availability in the article). The data were extracted by the three previously mentioned reviewers, and disagreements were resolved by consensus. Due to the heterogeneity of the study designs, the data were not meta-analyzed.

2.4 Quality assessment

Quality assessment was performed using the Newcastle-Ottawa Quality Assessment Scale (NOS) methodology for case-control and cohort studies. The score of each study was calculated based on three items: selection, comparability, and exposure/outcome. Studies with a score of 0–3 points were considered low-quality studies, those of 4–6 points were of moderate quality, and studies of 7–9 points were high-quality studies (17) **(Supplementary Material 2).**

In addition, it was verified whether the studies presented information regarding an assessment of the Hardy-Weinberg equilibrium (HWE) in the control groups (studies where both cases and controls were sick but differed in some other result, for example, response to treatment). A *P-value* of more than 0.05 was considered statistically significant.

3. RESULTS

We identified 1,201 articles in the databases from the keyword descriptors and their variations, and additional records identified through other sources. The PRISMA flow diagram of the literature analysis and manuscript selection is illustrated in **Figure 1**. We excluded 1,189 papers that did not meet one or more of the inclusion criteria or did not provide the required information. Finally, 12 manuscripts were selected (eight cohort studies and four case-control studies, with a total of 8,027 evaluated subjects), that met the eligibility criteria.

[Figure 1 is here]

The population investigated in the selected Val16Ala-SOD2 studies included Americans, Chinese, Czechs, Danes, Finns, and Serbs. The studies covered the following cancer types: breast cancer, prostate cancer, gastric cancer, acute lymphoblastic leukemia (ALL), urothelial bladder cancer, medulloblastoma, and supratentorial primitive neuroectodermal tumors. In general, the studies showed moderate to high quality (6–9 points). Only four articles included information about the Hardy-Weinberg equilibrium in which the *P-values* presented were >0.05 (14, 18, 19). The characteristics of these studies are summarized in **Table 2.**

[Table 2 is here]

The distribution of the genotypes/alleles and their association with the Val16Ala-SOD2 polymorphism in each study, as well as the results of the prognosis of cancer patients are presented in **Table 3.** Among the 12 included studies and the 15 clinical outcomes evaluated, eight studies (66.6%) identified a significant association between the Val16Ala-SOD2 polymorphism and the prognosis of patients with cancer.

[Table 3 is here]

The association between the Ala-SOD2 allele and clinical outcomes for cancer was described in seven studies. Ala-allele was associated with higher hepatotoxicity (19), ototoxicity (20), lower overall survival (3, 21), and lower progression-free survival (22) compared to the Val-allele. Other studies have indicated that the Ala-allele is associated with a lower risk of acute hematologic toxicity but no association with disease-free survival (23), and reduced risk of specific lethality without association with distant metastasis or biochemical recurrence (24).

One study described an association between the Val-allele and increased relapse-free survival and cancer-specific survival, however, no differences in the overall survival were reported (7). In another four studies, no statistically significant association was observed between polymorphism and overall survival (25), cancer recurrence (14), disease control rate (18), mortality or specific lethality, and circulating levels of antioxidants (26). For a better analysis of the results, the data were grouped by cancer type and are presented below.

Breast cancer

Breast cancer patients, i.e., mostly Caucasian women, were evaluated in four studies. Two studies showed an association between the Ala-allele and lower risk of hematological toxicity (grade 3 and 4 neutropenia, adaptive OR = 0.52, 95% CI, 0.29–0.92, P=0.03) in women who received adjuvant chemotherapy (23) and shorter progression-free survival for treatments containing cyclophosphamide (P=0.004), but not hormonal regimens (22).

Two studies did not identify an association between Val16Ala-SOD2 and the prognosis for disease-free survival (23) and breast cancer recurrence, (14) or the impact of the chemotherapy regimen or treatment with tamoxifen in this interaction. Only one study found that the Val-allele was protective, increased recurrence-free survival (P=0.014), and breast cancer-specific survival (P=0.026) in patients treated with tamoxifen for estrogen receptor-positive (ER+) breast cancer (7).

Gastric cancer

Gastric cancer patients were evaluated in three studies. The same researchers performed two investigations in Asian patients. In both studies by Xu *et al.* (2012) (3) and Xu e*t al.* (2015) (21), patients with the Ala-allele showed lower overall survival (P=0.009), and in the most recent study, this result was independent of the tumor stage (stage II or III, P=0.04 and P=0.015, respectively) for patients who received adjuvant chemotherapy. However, Geng *et al.* (2014) found no association between this polymorphism and disease control rate in Asian patients (18).

Prostate cancer

Prostate cancer patients were analyzed in two American studies (only one study reported that patients were Caucasian), with divergent results. In the study by Van Blarigan *et al.* (2014) (26), no statistically significant association was found between genotypes and the risk of lethal prostate cancer or prostate cancer-specific mortality. A similar result was observed for the impact of the circulating antioxidant levels in this interaction. However, in the study by Margalit *et al.*, (2015) (24), the Ala-allele was associated with a statistically significant reduction in the risk of lethal prostate cancer (HR 0.37 for Ala/Ala and HR 0.84 for Val/Ala, P=0.04), compared to the Val-allele. Only the study by Van Blarigan *et al.* (2014) reported the race of the participants, which was Caucasian (26).

Other cancers

Patients with medulloblastoma and primitive supratentorial neuroectodermal tumors of different ethnicities were evaluated in the study by Brown *et al.* (2015). The Ala-allele was associated with a greater chance of developing treatment-related ototoxicity (defined as using a hearing aid for more than one year) (OR=2.16, 95% CI, 1.06–4.38), maintaining a significant association after adjustment for clinical factors (age at diagnosis, sex, ethnic group, cumulative dose of cisplatin, and doses of craniospinal irradiation ≥34 Gy) and correction for multiple comparisons (OR=3.06, 95% CI, 1.30–7.20, FDR q = 0.040) (20).

Also, Alachkar *et al.*, (2017) evaluated ALL in Caucasian patients (19). Ala-allele homozygous had a higher frequency of hepatotoxicity (grade III or IV of both aspartate transaminase (AST) and alanine transaminase (ALT); grade IV of either AST or ALT) associated with asparaginase-based therapy than those with the Val-allele (OR = 2.53; 95% CI, 1.3–4.8, P= 0.006).

Finally, a study conducted by Nikic *et al.*, (2018) evaluated patients diagnosed with urothelial bladder cancer, and did not identify an association between SOD2 polymorphisms and overall survival for patients treated with cisplatin-based chemotherapy (25).

4. DISCUSSION AND CONCLUSIONS

In this systematic review, 66.6% of the studies suggested that the SOD2 Val16Ala polymorphism was associated with the prognosis of cancer patients (3, 7, 19-24). Although few reports have found associations between the Val allele and prognosis, most studies have shown that the Ala-allele is associated with the prognosis of different types of cancer.

However, these results must be interpreted with caution, as reports of the associations between polymorphisms and better or worse cancer prognosis remains controversial. The results of the original manuscripts have not yet established a consensus on the association between polymorphism and overall survival, specific mortality, or lethality (3, 21, 24-26).

In this context, the results of the reviewed studies may suggest a differential modulation of the SOD2 polymorphism depending on the tumor microenvironment and other carcinogenic factors. According to Wang et al. (2018), the SOD2 Val16Ala polymorphism can be modulated by internal and external factors, which could contribute to specific clinical outcomes (27).

To better interpret and discuss cancers heterogeneity, we grouped the results by types of cancer and the description of interactions with oncologic treatments. Breast cancer, gastric cancer, and prostate cancer have been the most studied cancer in the last decade. However, even after grouping the studies, we observed varying results for the same type of cancer. Due to the number of studies, the different clinical outcomes, and the stratification formats of the treatment groups, it was not possible to meta-analyze the data, which is considered a limitation of the study. However, in addition to the identified evidence, this systematic review presents the main hypotheses in the literature regarding the prognostic impact of Val16Ala-SOD2 for different types of cancer.

According to the literature, there may be differences in mitochondrial availability, expression, and antioxidant activity of the SOD2 enzyme between the Ala and Val alleles, influencing the clinical evolution of patients with cancer, with no prognostic impact of Val16Ala-SOD2. The Ala-allele would have a higher rate of dismutation, catalyzing the conversion of superoxide into H2O2, suggesting that

Ala/Ala and Ala/Val individuals may have higher SOD2 activity. However, this increased efficiency is not necessarily accompanied by GPX enzyme activity, resulting in excessive H2O2 generation, (4, 28) and may contribute to cell dysregulation, prevention of apoptosis, progression of the transformed phenotype, and poorer prognosis (3, 20, 28-32). It is noteworthy that the Ala-SOD2 allele is not uncommon, according to Ekoue, 25% of people are homozygous for this allele (28).

Another issue that may influence the results of the impact of the Ala-SOD2 allele on cancer progression concerns the reports stating that the Val-SOD2 allele can also influence the progression and aggressiveness of certain types of cancer. As the superoxide anion has a high affinity for nitric oxide, the uncontrolled reaction of these two molecules produces nitrosative molecules, such as peroxynitrite, and can also give rise to other ROS, including H2O2, leading to states of oxidative stress that can favor disease progression (4, 33).

In this context, two studies on breast cancer conducted by the same research group found divergent associations. While the Ala-allele was associated with breast cancer risk in Brazilian women and men (34), metastases in the axillary lymph nodes (LN+) were significantly associated with the Val-allele homozygous (35). Studies with greater control of intervening variables are needed for further clarification.

Intervenient factors such as blood levels of non-enzymatic and dietary antioxidants, smoking, alcohol consumption, consumption of hot beverages, ethnicity, pathological features of the tumor, chronic inflammation, and difference in the level of SOD2 expression according to the type of tumor tissue should also be considered when assessing the prognosis (23, 30, 33, 36-45). Thus, we must consider that the populations analyzed in the studies assess participants belonging to different ethnicities with different physiological states, lifestyles, and dietary habits.

On the other hand, it is possible that lifestyle-related factors, especially diet, physical activity, and stress control, could attenuate the impact of higher basal levels of H2O2 in patients with the Ala-allele; thus decreasing the influence of this genetic variant on the prognosis of the disease. The included studies assessed some of these factors, but used different methodologies, making it difficult to compare the results. Thus, patronized analyses of these factors should also be conducted in a larger, complementary study.

Another relevant question concerns the impact of oncological treatments on basal levels of H2O2 associated with SOD2 Val16Ala SNP, and whether this polymorphism can interfere with the efficacy, effectiveness, and safety of antitumor treatments and drug resistance signaling pathways. Cancer cells survive under low hypoxic stress to malignant progression and chemoresistance, while prolonged stress triggers cell death (19, 25, 46).

Thus, carriers of the Val allele would have a better prognosis due to the lower efficiency of the enzyme, increased ROS, and apoptosis of neoplastic cells compared to carriers of the Ala allele. This association was observed in patients who received adjuvant monotherapy with tamoxifen for ER+ breast cancer (7, 47). Clinically, patients with ER+ breast cancer are treated with anti-hormonal therapy, with several selective estrogen receptor-modulating molecules (MSREs), such as tamoxifen. However, studies have shown that the function of SOD2 can also be modulated by estrogen; therefore, it may be relevant to the progression and treatment of breast cancer (22, 48).

There is evidence that the Ala-SOD2 allele can also increase chemotherapy resistance. Chemotherapy drugs, such as platinum, taxane, and fluorouracil, can promote cancer cell death by inducing oxidative stress to highly toxic levels. However, the increased activity of Ala-SOD2 could reduce the concentration of the superoxide anion, protecting neoplastic cells from cell death by oxidative damage (21, 23, 47). Another hypothesis is that excessive generation of H2O2 would occur in this process, which is also associated with resistance to treatment. In treatments with cisplatin, negative regulation or inhibition of other antioxidant system components necessary for the metabolic reduction of H2O2 was identified (20). Excess ROS can lead to inactivation of the tumor suppressor PTEN (chromosome 10 deleted phosphatase and tensin homolog), oxidation, and activation of the PI3K/Akt pathway, which is a relevant signaling pathway related to drug resistance (49).

In conclusion, evidence suggests that SNP Val16Ala-SOD2 (especially Ala-SOD2) influences cancer prognosis to some degree, positively or negatively depending on the each study, due to the correlation of superoxide-hydrogen peroxide imbalance, although genetic and environmental factors can strongly influence this association. We emphasize the importance of this review by listing the main evidence and hypotheses regarding the prognostic impact of Val16Ala-SOD2 in cancer patients.

New studies need to be conducted on the basis of the evidence cited here. Further investigations are needed to elucidate possible differences in the role of polymorphisms in the progression of each type of cancer, allowing the projection of better therapeutic strategies and the applicability of this polymorphism as a potential prognostic biomarker.

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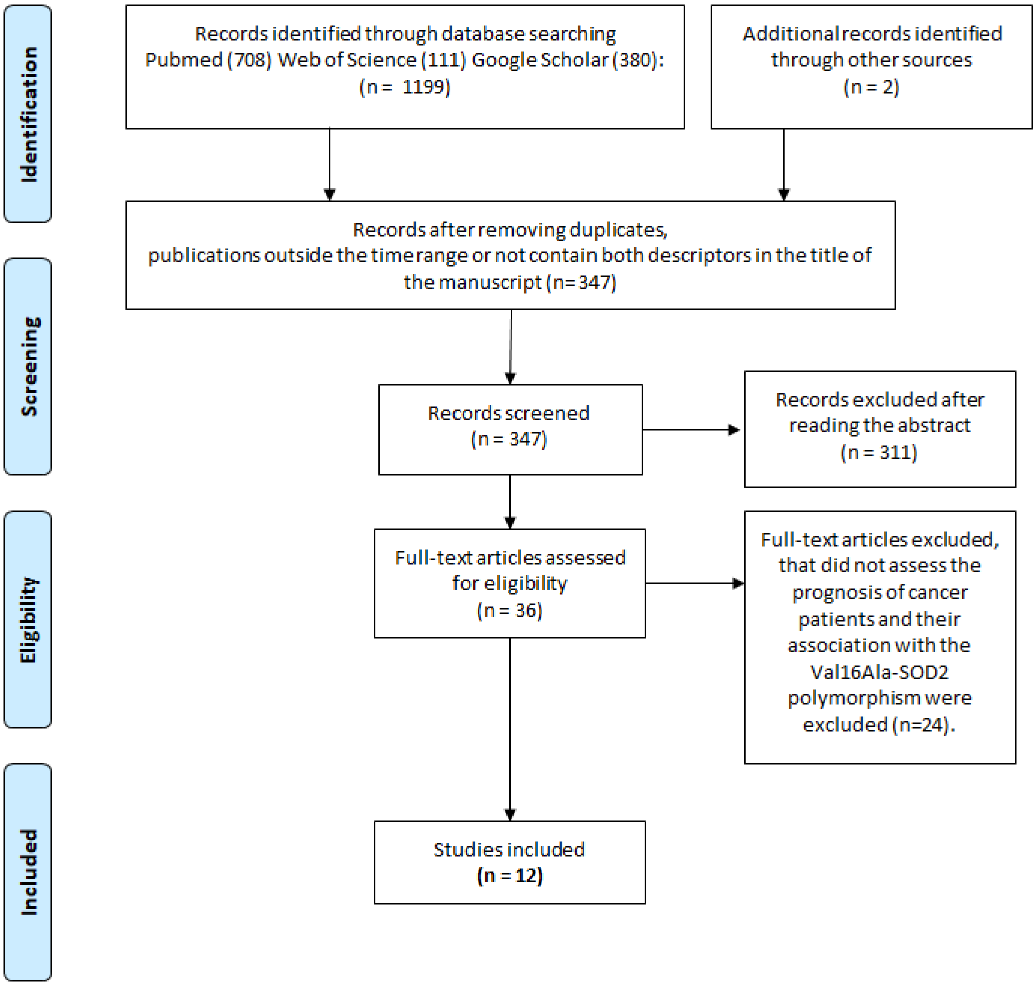
FIGURES LEGENDS

Figure 1. Flow Diagram PRISMA of study selection



TABLES

Table 1. Eligibility criteria for evaluated studies.

Study characteristics	**Inclusion criteria**	**Exclusion criteria**

Design	• Observational studies
	  o Cohort or Case-Control
	  o Human or Part with Human
		• Trial
		• Review
		• Totality cell culture
		• Animal model study

Publication	• Abstract and full text available • Any language • When multiple publications reported the same or overlapping data, we use the most recent or largest population, as recommended	• Dissertation • Conference proceeding, abstract or poster • Or it was a duplication of a previous study
Participants	• Female or Male • No age restriction • All cases were first diagnosed as cancer	• Not applicabe
Intervention	• Not applicable	• Not applicable
Comparator(s)	• Not applicable	• Not applicable
Outcomes	• Provide the allele or genotype frequencies of the SOD2 Val16Ala (rs4880) polymorphism • The study performed a quantitative analysis of the association between cancer and this polymorphism • The study had enough data to calculate the hazard ratio (HR) or odds ratio (OR) with a 95% confidence interval (CI), *P- value* (P)	• Genotype frequency data not available, incomplete or grouped in analysis of interaction with other polymorphisms • Statistical data from the quantitative analysis regarding the outcome, association between cancer and polymorphism, are not available.

Table 2. The characteristics of the studies included.

Author Year	Country	Ethnicity	Cancer Type	Design Study	Genotype method	HWE	NOS score
Yao et al 2010	EUA	American	Breast cancer	Case-Control	MALDI-TOF	NA	9
Xu et al 2012	China	Asian	Gastric cancer	Cohort	Multiplex SNaPshot	NA	8
Hubackova et al 2012	Czech republic	European	Breast cancer	Cohort	Real-time PCR -TaqMan	NA[1]	8
Cronin-Fenton et al 2014	Denmark	European	Breast cancer	Case-Control	Real-time PCR -TaqMan	P=0.07	7
Tengström et al 2014	Finland	European	Breast cancer	Cohort	PCR-RFLP	NA	9
Geng et al 2014	China	Asian	Gastric cancer	Cohort	Real-time PCR -TaqMan	P = 0.703	6
Van Blarigan et al 2014	EUA	American	Prostate cancer	Cohort	MassARRAY	NA[1]	6
Margalit et al 2015	EUA	American	Prostate cancer	Cohort	MassARRAY	NA[1]	6
Brown et al 2015	EUA	American	Medulloblastoma and supratentorial Primitive Neuroectodermal Tumors	Case-Control	HumanOmni1-Quad BeadCHip	P=0.57	7
Xu et al 2015	China	Asian	Gastric cancer	Cohort	Multiplex SNaPshot	NA	9
Alachkar et al 2017	EUA	American	Acute Lymphoblastic Leukemia (ALL)	Cohort	Real-time PCR -TaqMan	P=0.32	6
Nikic et al 2018	Serbia	European	Urothelial bladder cancer	Case-Control	Real-time PCR -TaqMan	NA	7

Legend: HWE: Hardy-Weinberg equilibrium ($P>0.05$); NA: Not available in the articles; NA[1]: values not available in the articles, but text reports that the rs4880 SNP was in HWE; PCR: polymorphism chain reaction real time; MALDI-TOF: matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry.

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Table 3. Association between SOD2 Val16Ala Polymorphism and prognosis for cancer.

Author / Year	Cancer Type	Outcomes	Treatment	Val/Val	Val/Ala	Ala/Ala	Study size	Finding
Yao et al 2010	Breast cancer	AHT and DFS	Surgery, adjuvant chemotherapy with or without tamoxifen treatment	Treated group: 25% Untreated group (not receive adjuvant therapy): 24%	Treated group: 50% Untreated group (not receive adjuvant therapy): 50%	Treated group: 25% Untreated group (not receive adjuvant therapy): 26%	Treated group: 458 Untreated group: 874	Ala/Ala was associated with a lower risk of AHT (OR= 0.52, P=0,03) for women receiving adjuvant chemotherapy. The results of the DFS curve were not statistically significant.
Hubackova et al 2012	Breast cancer	PFS	Surgery, adjuvant therapy, hormonal regimens	24%	53%	23%	320	Ala/Ala treated by regimens containing cyclophosphamide, but not hormonal regimens, had PFS significantly less than those with the Val allele (P=0.004). Negative expression of ER and PR there was also a similar association (P=0.001)
Xu et al 2012	Gastric cancer	OS	Surgery, Adjuvant chemotherapy or radiotherapy	74%	24%	2%	900	Val/Ala + Ala/Ala were correlated with a shorter OS (P=0.009).
Cronin -Fenton et al 2014	Breast cancer	Breast cancer recurrence	Surgery, adjuvant chemotherapy and adjuvant radiation therapy	Case: 30% Control: 32%	Case: 40% Control: 43%	Case: 30% Control: 25%	Case: 117 [a]Control: 209	No statistically significant association.
Tengström et al 2014	Breast cancer	OS, Breast cancer-specific survival, and	Surgery, chemotherapy, radiotherapy, and hormonal	28%	58%	14%	64	Val allele was associated with an increased RFS (P=0.014) and breast cancer-specific survival (P=0.026) in patients treated

		RFS.	treatment					with Tamoxifen ER+. The results of the OS were not statistically significant.
Geng et al 2014	Gastric cancer	DCR	EOF treatment	Controlled*: 82.6% Uncontroled: 57.2%	Controlled*: 17.4% Uncontroled: 33.3%	Controlled*: 0% Uncontroled: 9.5%	Controlled*: 86 Uncontroled: 21	No statistically significant association.
Van Blarigan et al 2014	Prostate cancer	Prostate cancer-specific mortality, risk of lethal prostate cancer, and circulating antioxidants level	NA	Cohort HPFS: 24% Cohort PHS: 25% / Total:25%**	Cohort HPFS: 49% Cohort PHS: 50% / Total:50%**	Cohort HPFS: 27% Cohort PHS: 25% / Total:25%**	Cohort HPFS: 1158 Cohort PHS: 1126 / Total: 2284**	No statistically significant association.
Margalit et al 2015	Prostate cancer	Risk of lethal prostate cancer, distant metastasis or biochemical recurrence	Radiation therapy and radical prostatectomy	Radiotherapy: 26% Radical Prostatectomy: 24%***	Radiotherapy: 48% Radical Prostatectomy: 50%***	Radiotherapy: 26% Radical Prostatectomy: 26%***	Radiotherapy: 707 Radical Prostatectomy: 981***	Ala allele was associated with a reduction in the risk of lethal prostate cancer (HR 0.37 for homozygous C / C and HR 0.84 for T / C genotype, P=0.04) compared to the Val/Val, in the patients treated with radiation therapy. No significant association for other outcomes, or radical prostatectomy, or validation cohort.
Brown et al 2015	Medulloblastoma and PNET	Ototoxicity	Chemotherapy, craniospinal irradiation, amifostine therapy	Case: 11% Control: 40%	Case: 58% Control: 40%	Case: 31% Control: 20%	Case: 26 [a]Control: 45	Ala allele is associated with a greater chance of a child developing treatment-related ototoxicity (OR=2.16, 95%CI:1.06-4.38).
Xu et al 2015		OS	Curative surgery,	Chemotherapy: 75% No	Chemotherapy: 23% No	Chemotherapy: 2% No	Chemotherapy: 201 No	Val/Ala and Ala/Ala increased the risk of death (HR=2.042,

	Gastric cancer		with or without adjuvant chemotherapy.	chemotherapy: 74%	chemotherapy: 23%	chemotherapy: 3%	chemotherapy: 299	95%CI:1.298-3.212) and correlated with shorter OS, when compared to the Val/Val, regardless of the tumor stage (II or III, P =0.04 and P=0.01) for patients who received adjuvant chemotherapy.
Alachkar et al 2017	ALL	Hepatotoxicity	Asparaginase	25%	46%	29%	190	Ala/Ala had a higher frequency of hepatotoxicity than those with the Val/Val or Va /Ala (Fisher's exact test =0.006; OR = 2.5)
Nikic et al 2018	Urothelial bladder cancer	OS	Chemotherapy	Case: 27% Control: 31%	Case: 52% Control: 47%	Case: 21% Control: 22%	Case: 33 [a]Control: 212	No statistically significant association.

Legend: ALL:Acute Lymphoblastic Leukemia. PNET: Supratentorial Primitive Neuroectodermal Tumors. AHT:Acute hematological toxicity. Disease-free survival: DFS. Progression-free survival: PFS. Overall survival: OS. Relapse-free survival: RFS. Disease control rate: DCR; Ototoxicity: use of a hearing aid device greater than 1 year removed from the completion of primary therapy. Hepatotoxicity: as grade 3/4 of both AST and ALT, grade 4 of either AST or ALT. EOF treatment: Epirubicin, oxaliplatin, and 5-FU. ER: estrogen receptors. PR: Progesterone receptor. *Patients with remission, partial remission and stable disease were considered as controlled. Patients with progressive disease were considered to be uncontrolled. **The distribution of genetic variants was similar between the HPFS and PHS cohorts and the analyzes presented were conducted in the combined population. ***Test cohort data. [a]Control group:Selection criteria must be consulted in each study.

Supplementary Material 1 - Synonym search descriptors

Query MEDLINE (Pubmed)

Search ("neoplasm"[MeSHTerms]) OR cancer*[Title]) OR carcinoma*[Title]) OR tumor*[Title]) OR tumors*[Title]) AND "superoxide dismutase 2"[MeSH Terms]) OR superoxide dismutase-2*[Title]) OR Mn-SOD*[Title]) OR SOD2*[Title]) OR SOD 2*[Title]) OR manganese superoxide dismutase*[Title]) OR mn-sod*[Title]) OR mn sod*[Title]) OR mitochondrial superoxide dismutase*[Title]) OR mitochondrial superoxide dismutase 2*[Title]) Sort by: Title Filters: Journal Article; Full text; Publication date from 2010/01/01 to 2020/01/01

Query Web of Science

(TI=(superoxide dismutase 2 OR superoxide dismutase-2 OR SOD2 OR manganese superoxide dismutase OR mn-sod OR mn sod OR mitochondrial superoxide dismutase 2 OR mitochondrial superoxide dismutase-2)) AND TIPOS DE DOCUMENTO: (Article)

Índices=SCI-EXPANDED, SSCI, A&HCI, CPCI-S, CPCI-SSH, ESCI Tempo estipulado=2010-2020)

AND

(TI=neoplasms OR cancer OR cancers OR neoplasia OR carcinoma OR tumor OR tumors)) AND TIPOS DE DOCUMENTO: (Article)

Índices=SCI-EXPANDED, SSCI, A&HCI, CPCI-S, CPCI-SSH, ESCI Tempo estipulado=2010-2020

Google Scholar

allintitle: (cancer OR neoplasm OR neoplasia OR carcinoma OR tumor OR tumores) (SOD2 OR “mnSOD” OR “Mn SOD” OR SOD2 OR SOD OR “mitochondrial superoxide dismutase” OR “manganese superoxide dismutase” OR “superoxide dismutase”

Supplementary Material 2 - Methodological quality of the included studies according to the Newcastle-Ottawa Scale (NOS)

		Selection				Comparability	Outcome			
Cancer	**Cohort Study**	**Representation of the exposure cohort (0 or 1)**	**Selection of the non exposed cohort (0 or 1)**	**Ascertainment of exposure (0 or 1)**	**Demonstration that outcome of interest was not present at start of study (0 or 1)**	**Comparability of cohorts on the basis of the design or analysis (0, 1 or 2)**	**Assessment of outcome (0 or 1)**	**Follow-up adequacy (0 or 1)**	**Adequacy of follow up of cohorts (0 or 1)**	**Total score**
Breast cancer	Yao et al 2010	*	*	*	*	**	*	*	*	9
Breast cancer	Hubackova et al 2012	*	*	*	*	*	*	*	*	8
Breast cancer	Tengström et al 2014	*	*	*	*	**	*	*	*	9
Prostate cancer	Van Blarigan et al 2014	-	*	*	*	*	*	*	-	6
Prostate cancer	Margalit et al 2015	-	-	*	*	**	*	-	*	6
Gastric cancer	Xu et al 2012	*	*	*	*	*	*	*	*	8
Gastric cancer	Xu et al 2015	*	*	*	*	**	*	*	*	9
Gastric cancer	Geng et al 2014	*	*	*	*	*	*	-	-	6
ALL	Alachkar et al 2017	*	*	*	*	*	*	-	-	6

Cancer	Case-Control Study	Selection					Comparability	Outcome		Total score
		Definition adequate of the Cases (0 or 1)	**Representativeness of the Cases (0 or 1)**	**Selection of the Controls (0 or 1)**	**Definition adequate of the Cases (0 or 1)**	**Comparability of cases and controls on the basis of the design or analysis (0, 1 or 2)**	**Assessment of outcome (0 or 1)**	**Same method of ascertainment for cases and controls (0 or 1)**	**Non-Response rate (0 or 1)**	
Breast cancer	Cronin-Fenton et al 2014	*	*	-	*	**	*	*	-	7
Medulloblastoma and supratentorial Primitive Neuroectodermal Tumors	Brown et al 2015	*	*	-	*	*	*	*	*	7
Urothelial bladder cancer	Nikic et al 2018	*	*	-	*	*	*	*	*	7

Lgend: ALL, Acute Lymphoblastic Leukemia.The star system (*) was used to allow a semi-quantitative assessment of study quality. A study was awarded a maximum of one star (*) for each numbered item within the selection and outcome categories. A maximum of two stars (**) was awarded for comparability. The Total score NOS ranged from zero (-) to nine stars or points. Studies with a score of 0-3 points were considered to be low-quality studies; 4-6 points, studies of moderate-quality; and 7-9 points, high-quality studies

4.2. ARTIGO II

“Impact of the association of Val16Ala-SOD2 SNP and SOD2 expression in the prognosis of patients with esophageal cancer.”

Aniúsca Vieira dos Santos, Antonella Jacobsen Kaul, Giovana Tavares dos Santos, Maiquidieli Dal Berto, Giuliano Rizzoto, Adriana Vial Roehe, Rita de Cassia Sant’Anna Alves, Andreas Timóteo Lutz, Plauto Erasmo Beck, Rafael José Vargas Alves, Ivana Beatrice Mânica da Cruz, e Claudia Giuliano Bica.

Enviado para publicação na Revista

“Journal of Cancer Research and Clinical Oncology”

Impact of the association of Val16Ala-SOD2 SNP and SOD2 expression in the prognosis of patients with esophageal cancer.

Short title: Val16Ala-SOD2 polymorphism and esophageal cancer

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ABSTRACT

Introduction: Esophageal cancer is a serious public health problem worldwide, and it is necessary to identify biomarkers related to its risk and progression. Previous studies indicate that a polymorphism found in the gene for the enzyme Superoxide dismutase 2 of esophageal cancer. However, further investigations in other populations are needed to clarify the role of this polymorphism in disease risk and progression. **Objective:** Therefore, the present study evaluated the role of the Val16AlaSOD2 SNP in the risk of esophageal cancer and in the survival of Brazilian patients. **Methods:** Observational study of patients with esophageal cancer and healthy individuals. Samples from tumors previously fixed with formalin were used to perform Val16Ala-SNP genotyping, and SOD2 expression tissue levels by immunohistochemistry. Risk and overall survival analyzes were performed. **Results:** The Val16Ala SNP (Ala-SOD2) was associated with an increased risk of esophageal cancer (RR 2.18, 95%CI 1.23-3.86), regardless of age and gender, but did not have a significant effect on patient survival. Meanwhile, weak SOD2 expression was significantly associated with poor overall survival after treatment, independent of other clinicopathological variables (HR, 0.41; 95% CI, 0.22-0.79 P = 0.07). **Conclusions:** Val16Ala-SOD2 (Ala-allele) has been associated with esophageal cancer, and the immunohistochemistry expression of SOD2 was an independent prognostic marker.

KEYWORDS: superoxide dismutase 2, Val16Ala-SOD2, biomarker, esophageal cancer

BACKGROUND

Esophageal cancer is the seventh most common cancer, with a five-year survival rate of less than 20% (Bray et al., 2021, Zhao et al., 2019). In Brazil, the estimated annual incidence rate is 11,390 cases for 2020-2022, mostly in the country's southern region (INCA, 2019).

In this context, the role of antioxidant enzymes in pro and antitumor activities has been evaluated in the search for new predictive and prognostic biomarkers in esophageal cancer. The main factor for this is the involvement of genetic alterations (Uhlenhopp et al., 2020) in the pathogenesis of cancer, which may be caused by DNA damage associated with excess reactive oxygen species (ROS) (Sharifi-Rad et al., 2020).

Among these enzymes, Superoxide Dismutase (SOD2) stands out evaluated in esophageal cancer by role in the front line of cellular defense against excess ROS (Cheng et al., 2009; di Martino et al., 2007; Murphy et al., 2017; Sun et al., 2010). This enzyme catalyzes the conversion of the superoxide anion (O2 • -) to hydrogen peroxide (H2O2), which can be further neutralized to H2O and oxygen by catalase (CAT) and glutathione peroxidase (GPX) (Kim et al., 2017).

Differences in tissue SOD2 expression have been observed in esophageal cancer compared to normal tissue (Janssen et al., 2000). However, the role of the enzyme in the carcinogenic process is still contradictory (Hermann et al., 2005; Ma et al., 2014; Sun et al., 2011; Toh et al., 2000; Zuo et al., 2019). The Val16Ala (rs4880) SOD2 polymorphism has been widely analyzed in different types of cancer. Val16Ala is a single nucleotide polymorphism (SNP) (substitution T → C at codon 16 at position 9), resulting in the replacement of valine (Val) by alanine (Ala) (Tengström et al., 2014).

A dysregulation of enzyme expression and activity was identified between the Ala and Val alleles (McAtee & Yager, 2010; Sutton et al., 2003), and associated with risk for different types of cancer (Ekoue et al., 2017; Wang et al., 2018), but its role of esophageal cancer is not fully understood. In this context, whether tissue expression levels of SOD2 and Val16Ala-SNP can be used as biomarkers to diagnose or predict esophageal cancer prognosis remains unknown.

Therefore, this study aims to evaluate 1) the association of Val16Ala-SOD2 SNP with the risk of esophageal cancer, and 2) the association the Val16Ala-SOD2 SNP and SOD2 expression in the prognosis of patients with esophageal cancer.

METHODS

This observational study is according to REporting recommendations for tumour MARKer prognostic studies (REMARK) (McShane et al., 2005).

Study subjects

The patients with esophageal cancer from undergoing esophagectomy, who were treated at the Santa Casa Hospital Complex (CHSC) from January 2010 to December 2017 were included. Medical records, images, and pathological tests were reviewed to collect demographic and clinical information. For genotype analysis and immunohistochemistry (IHC) of esophageal cancer patients, biological material (formalin-fixed paraffin-embedded tissue - FFPE) available were included. The esophageal cancer samples used were reviewed and classified by 2 independent pathologists. Demographic and clinicopathological variables initially examined, SOD2 rs4880 genotypes, and SOD2 IHC levels are shown in **Supplementary Material.**

Information on the genotype of healthy individuals residing in the same geographic region as the patients with esophageal cancer in this study (Rio Grande do Sul, Brazil), matched for age and sex, was identified from a SNP SOD2 rs4880 database. Demographic information and the SOD2 rs4880 genotyping protocol of individuals in this database are available in the study by Jung et al 2019. The database does not include SOD2 IHC information and, therefore, this variable was not evaluated in the analysis of risk for esophageal cancer in our study.

This study is in accordance with the Declaration of Helsinki, the Universal Declaration of Bioethics and Human Rights and Resolution 466/2012 of the National Health Council of Brazil. The study was approved by the Research Ethics Committees, and the informed consent of the participants or legal representatives was obtained.

Esophageal Cancer - Analyses

1. *SOD2 Immunohistochemistry (IHC)*

We used Anti-SOD2/MnSOD, ab13534, Abcam® (Rabbit Polyclonal Antibody, 1: 600 dilution; Control: Colon human) in esophageal tumors. Tissue sections were heated (75°C, 30 min), deparaffinized (three washes in xylenes), and rehydrated by successive washes in different concentrations of alcohols and deionized water. For immunohistochemical staining, antigen retrieval was performed at 98°C for 40min in pH 9.0 TRIS-EDTA. Endogenous peroxidase activity was blocked with hydrogen peroxide (5%) in methanol (3 × 10 min). Afterwards, the slides were washed (3x) with phosphate-buffered saline (PBS) and incubated in a solution to block non-specific binding (1% bovine serum albumin for 1 h).

A negative control (bovine serum albumin 1%) was substituted for the primary antibody. All primary antibodies and controls were incubated for 1h at room temperature and then submitted to temperatures of 4°C overnight. After that, the slides were kept at room temperature for 1h, in sequence, and were washed thrice with PBS. The sections were incubated with the EnVision + Dual Link System-HPR (Agilent DAKO) for 30 min, then washed again with PBS. Staining was completed by incubating the specimens with DAKO Liquid DAB + Substrate Chromogen System for 5 min.

Finally, tissue sections were counterstained with haematoxylin-harris solution and slides mounted in non-aqueous medium. Then, slides were mounted in a non-aqueous medium. Two pathologists not knowing the patients' information were responsible for assessing the results. IHC was successful in 120 specimens, and expression levels were determined based on the percentage of positive cells and staining intensity.

According to the Immunoreactivity Score (IRS) (Fedchenko,Reifenrath 2014), the percentage of positive cancer cells was divided into five levels: 0 (no positive cells), 1 (10% of positive cells), 2 (10-50% positive cells) 3 (51-80% positive cells) and 4 (>80% positive cells). The intensity of staining was classified as: 0 (no staining), 1 (weak staining); 2 (moderate staining); and 3 (intense staining). The final score of SOD2 expression (multiplication of percentage of positive cells and intensity of staining) was graded as (0 – 1) negative, (2-3) weak, (4-8) for moderate, and (9-12) strongly positive **(Figure 1).**

2. *Genotype SOD2 (rs4880) - Real Time-PCR (qPCR)*

SOD2 genotyping was performed for esophageal cancer tissue. Genomic DNA was extracted from 10 micrometers of tissue slices of formalin-fixed paraffin-embedded (FFPE) with the Magnetic Method to Purify DNA from FFPE Tissue/ MagneSil® Genomic, Fixed-Tissue System (PROMEGA) kit following the manufacturer specifications. All DNA samples were quantified using NanoDrop 2000 (ThermoScientific) and maintained at − 20 °C until the Polymerase chain reaction (PCR).

Isolated DNA was subjected StepOnePlus Real-Time PCR System in final volume of 10μL, containing 1μL of DNA sample (10-50ng/μL), 0,25μL of probe TaqMan™ SNP Genotyping Assay, human rs4880 (Assay ID: C_8709053_10), 5μL of TaqMan™ Genotyping Master Mix and 3,75μL of H_2O ultrapure (DNAse and RNAse free). The reaction consisted of 60ºC of an initial denaturation step of 30s at 95ºC for 10min, followed by 50 cycles of 92ºC for 15s, 60ºC for 1min30s and a final extension step at 60ºC for 30s. *SOD2* rs4880 was successfully genotyped in 114 specimens. Genotypes were determined for the Val16Ala-SOD2 polymorphism as VV(Val/Val or TT), AV(Ala/Val or CT), AA (Ala/Ala or CC). Genotypes were also grouped for the allele dose effect test (AV + AA vs. VV, and AA vs. AV + VV), described in previous studies (da Cruz Jung et al., 2020; Taufer et al., 2005).

Statistical Analysis

Chi-square ($\chi 2$) analysis was used to estimate the Hardy–Weinberg equilibrium. Correlation of frequencies between gender (female and male) of cases and controls was done by $\chi 2$. Age was compared among genotypes using analysis of variance (ANOVA) followed by a Bonferroni post hoc test. Considering some differences related to gender and age, a multivariate analysis was performed using logistic regression (backward stepwise Wald method) to determine the effect of these variables on the association between esophageal cancer and SOD2 rs4880 SNP.

The associations of each genotype or SOD2 expression levels, with clinicopathologic data and clinical endpoints (death and recurrence of esophageal cancer) were compared using Pearson chi-square or Fisher exact tests for categorical variables, and Student t or Mann-Whitney U tests for continuous data. Survival curves were constructed according to the Kaplan-Meier method and compared using the log-rank test.

The overall survival time was defined to be the time from the date of the beginning of the treatment (neoadjuvant more surgery or only surgery) to the date of death/last follow-up (May 14, 2021). Cox proportional hazards regression analysis was used to determine hazard ratios for OS. Variables with $P < 0.20$ and clinically relevant were included in the multivariate analyses. The SPSS statistical package for Windows (IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp) was used for data analysis. Statistic tests were two-sided, and levels of significance were 0.05.

RESULTS

A total of 340 individuals, 126 patients with esophageal cancer and 114 healthy subjects were evaluated. Regarding esophageal cancer patients, 122 had samples available, SOD2 rs4880 was successfully genotyped in 114 samples (a loss of 6.5%), and SOD2 expression in 120 samples (a loss of 1.6%).

Associations of SOD2 rs4880 with Esophagus Cancer Risk

For the SOD2 rs4880 genotypes, 114 patients with esophageal cancer and 114 healthy individuals were evaluated. The frequency of homozygous genotypes was VV = 20% (n = 46), AA = 31.7% (n = 73) and heterozygous AV = 109 (47.3%) with allele frequencies of A = 0.559 and V = 0441, Hardy-Weinberg equilibrium ($\chi 2$ = 0.2008). For better data display, patients will be described as cases and information from healthy individuals (database) as controls. The frequency of SOD2 rs4880 genotypes of cases and control is shown in Table 1.

No significant differences were found between control men (n = 81, 49.4%) and esophageal cancer (n = 83, 50.6%), and control women (n = 33, 51.6%) and with esophageal cancer (n = 31, 48.4%) (P = 0.768). Mean age was also between similar controls (63.1 ± 9.2) and cases (63.0 ± 9.4) (P = 0.927). The frequency of the AA genotype was higher in cases than in controls with a recessive pattern (allele dose effect, P = 0.007) **(Table 1)**. The relative risk of carriers of the AA genotype to develop esophageal cancer was corrected to 2.18 (95%CI 1.23-3.86), and logistic regression analysis showed that this association is independent of the gender and age of the patients (VV vs AV+AA P=0.741, AA vs AV+VV P=0.008, Odds Ratio= 1.455)

[Table 1 is here]

Patient Characteristics Related to SOD2 rs4880 Genotypes and SOD2 IHC

The VV variant compared to the AV and AA variant did not present statistically significant differences associated with demographic characteristics and clinicopathological variables **(Table 2).** A second analysis was then performed to test the allele dose effect (AV+AA vs. VV and AA vs. AV+VV) associated with clinical variables, but there were no statistically significant differences.

About SOD2 IHC, expression levels SOD2 in esophageal cancer were 14 (11.5%) for weak, 57 (46.7%) for moderate and 49 (40.2%) for strongly positive. The SOD2 expression weak compared with that for the SOD2 moderate + strongly positive, was associated significantly with tumor size (*P*<0.001) or location (*P*=0.045), and recurrence (local relapse or metastatic disease, *P*=0.045). Most patients with weak expression for SOD2 had larger tumor size (57.1% T3, 21.4% T4), and location in the mid-chest (50%). In contrast, patients with moderate to strongly positive SOD2 expression had smaller tumor sizes (12.3% T1 and 34% T2), mainly at the gastroesophageal junction (46.7%). Regarding the clinical outcome of recurrence, few cases with weak SOD2 expression in tumor tissue (16.7%) had disease recurrence. In cases of moderate to strongly positive expression, 52.9% had relapse while 47.1% did not relapse.

[Table 2 is here]

Associations of SOD2 rs4880 and SOD2 IHC with Overall Survival

The median overall survival time (OS) was 16 months (N=118, 95%CI 10.3-21.7). There were no significant associations between the *SOD2* rs4880 genotypes and esophagus cancer OS (N=108, log-rank test 1.261; *P* = 0.53). The median survival of patients who carried a *SOD2* rs4880 AV genotype was only 12 months, whereas that of patients who carried the VV and AA genotypes was 16 and 17 months, respectively.

However, for SOD2 expression levels, tumors SOD2 weak compared with those for the SOD2-moderate or strongly positive were associated significantly with shorter OS (N= 112, log-rank test 6.089; *P* = 0.014) **(Figure 2)**. The median survival of the patients with tumor SOD2 weak was only six months, whereas that of SOD2 moderate or strongly positive was 17 months.

[Figure 2 is here]

In Cox univariate regression analysis, clinical stage III (stage III vs stage I + II HR:1.66, P = 0.015), tumor invasion (T3 vs. T1 HR: 3.00, P = 0.006, T4 vs. T1 HR: 7,40, P = 0.002), extranodal extension (HR: 2.14, P = 0.006), lymphovascular invasion (HR:2.16, P = 0.003), regional lymph node resection (HR: 1.07, P < 0.001), no neoadjuvant therapy before surgery (HR: 1.84, P = 0.036), were correlated with the risk of lower overall survival. These variables did not maintain statistical significance in the multivariate Cox regression **(Table 3).**

However, moderate/strongly positive *SOD2* expression in immunohistochemistry was a protective factor for overall survival (HR: 0.48, P = 0.019). In addition, multivariate Cox regression revealed that *SOD2* expression level was an independent prognostic marker for posttreatment overall survival in patients with esophagus cancer (HR, 0.41; 95% CI, 0.22-0.79 *P* = 0.07) **(Table 3).**

[Table 3 is here]

DISCUSSION AND CONCLUSION

In this study, we investigated the clinical significance of Superoxide Dismutase-2 (Val16Ala-SNP, and the immunohistochemical expression), in esophageal cancer. The Val16Ala SNP (AA-SOD2) was associated with an increased risk of esophageal cancer, regardless of age and gender, but did not have a significant effect on patient survival. Meanwhile, weak SOD2 immunohistochemical expression was significantly associated with shorter overall survival time after treatment, while moderate/strongly positive expression was a protective factor. The immunohistochemical expression of SOD2 was a prognostic marker, independent of other clinicopathological variables.

Our results show that Val16Ala-SOD2 and the level of SOD2 expression in the tumor have potential as independent biomarkers in esophageal cancer. As far as we know, this is the first study to investigate the associations between the rs4880, immunohistochemical expression of SOD2, and the survival of patients with ESSC and Esophageal Adenocarcinoma.

In previous studies (Cheng et al., 2009; di Martino et al., 2007; Murphy et al., 2007; Pinto et al., 2003; Sun et al., 2009), only Cheng et al 2009 had identified a significant association between polymorphism and risk of ESSC (Cheng et al., 2009). This association was evidenced when Sun et al. al 2013 performed a meta-analysis of four studies, and Ala-Allele was associated with esophageal cancer risk in all comparison models, corroborating the results of our study (Sun et al., 2013). However, the association of this polymorphism with the prognosis of patients with cancer is not well determined. Evidence of this association for esophageal cancer is even more scarce, especially in the Brazilian population, emphasizing the importance of this study.

Regarding the expression of SOD2 in cancer and its associations with prognosis, there are still controversial results in the literature. Corroborating our results, studies evaluating the ESSC found that low SOD2 mRNA and protein expression was associated with tumor invasion (Sun et al., 2011; Toh et al., 2000). In addition, low SOD2 expression was associated with progression from Barrett's esophagus to esophageal adenocarcinoma (Hermann et al., 2005). In contrast, two studies identified SOD2 overexpression in ESSC as being associated with a worse prognosis (Ma et al., 2014; Zuo et al., 2019). Although prognostic differences for SOD2 were observed in these studies, our results show low SOD2 expression as an indicator of poor survival, regardless of histological subtype and neoadjuvant treatment.

Several studies have indicated the importance of oxidant-antioxidant balance in initiation, promotion and resistance to cancer therapy (Rodic & Vincent, 2018), but a dichotomous role for SOD2 in tumor biology modulated by diverse factors (genetic, immunological, environmental or tumor-associated) (Ekoue et al., 2017; Talarico et al., 2021). Studies evaluating Va16Ala-SNP propose that in individuals with Allele-Ala there would be greater expression and activity of SOD2, favoring the generation and accumulation of H202, which would induce additional cell damage and promote tumor development (Che et al., 2016; Ekoue et al., 2017; Rodic & Vincent, 2018; Talarico et al., 2021). Ekoue et al 2017 proposed that high levels of SOD2 result in excess H2O2 and contribute to the progression of the transformation phenotype unless H2O2 is removed by the activity of a ROS detoxifying enzyme such as CAT or GPX.

Thus, it is estimated that cases of SOD2 overexpression in tumors may be associated with a better prognosis when other enzymes compensate for the excessive generation of H202 by removing it. In vitro studies have shown that SOD2 overexpression is related to increased cell differentiation, decreased cell growth or proliferation, and reversion from a malignant to non-malignant phenotype (Dhar & St Clair, 2012; Kinnula & Crapo, 2004). Regarding the cases of lower SOD2 expression associated with worse prognosis, a hypothesis raised by Xu et al 2012 and corroborated our results, is that the lower enzymatic expression or activity of SOD2 may allow a longer exposure of abnormal cells to the toxic effects of excessive ROS (in this case anion superoxide) contributing to poor survival.

Although the role of the SOD2 gene in cancer is not fully understood, it is noteworthy that the changes caused by the carcinogenic process may not only perturb protection from potential ROS toxicity but also misregulate many redox-sensitive pathways that contribute to disease outcomes. In addition, physiological, behavioral, disease-related, and inherent cancer treatment factors can influence the antioxidant action and patient survival (Kim et al., 2017; Rodic & Vincent, 2018;

Talarico et al., 2021; Wang et al., 2018).

As for the limitations of this study, it was not possible to analyze the correlation of SOD2 expression with some intervening variables, such as smoking, alcohol consumption or mate consumption. Another limiting factor was the impossibility of analyzing some tissues included in paraffin, resulting in a smaller number of samples. As we have not evaluated other antioxidant components, the importance of further studies is highlighted.

In conclusion, SNP Val16Ala-SOD2 and immunohistochemical expression of SOD2 were independent biomarkers of esophageal cancer. These data highlight the importance of the antioxidant system in the carcinogenic process, and further studies on factors that modulate it, favoring the future development of broader therapies for esophageal cancer.

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FIGURE LEGENDS

Figure 1. Immunohistochemical SOD2 expression in esophageal cancer A) Control positive B) Control negative C) Weak expression D) Moderate expression E) Strongly positive expression (Original magnification 200x).

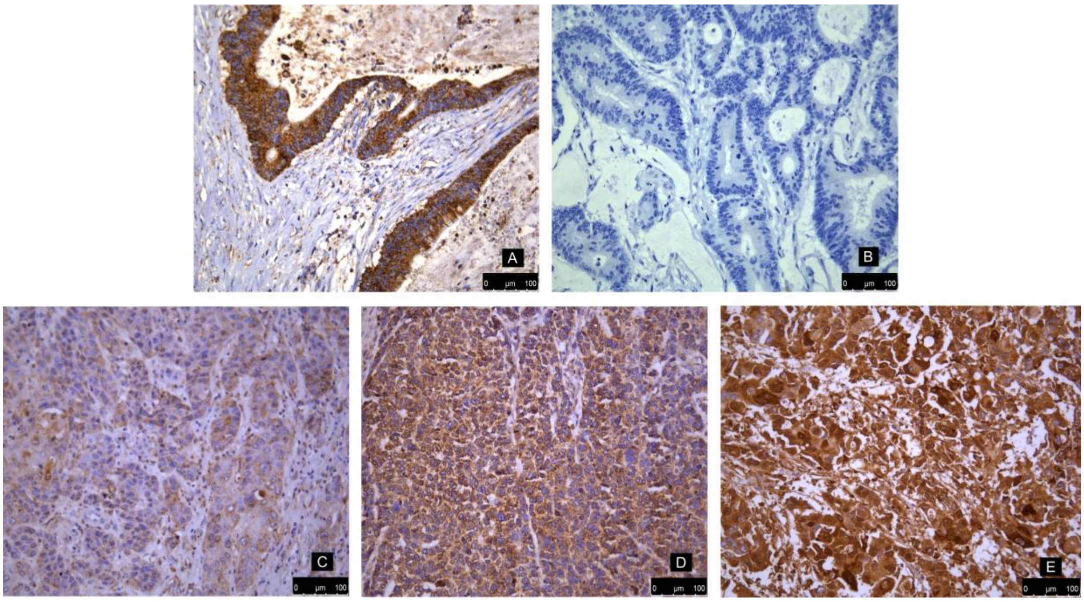
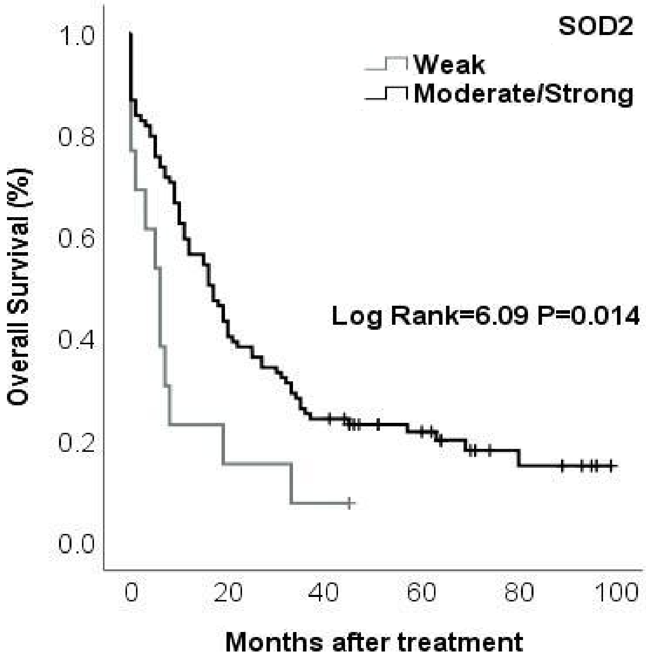


Figure 2. Kaplan-Meier estimates of overall survival among the esophageal cancer patients, according to tumor SOD2-expression.



TABLES

Table 1. Genotypic frequencies of the Val16Ala-SOD2 polymorphism between healthy subjects (Control) and esophageal cancer patients (Cases).

Genetic SOD2 Rs4880		**Control**	**Cases**	***P-value***
Genotype	VV	24 (21.1)	22 (19.3)	**0.021**
	AA	27 (23.7)	46 (40.4)	
	AV	63 (55.3)	46 (40.4)	
Allele-dose effect	AA+ AV	90 (78.9)	92 (80.7)	0.741
	VV	24 (21.1)	22 (19.3)	
	VV+AV	87 (76.3)	68 (59.6)	**0.007**
	AA	27 (23.7)	46 (40.4)	

Reported as n and % unless otherwise specified.
Abbreviation: V, Valine ; A, Alanine
Note: Statistical comparisons were performed by Chi-squared test.

Table 2. Clinical Correlations of *Superoxide Dismutase 2* (*SOD2*), Val16Ala-SNP (rs4880) and expression tissue levels by immunohistochemistry, for Esophageal Cancer.

Clinicopathological Variables		**SOD2 rs4880** (n = 114)							**SOD2 expression levels** (n= 122)				
		VV		**AV**		**AA**		***P-value***	**Weak**		**Moderate/ Strongly Positive**		***P-value***
Age, years	Mean ± SD	65.9	±9.7	60.8	±9.8	63.4	±8.2	0.098	63	±12	63	±9	0.957
Lymph node resected	Median. P25;P75	0	0.0; 2.0	1.0	0.0; 2.0	0	0; 3	0.687	0	0; 2	1	0; 2	0.207
Surgery time	Median. P25;P76	382.5	298; 540	360.0	225; 450	410.0	285; 498	0.092	420	285; 468	373	260; 485	0.505
Gender	Female	9	40.9%	12	26.1%	10	21.7%	0.245	5	35.7%	27	25.5%	0.520
	Male	13	59.1%	34	73.9%	36	78.3%		9	64.3%	79	74.5%	
Histopathologic cell type	Adenocarcinoma	8	36.4%	17	37.0%	19	41.3%	0.887	3	21.4%	41	38.7%	0.208
	ESCC	14	63.6%	29	63.0%	27	58.7%		11	78.6%	65	61.3%	
TNM Clinic Stage¹	I-II	13	61.9%	25	55.6%	23	52.3%	0.766	11	78.6%	53	52.0%	0.060
	III	8	38.1%	20	44.4%	21	47.7%		3	21.4%	49	48.0%	
Tumor cell differentiation	G1 - Well	1	4.8%	0	0.0%	4	8.9%	0.375	0	0.0%	6	5.8%	0.447
	G2 - Moderate	13	61.9%	30	66.7%	26	57.8%		7	53.8%	65	62.5%	
	G3 - Poor	7	33.3%	15	33.3%	15	33.3%		6	46.2%	33	31.7%	
Tumor invasion (T)	T1	4	18.2%	3	6.5%	6	13.0%	0.721	1	7.1%	13	12.3%	**0.001**
	T2	6	27.3%	16	34.8%	11	23.9%		2	14.3%	36	34.0%	
	T3	11	50.0%	25	54.3%	28	60.9%		8	57.1%	56	52.8%	
	T4	1	4.5%	2	4.3%	1	2.2%		3	21.4%	1	0.9%	
Tumor location²	Cervical	0	0.0%	0	0.0%	1	2.2%	0.862	1	7.1%	0	0.0%	**0.010**
	Middle Thoracic	8	38.1%	14	30.4%	12	26.1%		7	50.0%	30	28.6%	

	Lower Thoracic	5	23.8%	10	21.7%	11	23.9%		1	7.1%	26	24.8%	
	EGJ	8	38.1%	22	47.8%	22	47.8%		5	35.7%	49	46.7%	
Neoadjuvante therapy[3]	No	20	90.9%	42	91.3%	35	76.1%	0.085	11	78.6%	88	83.0%	0.710
	Yes	2	9.1%	4	8.7%	11	23.9%		3	21.4%	18	17.0%	
Histoviability	No	0	0.0%	1	2.2%	2	4.3%	0.560	1	7.1%	2	1.9%	0.313
	Yes	22	100.0%	45	97.8%	44	95.7%		13	92.9%	104	98.1%	
Surgery types	Transhiatal	6	27.3%	21	48.8%	23	52.3%	0.220	5	38.5%	48	47.5%	0.743
	Transthoracic	14	63.6%	16	37.2%	18	40.9%		7	53.8%	43	42.6%	
	Others	2	9.1%	6	14.0%	3	6.8%		1	7.7%	10	9.9%	
Surgical Margin: R Category	R0	16	72.7%	35	76.1%	33	71.7%	0.888	10	71.4%	80	75.5%	0.748
	R1-R2	6	27.3%	11	23.9%	13	28.3%		4	28.6%	26	24.5%	
Smokers / Smoking status	No	1	7.7%	6	14.3%	4	10.3%	0.744	3	30.0%	9	9.9%	0.156
	Yes	4	30.8%	14	33.3%	9	23.1%		3	30.0%	27	29.7%	
	Former Smoker	8	61.5%	22	52.4%	26	66.7%		4	40.0%	55	60.4%	
Alcoholic beverages drinking status*	No	4	30.8%	12	36.4%	11	39.3%	0.790	4	40.0%	26	37.7%	0.702
	Yes	4	30.8%	8	24.2%	4	14.3%		3	30.0%	14	20.3%	
	Former regular drinker	5	38.5%	13	39.4%	13	46.4%		3	30.0%	29	42.0%	
SOD2 expression levels	Weak	2	9.1%	4	8.9%	7	15.6%	0.565	-	-	-	-	-
	Moderate or Strongly positive	20	90.9%	41	91.1%	38	84.4%		-	-	-	-	-
SOD2 rs4880													
Genotype	VV	-	-	-	-	-	-	-	2	15.4%	20	20.2%	0.565
	AV	-	-	-	-	-	-	-	4	30.8%	41	41.4%	
	AA	-	-	-	-	-	-	-	7	53.8%	38	38.4%	
Allele-dose effect	AV+AA	-	-	-	-	-	-	-	2	15.4%	20	20.2%	0.681
	VV	-	-	-	-	-	-	-	11	84.6%	79	79.8%	
	AA	-	-	-	-	-	-	-	7	53.8%	38	38.4%	0.285
	AV+VV	-	-	-	-	-	-	-	6	46.2%	61	61.6%	

Death	No	2	9.5%	6	13.3%	11	26.2%	0.161	1	7.7%	19	19.2%	0.457
	Yes	19	90.5%	39	86.7%	31	73.8%		12	92.3%	80	80.8%	
Recurrence	No	13	59.1%	20	44.4%	28	66.7%	0.107	10	83.3%	54	52.9%	**0.045**
	Yes	9	40.9%	25	55.6%	14	33.3%		2	16.7%	48	47.1%	

Reported as n and % unless otherwise specified. Abbreviation: V, Valine ; A, Alanine. ESCC, Esophageal squamous cell carcinoma; EGJ, Esophagogastric junction. [1]AJCC 8th, seventh edition of the American Joint Commission on Cancer (AJCC) Cancer Staging Manual (TNM classification)
[2]Location of cancer primary site is defined by cancer epicenter.[3] Neoadjuvant chemotherapy or radiotherapy before surgery.
*Excessive consumption of alcoholic beverages. Note: Statistical univariate comparison between two elderly groups was performed by Chi-squared or Fisher's exact test, and Student t or Mann-Whitney U tests for continuous

Table 3. Analysis of Overall Survival in the Patients with Esophageal Cancer

Variables	Overall Survival			
	Univariate analysis			
	P-value	**HR**	**95% CI**	
Age, years	0,748	1,00	0,98	1,03
Gender (Male vs. Female)	0,668	1,10	0,70	1,74
Histopathologic cell type (ESSC vs. Adenocarcinoma)	0,476	0,86	0,57	1,30
TNM Clinic Stage[1] (III vs. I-II)	**0,015**	**1,66**	**1,10**	**2,50**
Tumor cell differentiation (G2 vs. G1)	0,602	1,36	0,43	4,36
Tumor cell differentiation (G3 vs. G1)	0,519	1,48	0,45	4,81
Tumor invasion (T3 vs. T1)	**0,006**	**3,00**	**1,36**	**6,63**
Tumor invasion (T4 vs T1)	**0,002**	**7,40**	**2,09**	**26,23**
Extranodal extension (Yes vs. No)	**0,006**	**2,14**	**1,25**	**3,66**
Lymphovascular invasion (Yes vs No)	**0,003**	**2,16**	**1,31**	**3,58**
Tumor location[2] (Middle Thoracic vs. Cervical)	0,287	0,34	0,04	2,50
Tumor location[2] (Lower Thoracic vs. Cervical)	0,415	0,43	0,06	3,25
Tumor location[2] (EGJ vs. Cervical)	0,322	0,36	0,05	2,69
Neoadjuvant therapy* (No vs. Yes)	**0,036**	**1,84**	**1,04**	**3,24**
Surgery (Transthoracic vs. Transhiatal)	0,604	1,12	0,72	1,74
Surgical Margin: R Category (R1-R2 vs. R0)	0,069	1,51	0,97	2,36
Lymph node resection	**0,001**	**1,07**	**1,03**	**1,12**
SOD2rs4880 (AV+AA vs. VV)	0,521	0,85	0,51	1,41
SOD2rs4880 (AA vs. VV + AV)	0,284	0,79	0,51	1,22
SOD2 expression:				
Moderate/Strongly positive vs. Weak	**0,019**	0,48	0,26	0,89
Moderate vs. Weak	**0,043**	**0,51**	**0,27**	**0,98**
Strongly positive vs. Weak	**0,016**	**0,44**	**0,23**	**0,86**
	Multivariate analysis			
	P-value	**HR**	**95% CI**	
TNM Clinic Stage[1] (III vs. I-II)	0,204	1,36	0,84	2,20
Neoadjuvant therapy* (No vs. Yes)	0,210	1,50	0,79	2,84
Surgical Margin: R Category (R1-R2 vs. R0)	0,449	1,22	0,73	2,02
Lymph node resection	0,067	1,05	1,00	1,10
SOD2 expression Moderate/Strongly positive vs. Weak	**0,007**	**0,41**	**0,22**	**0,79**

CI – confidence interval; HR – Hazard ratio; V - Valine (Val); A - Alanine (Ala). ESCC - Esophageal squamous cell carcinoma. EGJ: Esophagogastric junction. *SOD2* – superoxide dismutase 2.

[1]AJCC 8th, seventh edition of the American Joint Commission on Cancer (AJCC) Cancer Staging Manual (TNM classification). [2]Location of cancer primary site is defined by cancer epicenter.[3] Neoadjuvant chemotherapy or radiotherapy before surgery. *Excessive consumption of alcoholic beverages.

Note: Cox proportional hazards regression analysis was used to determine hazard ratios for Overall Survival.

Supplementary Material

Table 1. Patients Characterization for Clinicopathological variables.

Variables		**Total (n=126)**		**%valid**
Age, years	Mean ± SD	63.3	±9.6	-
Surgery time	Median,P25;P75	386.5	268;485	-
Number of Lymph node resected	Median,P25;P75	0.0	0;2	-
Smoking Pack-years	Median,P25;P75	40.0	25;66	-
Gender	Female	34	27.0%	27.0%
	Male	92	73.0%	73.0%
Histopathologic cell type	Adenocarcinoma	46	36.5%	36.5%
	ESCC	80	63.5%	63.5%
TNM Clinic Stage[1]	I	6	4.8%	4.9%
	II	63	50.0%	51.6%
	III	53	42.1%	43.4%
	Missing	4	3.2%	
Tumor cell differentiation	G1 - Well	6	4.8%	4.9%
	G2 - Moderate	73	57.9%	59.8%
	G3 - Poor	43	34.1%	35.2%
	Missing	4	3.2%	
Tumor invasion (T)	T1	15	11.9%	11.9%
	T2	40	31.7%	31.7%
	T3	67	53.2%	53.2%
	T4	4	3.2%	3.2%
Tumor location[2]	Cervical	1	0.8%	0.8%
	Middle Thoracic	39	31.0%	31.2%
	Lower Thoracic	29	23.0%	23.2%
	EGJ	56	44.4%	44.8%
	Missing	1	0.8%	
Extranodal Extension	No	61	48.4%	71.8%
	Yes	24	19.0%	28.2%
	Missing	41	32.5%	
Lymphovascular Invasion	No	40	31.7%	47.1%
	Yes	45	35.7%	52.9%
	Missing	41	32.5%	
Neoadjuvant therapy[3]	No	102	81.0%	81.0%
	Yes	24	19.0%	19.0%
Histoviability	No	5	4.0%	4.0%
	Yes	121	96.0%	96.0%
HER2*	Negative	6	10.7%	75.0%
	Positive	1	1.8%	12.5%
	Indeterminate	1	1.8%	12.5%
	Missing	48	85.7%	
Surgery types	Transhiatal	55	43.7%	46.2%
	Transthoracic	52	41.3%	43.7%
	Others	12	9.5%	10.1%
	Missing	7	5.6%	
Surgical Margin: R Category	R0	95	75.4%	75.4%

	R1-R2	31	24.6%	24.6%
Smokers / Smoking status	No	13	10.3%	12.4%
	Yes	31	24.6%	29.5%
	Former Smoker	61	48.4%	58.1%
	Missing	21	16.7%	
Alcoholic beverages drinking status**	No	32	25.4%	39.0%
	Yes	17	13.5%	20.7%
	Former regular drinker	33	26.2%	40.2%
	Missing	44	34.9%	
Mate drinking status§	No	1	0.8%	16.7%
	Yes	5	4.0%	83.3%
	Missing	120	95.2%	
SOD2 rs4880 genotypes	VV	22	17.5%	19.3%
	AV	46	36.5%	40.4%
	AA	46	36.5%	40.4%
	Missing	12	9.5%	
Dominant Model	AV+AA	22	17.5%	19.3%
	VV	92	73.0%	80.7%
	Missing	12	9.5%	
Recessive Model	AA	46	36.5%	40.4%
	AV+VV	68	54.0%	59.6%
	Missing	12	9.5%	
SOD2 expression levels	Weak	14	11.1%	11.7%
	Moderate	57	45.2%	47.5%
	Strongly positive	49	38.9%	40.8%
	Missing	6	4.7%	

Legend: Reported as n and % unless otherwise specified. Abbreviation: SD, Standard deviation; ESCC, Esophageal squamous cell carcinoma; EGJ, Esophagogastric junction. V - Valine (Val); A - Alanine (Ala). [1]AJCC 8th, seventh edition of the American Joint Commission on Cancer (AJCC) Cancer Staging Manual (TNM classification) [2]Location of cancer primary site is defined by cancer epicenter.[3] Neoadjuvant chemotherapy or radiotherapy before surgery *HER2, human epidermal growth factor receptor 2 (Adenocarcinoma Only). **Excessive consumption of alcoholic beverages
§Yerba mate tea or *chimarrão*.

4.3. ARTIGO III

"HPV16 and p16 in esophageal cancer:

what is the prognostic impact?"

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Enviado para publicação na Revista "Human Pathology"

HPV16 and p16 in esophageal cancer: what is the prognostic impact?

Short title: HPV16 and p16 in the esophageal cancer

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ABSTRACT

Background: Esophageal cancer is the seventh most common cancer in the world and the sixth leading cause of cancer death. Due to aggressiveness, it is essential to identify an adequate prognostic biomarker to predict long-term survival. Human papillomavirus (HPV) infection, particularly HPV16, have been investigated as a potential factor in the genesis and progression of esophageal cancer. Although many studies use the p16 protein as an indirect marker of HPV infection, the correlation between HPV and p16 in esophageal cancer is not fully understood, especially its prognostic impact. **Objective:** to evaluate the association between HPV16 and p16 in esophageal cancer and its correlation with patient survival. **Methods:** A retrospective cohort with patients with esophageal cancer, where the presence of HPV16 in tumor tissue was evaluated by polymerase chain reaction (PCR) and tissue expression of p16 by immunohistochemistry (IHC). Correlation analysis was performed with clinicopathological variables and overall survival. **Results:** 126 patients were included, with a mean follow-up of 13 months. All cases evaluated by PCR were negative for HPV16. Expression IHC p16 was not associated with overall survival (P = 0.563). **Conclusion:** Positive p16 was not associated with HPV16 infection, and to not impact the prognosis of patients with esophageal carcinoma or adenocarcinoma. These results contribute to a greater understanding of the clinical significance of HPV16 and p16 in esophageal cancer and encourage further studies on other factors that influence p16 expression in this cancer.

KEYWORDS: HPV, p16, esophageal cancer, biomarkers, prognosis.

1. BACKGROUND

Esophageal cancer is the seventh most common cancer in the world and the sixth leading cause of cancer death [1]. Characterize yourself by the high mortality rate associated with diagnosis in advanced stages of the disease. Globally, about 87% of all esophageal cancers are squamous cell carcinoma with 11% constituting adenocarcinoma [2, 3].

The five-year survival rate for esophageal cancer patients with resectable disease in the past 30 years is less than 20% [4], and this is due to diagnosis in advanced stages of the disease, low treatment remission rates and high local recurrence rates [5]. From this context, it became essential to identify a prognostic biomarker to predict the long-term survival of patients with esophageal cancer.

Persistent human papillomavirus (HPV) infection, especially high-risk oncogenic types (ie, HPV16, HPV18), has been investigated as a potential factor in the genesis and progression of esophageal cancer [5-10]. It was found in other types of cancer, such as gynecological tumors, that the viral oncoproteins E6 and E7 could interact and inhibit the action of proteins involved in cell cycle control and tumor suppressors. E6 and E7 would inactivate p53 and pRb (retinoblastoma protein), respectively, resulting in increased expressions of p16 (or p16INK4A) and p53 [11].

Although many studies use the p16 protein as an indirect marker of HPV infection, the correlation between HPV and p16 in esophageal cancer is not fully stablished [5, 12-15]. Thus, it is necessary to identify whether viral infection by a type of high oncogenic risk HPV and tissue expression of p16 in the tumor may have an impact on the survival of patients with esophageal cancer, being potential prognostic biomarkers. In this context, the aim of this study was to evaluate the association between HPV16 and p16 in esophageal cancer and its correlation with patient survival.

2. METHODS

This study is according to REporting recommendations for tumour MARKer prognostic studies (REMARK) [16].

2.1 Study subjects

The patients with esophageal cancer from undergoing esophagectomy, who were diagnosed and treated in a regional referral cancer center in the south of Brazil, from January 2010 to December 2017 were selected. Medical records, imaging and pathological examinations were reviewed to collect demographic and clinical information, as well as tumor characteristics. For genotype and immunohistochemistry (IHC) analysis included patients who had biological material (tumor tissue formalin-fixed, paraffin-embedded - FFPE) available. The esophagus cancer samples used were reviewed and classified by 2 independent pathologists.

Regarding the ethical and legal aspects, this study is in accordance with the Declaration of Helsinki, the Universal Declaration of Bioethics and Human Rights and Resolution 466/2012 of the National Health Council of Brazil. The study was approved by the Research Ethics Committees of the participating institutions, and informed consent was obtained from participants or legal representatives.

2.2 p16 - Immunohistochemistry (IHC)

We used Anti-CDKN2A/p16INK4a, ab108349, Abcam® (Rabbit Monoclonal Antibody, Clone number EPR1473, 1:500 dilution, positive control: cervical carcinoma) in esophageal tumors. Tissue sections were heated (75°C, 30 min), deparaffinized (three washes in xylenes), and rehydrated by successive washes in different concentrations of alcohols and deionized water. For immunohistochemical staining, antigen retrieval was performed at 98°C for 40 min in pH 9.0 TRIS-EDTA. Endogenous peroxidase activity was blocked with hydrogen peroxide (5%) in methanol (3 × 10 min). Afterwards, the slides were washed (3x) with phosphate-buffered saline (PBS) and incubated in a solution to block non-specific binding (1% bovine serum albumin for 1 h).

For negative control bovine serum albumin 1% was substituted for the primary antibody. The primary antibody and control were incubated for 1 h at room temperature and then submitted to temperatures of 4°C overnight. After that, the slides were kept at room temperature for 1 h, in sequence, and were washed thrice with PBS.The sections were incubated with the EnVision + Dual Link System-HPR (Agilent DAKO®/ Santa Clara/USA) for 40 min. Staining was completed by incubating the specimens with DAKO® Liquid DAB + Substrate Chromogen System for 5 min.

Finally, tissue sections were counterstained with haematoxylin-harris solution and slides mounted in non-aqueous medium. p16 IHC was considered positive when it showed nuclear/nuclear and cytoplasmic staining in more than 70% of the tumor tissue [10, 17] **(Figure 1).** All scoring were conducted blindly by two observers (AVS and AVR).

(Figure 1)

2.3 HPV16 - DNA Extraction and PCR

Genomic DNA was extracted from 4-6 FFPE 10-μm sections with the Magnetic Method to Purify DNA from FFPE Tissue/ MagneSil® Genomic, Fixed-Tissue System (PROMEGA/Medison/USA) kit following the manufacturer specifications. Positive internal control for DNA extraction was used. All DNA samples were quantified using NanoDrop 2000 (ThermoScientific®) and maintained at − 20 °C until the Polymerase chain reaction (PCR).

The HPV16 DNA, was detected by PCR using specific HPV16 primer sets annealing in the E6 gene (F- 5' CAAGCAACAGTTACTGCGA 3', and R- 5' CAACAAGACATACATCGACC 3', 282-bp DNA fragment), according to published protocols [18, 19]. The reaction mixture was prepared as follows: 1.1X Platinum PCR SuperMix (Invitrogen™ Life Technologies®) (20.5μL), 0.2μM (1μL) each primer, 2.5μL of DNA was used in each reaction, final volume of 25uL. The thermal cycling conditions consisted of initial denaturing for 2 min at 94°C, followed by 40 amplification cycles for 1 min at 94°C, 1 min at 60°C and 1 min at 72°C. Each PCR reaction included negative (water) and positive controls (DNA extracted from cervical samples HPV16 positive).

The PCR products were electrophoresed using a 1.5% agarose gel in 1× TBE buffer, stained with ethidium bromide and photographed under UV-transillumination. A low DNA mass ladder was used as a base-pair molecular weight pattern (DNA ladder 100 pb, Invitrogen ™ Life Technologies®/Carlsbad, Califórnia/USA) **(Figure 2).** Rigorous efforts were made to avoid cross-contamination at all stages of sample processing and analysis. A new microtome slide was used each time a new case was sectioned, and the microtome components were cleaned with ethanol after each sample. Sterile materials were also used and all recommendations for decontamination and biosafety of the laboratory environment were followed.

(Figure 2)

2.4 Statistical Analysis

Descriptive analysis of the cohort was initially performed, including HPV16 and p16 frequencies. The associations of p16 with demographic features and clinicopathologic data were compared using Pearson chi-square or Fisher exact tests for categorical variables and Student t or Mann-Whitney U tests for continuous data. Surgical time was the only variable with no expressive loss of cases with p<0.20 and crude and adjusted for sex and age estimates of Relative Risk (RR) with 95%CI were obtained by Poisson regression analysis with robust error variance.

Survival curves were constructed according to the Kaplan-Meier method and compared using the log-rank test. The survival time was defined to be the time from the date of the beginning of the treatment (neoadjuvant more surgery or only surgery) to the date of death/last follow-up (May 14, 2021). The SPSS statistical package for Windows (IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp) was used for data analysis. Statistic tests were two-sided, and levels of significance were < 0.05.

3. RESULTS

A retrospective cohort included 126 patients, who had a median follow-up of 13 months (range, 0-99 months). The mean patient age was 63 years (range, 41-87 years, SD = 9.8). Among patients 80 (63.5) were diagnosed with esophageal squamous cell carcinoma (ESCC). Only 24 (19.0%) had received neoadjuvant chemotherapy or radiotherapy. The demographic features and clinicopathologic data are summarized in **Table 1.**

[Table 1 is here]

Among 126 patients with esophagus cancer, 122 available were specimens. PCR was successful in 114 specimens and p16 for IHC in 119 specimens. No esophageal cancer sample was positive for HPV16. Tissue expression of p16 in the tumor was identified in 6 samples (5%) but did not show associations with demographic and clinicopathological variables. Thus, there was no correlation between HPV16 and positive p16 in our study **(Table 1).**

For clinical outcomes, p16-positive patients did not show a statistically significant difference from p16-negative patients regarding death (*P*=0.295) and recurrence (*P*=0.231) **(Table 2).** The median overall survival time (OS) was 16 months (N=111, 95%CI 11.2-20.8). The median OS of patients who were positive p16 was only 6 months (95%CI, 0-45.6), whereas that of patients who were negative p16 were 16 months (95%CI, 11-21), but this difference was not statistically significant (*P*=0.563) **(Figure 3).**

[Table 2 is here]

[Figure 3 is here]

4. DISCUSSION AND CONCLUSIONS

In this study, HPV16 and p16 were not associated with survival of patients with ESCC or adenocarcinomas, therefore suggesting that these factors have no prognostic impact on esophageal cancer. It is noteworthy that there is a growing number of studies seeking evidence of this of HPV and p16 correlation in esophageal carcinomas (ESSC) [12, 15, 20], including studies in the Brazilian population [5, 7-10], with few studies on adenocarcinomas [21]. Thus, studies such as this one that assess the two main subtypes of esophageal cancer are essential, due to histological and tumor heterogeneity.

In our study, we did not identify positive cases for HPV16. This result corroborates Antunes et al. 2013 [9], in which no positive case for HPV was identified in the ESCC of Brazilian patients. Other studies have identified an HPV positivity rate <16% for the same tumor type and population [5, 7, 8, 10]. However, there is evidence that even in HPV-positive cases, including HPV16, the viral infection may not influence patient survival [5, 8].

In contrast to the results for cervical and oropharyngeal cancer, viral infection is not necessarily associated with p16 expression in esophageal cancer [10, 12, 15]. In systematic reviews, Ludmir et al 2015 concluded that the rate of HPV-positive with expression of p16 in these ESCC is below 5% of cases [15]. Iyer et al 2011 when evaluating esophageal adenocarcinomas, also found no statistical difference for p16 expression between positive or negative cases of HPV [21].

Therefore, different types of oncogenic stresses may be the causes of p16 expression in cancer, such as DNA damage, physiological aging and the action of HPV oncoproteins. Witkiewicz et al. 2001 proposed two models to explain this protein expression in carcinogenesis [22]. In the first model, oncogenic stresses induce the expression of p16, which, being a tumor suppressor protein, would limit abnormal cell proliferation. Still, this result can be circumvented by the loss of pRb, a secondary event that facilitates disease progression. While in a second model, the loss of pRB produces oncogenic stress that induces p16 expression. Since pRB is already compromised, p16 induction cannot halt cancer progression and therefore tumors develop with high levels of p16 [22].

Though, a correlation of p16 expression with survival and clinical outcomes in patients with esophageal cancer is still contradictory. In studies with ESCC, Cao et al. (2014) found that p16-positive patients had better overall survival rates and 5-year progression-free survival than the p16-negative group and, similarly, Kumar et al. (2015) found that the expression of p16 in the tumor correlates with the highest rate of pathological complete remission in patients to neoadjuvant chemotherapy[14, 23].

In contrast to these studies, da Costa et al (2017) demonstrated that an expression of p16 in ESCC did not have predictive value for cancer-specific and progression-free survival, a result also identified by Ishida et al (2021) in which p16 expression was not predictive of clinical outcome [5, 24]. This study corroborates our result, in which there was no statistically significant association between the isolated expression of p16 and death, recurrence or overall survival, not differing between ESCC and esophageal adenocarcinomas. Thus, it is suggested that the patient's prognosis may be associated with other genetic, environmental or tumor microenvironment-related factors.

Regarding the limitations of this study, it was not possible to identify other oncogenic HPV types that could affect p16 expression, suggesting additional studies. Although HPV16, analyzed in our study, is one of the most frequent types of HPV with high oncogenic risk and associated with other types of cancer such as cervical cancer, anus and oropharynx [25].

In conclusion, HPV16 and expression immunohistochemical p16 were not associated with the survival of patients with esophageal cancer. Our results contribute to a greater understanding of the clinical significance of HPV16 infection and the expression of p16 in the prognosis of patients with esophageal cancer. Considering this study and evidence from the literature, larger studies are needed to evaluate other tumor suppressor proteins and risk factors (genetic or environmental) that may influence p16 expression and prognosis of patients with esophageal cancer.

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Conflict of Interest: The authors declare that they have no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent: Informed consent was obtained from all individual participants of each study included in the review.

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FIGURE LEGENDS

Figure 1. p16 expression in esophageal cancer A) Control positive B) Control negative C) p16 positive (≥70%) D) p16 negative (<70%) (Original magnification x200).

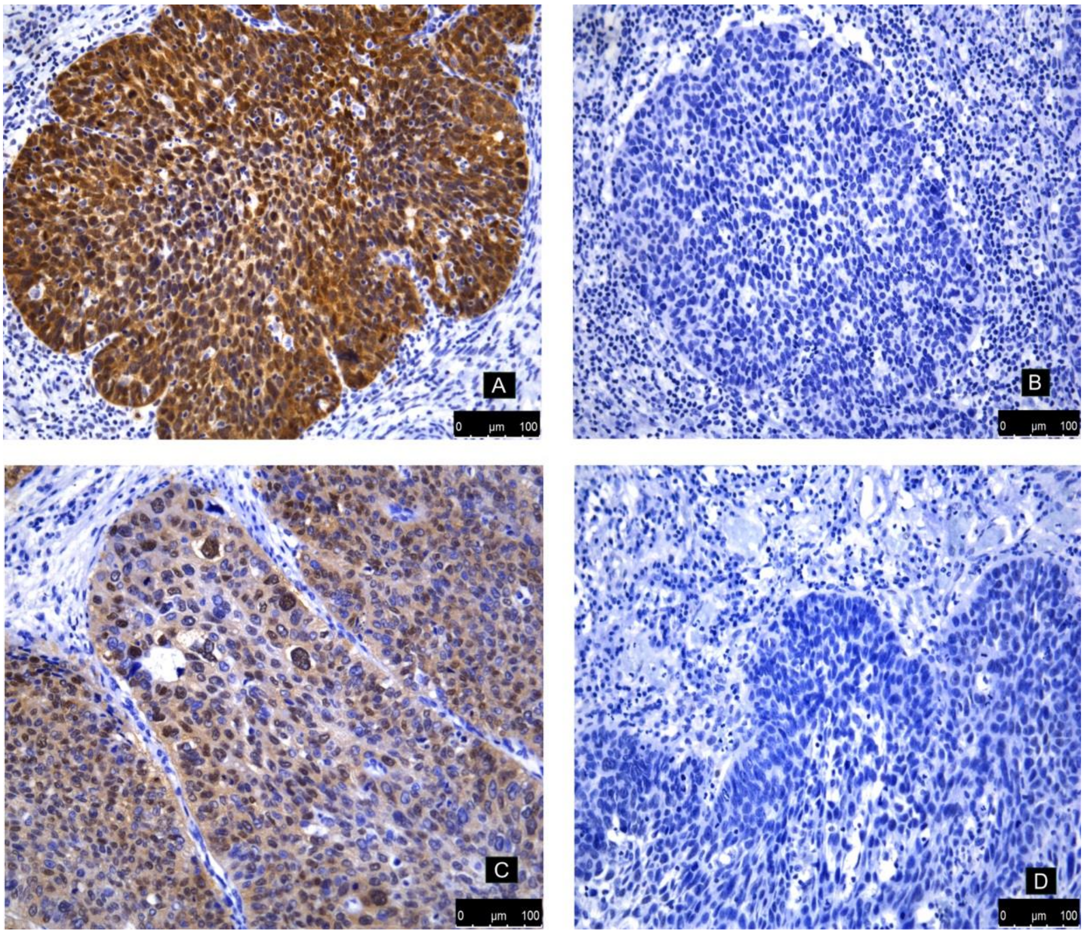


Figure 2. HPV16 detection by PCR. Amplification products with HPV16 primers. For 01 at 06 Samples HPV16 negatives, there were no amplification DNA bands for all esophageal cancer samples. C+ (Control Positive), C- (Control Negative). Molecular weight in base pairs (bp) is on the right) (Lane 10).

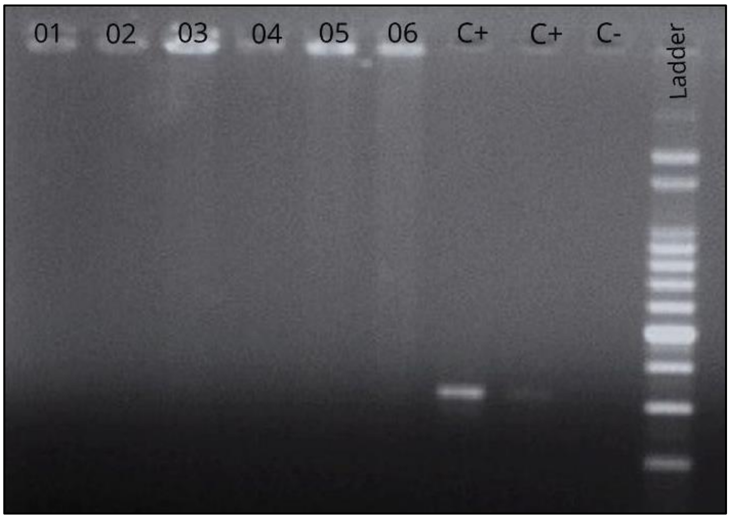
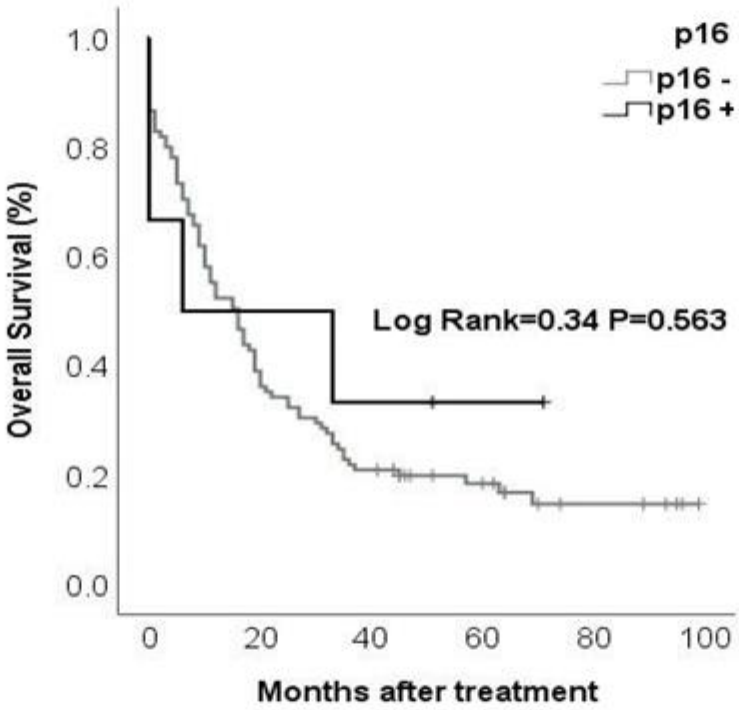


Figure 3. Kaplan-Meier estimates of overall survival among the esophageal cancer patients, according to tumor p16-expression.



TABLES

Table 1. Patients Characterization for Clinicopathological variables.

Variables		**Total (n=126)**		**%valid**
Age,years	Mean ± SD	63.3	±9.6	-
Surgery time	Median,P25;P75	386.5	268;485	-
Number of Lymph node resected	Median,P25;P75	0.0	0;2	-
Smoking Pack-years	Median,P25;P75	40.0	25;66	-
Gender	Female	34	27.0%	27.0%
	Male	92	73.0%	73.0%
Histopathologic cell type	Adenocarcinoma	46	36.5%	36.5%
	ESCC	80	63.5%	63.5%
TNM Clinic Stage[1]	I	6	4.8%	4.9%
	II	63	50.0%	51.6%
	III	53	42.1%	43.4%
	Missing	4	3.2%	
Tumor cell differentiation	G1 - Well	6	4.8%	4.9%
	G2 - Moderate	73	57.9%	59.8%
	G3 - Poor	43	34.1%	35.2%
	Missing	4	3.2%	
Tumor invasion (T)	T1	15	11.9%	11.9%
	T2	40	31.7%	31.7%
	T3	67	53.2%	53.2%
	T4	4	3.2%	3.2%
Tumor location[2]	Cervical	1	0.8%	0.8%
	Middle Thoracic	39	31.0%	31.2%
	Lower Thoracic	29	23.0%	23.2%
	EGJ	56	44.4%	44.8%
	Missing	1	0.8%	
Extranodal Extension	No	61	48.4%	71.8%
	Yes	24	19.0%	28.2%
	Missing	41	32.5%	
Lymphovascular Invasion	No	40	31.7%	47.1%
	Yes	45	35.7%	52.9%
	Missing	41	32.5%	
Neoadjuvant therapy[3]	Cirurgia	102	81.0%	81.0%
	Neoadjuvância	24	19.0%	19.0%
Histoviability	No	5	4.0%	4.0%
	Yes	121	96.0%	96.0%
HER2*	Negative	6	10.7%	75.0%
	Positive	1	1.8%	12.5%
	Indeterminate	1	1.8%	12.5%
	Missing	48	85.7%	
Surgery types	Transhiatal	55	43.7%	46.2%
	Transthoracic	52	41.3%	43.7%

	Others	12	9.5%	10.1%
	Missing	7	5.6%	
Surgical Margin: R Category	R0	95	75.4%	75.4%
	R1-R2	31	24.6%	24.6%
Smokers / Smoking status	No	13	10.3%	12.4%
	Yes	31	24.6%	29.5%
	Former Smoker	61	48.4%	58.1%
	Missing	21	16,7%	
Alcoholic beverages drinking status**	No	32	25,4%	39,0%
	Yes	17	13,5%	20,7%
	Former regular drinker	33	26,2%	40,2%
	Missing	44	34,9%	
Mate drinking status§	No	1	0,8%	16,7%
	Yes	5	4,0%	83,3%
	Missing	120	95,2%	
HPV16	Negative	114	90.5%	100%
	Positive	0	0%	0%
	Missing	12	9.5%	
p16	Negative	113	89,7%	95.0%
	Positive	6	4.8%	5.0%
	Missing	7	5.5%	

Reported as n and % unless otherwise specified. Abbreviation: SD, Standard deviation; ESCC, Esophageal squamous cell carcinoma; EGJ, Esophagogastric junction; p16: expression in tumor tissue by immunohistochemistry technique.
[1]AJCC 8th, seventh edition of the American Joint Commission on Cancer (AJCC) Cancer Staging Manual (TNM classification). [2]Location of cancer primary site is defined by cancer epicenter.
[3] Neoadjuvant chemotherapy or radiotherapy before surgery.*HER2, human epidermal growth factor receptor 2 (Adenocarcinoma Only).**Excessive consumption of alcoholic beverages. §Yerba mate tea or chimarrão.

Table 2. Clinical Correlations of p16 expression in the Esophageal Cancer, univariate analyses.

Variables		p16				
		Negative		**Positive**		***P-value***
Gender	Female	29	90.6%	3	9.4%	0.341
	Male	84	96.6%	3	3.4%	
Histopathologic cell type	Adenocarcinoma	40	93.0%	3	7.0%	0.666
	ESCC	73	96.1%	3	3.9%	
TNM Clinic Stage[1]	I-II	60	93.8%	4	6.3%	0.692
	III	49	96.1%	2	3.9%	
Tumor cell differentiation	G1 - Well	6	100.0%	0	0.0%	0.836
	G2 - Moderate	67	94.4%	4	5.6%	
	G3 - Poor	37	94.9%	2	5.1%	
Tumor invasion (T)	T1	13	92.9%	1	7.1%	0.695
	T2	35	92.1%	3	7.9%	
	T3	61	96.8%	2	3.2%	
	T4	4	100.0%	0	0.0%	
Tumor location[2]	Cervical	1	100.0%	0	0.0%	0.843
	Middle Thoracic	36	97.3%	1	2.7%	
	Lower Thoracic	25	92.6%	2	7.4%	
	EGJ	50	94.3%	3	5.7%	
Neoadjuvant therapy[3]	No	94	95.9%	4	4.1%	
	Yes	19	90.5%	2	9.5%	0.286
Histoviability	No	3	100.0%	0	0.0%	1.000
	Yes	110	94.8%	6	5.2%	
Surgical Margin: R Category	R0	83	93.3%	6	6.7%	0.335
	R1-R2	30	100.0%	0	0.0%	
Smokers / Smoking status	No	11	100.0%	0	0.0%	0.440
	Yes	27	90.0%	3	10.0%	
	Former Smoker	56	94.9%	3	5.1%	
Alcoholic beverages drinking status**	No	28	93.3%	2	6.7%	0.820
	Yes	16	94.1%	1	5.9%	
	Former regular Drinker	30	96.8%	1	3.2%	

Reported as n and % unless otherwise specified. Abbreviation: ESCC - Esophageal squamous cell carcinoma. EGJ: Esophagogastric junction. p16: expression in tumor tissue by immunohistochemistry technique.
[1]AJCC 8th. seventh edition of the American Joint Commission on Cancer (AJCC) Cancer Staging Manual (TNM classification). [2]Location of cancer primary site is defined by cancer epicenter.[3] Neoadjuvant chemotherapy or radiotherapy before surgery. *HER2, human epidermal growth factor receptor 2 (Adenocarcinoma Only).**Excessive consumption of alcoholic beverages.

Table 3. Association of p16 expression in esophageal cancer with disease outcomes. Univariate analyses.

		p16				
		Negative		**Positive**		***P- value***
Death	No	18	17,1%	2	33,3%	0,295
	Yes	87	82,9%	4	66,7%	
Recurrence	No	59	55,1%	5	83,3%	0,231
	Yes	48	44,9%	1	16,7%	

Reported as n and % unless otherwise specified.
p16: expression in tumor tissue by immunohistochemistry technique.

5. CONCLUSÕES

Através dos três artigos científicos apresentados, foi possível atingir todos os objetivos propostos nesta tese. A revisão sistemática proposta no Artigo I identificou evidências na literatura da associação entre o polimorfismo de SOD2 Val16Ala, especialmente do alelo Ala, e o prognóstico de pacientes com câncer.

Os artigos originais revisados mostraram resultados ainda divergentes conforme o tipo de câncer e o desfecho clínico avaliado. Para tanto, uma hipótese sugerida é a existência de uma modulação diferencial de SOD2 Val16Ala, associada ao (Des)equilíbrio Superóxido-Peróxido de hidrogênio, e influenciada pelo microambiente tumoral, tratamento oncológico e fatores ambientais. Por ser o primeiro artigo a revisar evidências da literatura sobre a associação do polimorfismo Val16Ala e o prognóstico de pacientes oncológicos, verificamos a importância de estudos mais amplos avaliando esta associação em diferentes tipos de câncer.

Entendendo a heterogeneidade tumoral, a influência de SOD2 no processo carcinogênico, e a importância do polimorfismo Val16Ala, foi realizado o Artigo II com enfoque no câncer de esôfago. Nesse estudo observacional o polimorfismo supracitado foi identificado como um biomarcador tumoral preditivo, associado ao maior risco de câncer de esôfago, independente de idade e sexo. Assim, uma hipótese é que os indivíduos com alelo Ala podem ter maior atividade de SOD2, favorecendo a geração e acúmulo de peróxido de hidrogênio, o que induz dano celular adicional e promoveria o desenvolvimento de tumor.

Entretanto, uma associação direta estatisticamente significativa entre Val16Ala e expressão imunohistoquímica de SOD2 no câncer de esôfago ou do polimorfismo com a sobrevida dos pacientes não foi identificada. Assim, estudos prospectivos mais amplos devem ser realizados para confirmar essas associações.

A partir dos resultados do Artigo II, também verificamos que a expressão imunohistoquímica de SOD2 no câncer de esôfago foi um biomarcador prognóstico independente. De modo que, a baixa expressão imunohistoquímica de SOD2 no tecido tumoral foi associada a uma menor sobrevida de pacientes com câncer de esôfago. Esses resultados evidenciam a influência do equilíbrio oxidante e antioxidante na carcinogenese, especialmente no câncer de esôfago, e corroboram com as evidências identificadas no Artigo I.

Portanto, uma hipótese sugerida é que a baixa expressão de SOD2 pode permitir maior exposição de células anormais aos efeitos tóxicos do superóxido, contribuindo para um pior prognóstico. Por outro lado, a superexpressão de SOD2 pode contribuir para a transformação celular e progressão da doença ao favorecer o acúmulo de peróxido de hidrogênio, a menos que essa espécie reativa seja convertida em água e oxigênio pela ativação de outros componentes do sistema antioxidante.

Assim, sugere-se que os casos de superexpressão de SOD2 em câncer de esôfago estão associados a um melhor prognóstico quando outras enzimas compensam a geração excessiva do peróxido de hidrogênio removendo-o. Nesse contexto, estudos complementares devem ser realizados para avaliar demais fatores que influenciam a expressão de

SOD2 no câncer de esôfago, assim como a expressão de outras enzimas antioxidantes nesses tumores.

No Artigo III, ao avaliarmos a infecção viral por HPV16 em tumores esofágicos verificamos que todos os casos foram negativos para esse tipo de alto risco oncogênico de HPV, não sendo correlacionado com os seis casos da expressão imunohistoquímica da proteína p16 ou, como um fator prognóstico para câncer de esôfago.

Além disso, compreendendo a importância de p16 para o controle do ciclo celular e na supressão tumoral, avaliamos a correlação da expressão imunohistoquímica da proteína com a sobrevida dos pacientes. Porém, não identificamos uma correlação estatisticamente significativa, sugerindo que outros fatores podem influenciar na expressão de p16 e no prognóstico dos pacientes.

Portanto, diante destes resultados, salientamos a importância dos três artigos produzidos, especialmente pela identificação da expressão imunohistoquímica de SOD2 no câncer de esôfago como biomarcador independente de prognóstico, estimulando a realização de estudos prospectivos maiores, que futuramente podem contribuir para a medicina personalizada.

6. CONSIDERAÇÕES FINAIS

O presente estudo faz parte de um projeto maior intitulado "Identificação e associação dos fatores de risco para pacientes com câncer de esôfago atendidos no Hospital Santa Rita - Porto Alegre/RS", aprovado pelo Comitê de Ética em Pesquisa (CEP) da Irmandade da Santa Casa de Misericórdia de Porto Alegre - ISCMPA (Parecer N° 2.226.604/2017) e registrado na Comissão de Pesquisa (ComPesq) UFCSPA (Parecer Nº 090/2018). Deste projeto maior também foram desenvolvidos outros trabalhos de iniciação científica, conclusão de curso de graduação (TCC) e residência (TCR), orientados pela Profª Claudia Bica e coorientados pela doutoranda Aniúsca Vieira dos Santos.

A ISCMPA é um centro de referência no diagnóstico e tratamento de pacientes com câncer. A parceria entre os médicos do Ambulatório de Oncologia da ISCMPA e pesquisadores do PPG-Patologia UFCSPA originou em 2018 o Grupo de Pesquisa Translacional em Oncologia.

A pesquisa translacional, que transfere conhecimentos das ciências básicas para a clínica, corrobora com maior entendimento de mecanismos ou desenvolvimento de instrumentos diagnósticos ou terapêuticos de uso clínico em oncologia. Assim, O GPTO tem por objetivo o desenvolvimento de estudos promissores nas linhas de pesquisa de biomarcadores tumorais e processo saúde e doença.

Entre esses projetos, destaca-se o presente estudo sobre a avaliação de biomarcadores prognósticos em câncer de esôfago. Os artigos que compõe esta tese contribuem para com a literatura atual e fornecem dados relevantes sobre potenciais biomarcadores, especialmente SOD2. Como perspectivas futuras, o embasamento teórico fornecido pelo artigo I de revisão sistemática, assim como os dados e hipóteses gerados a partir dos artigos II e III, estimulam a continuidade de pesquisas e maiores estudos sobre SOD2, p16 e HPV no câncer de esôfago.

Esses dados refletem a busca por estudos de qualidade e relevantes em biomarcadores para câncer de esôfago, apesar das limitações impostas pela pandemia desde 2020, restrições orçamentárias, e inerentes a estudos retrospectivos (como a dificuldade no rastreamento de materiais biológicos arquivados e a ausência de algumas informações sociodemográficas e clinicopatológicas nos registros médicos).

A partir dos dados gerados concluímos que a maior compreensão sobre os fatores (genéticos, ambientais e comportamentais) e os processos fisiológicos (como o estresse oxidativo e processos antioxidantes) interligados à gênese e progressão do câncer de esôfago é fundamental, indicando a pesquisa translacional como um caminho para a medicina personalizada baseada em evidências.

7. BIOGRAFIA

Minha trajetória acadêmica iniciou em 2010, no curso de graduação em biomedicina da Universidade de Cruz Alta (UNICRUZ, Cruz Alta- RS, Brasil) onde me graduei como Biomédica Habilitada nas áreas de Análises Clínicas e Citopatologia Oncótica, participando de projetos de pesquisa com enfoque em câncer de colo de útero e infecção por HPV, orientados pelas citologistas Prof. Dra. Janaina Coser e Prof. Dra Janice Zanella, assim como no projeto extensionista intitulado "Liga Acadêmica de Oncologia Preventiva".

- **AV Santos,** A Silva, J Malheiros, J Zanella, J Coser. Análise Da Representação Da Junção Escamo Colunar Em Mulheres Menopausadas [Trabalho de Conclusão de Curso]. Unicruz, 2014.
- A Silva, **AV Santos,** J Malheiros, J Coser, JP Zanella. A Técnica De Papanicolau No Diagnóstico Da Microbiota Cérvico-vaginal. [Trabalho de Conclusão de Curso]. Unicruz, 2014.

A partir de 2014, ingressei na UFCSPA como aluna voluntária no grupo de pesquisa orientado pela Prof. Dra. Claudia Bica, vinculado à Pós-Graduação em Patologia (PPG Patologia), intensificando a minha participação em projetos de pesquisa sobre carcinogênse no Laboratório de Pesquisa em Patologia. Em 2015, iniciei o meu mestrado na linha de pesquisa processo saúde e doença, no mesmo PPG e sob orientação da Prof. Claudia Bica e coorientação do médico citologista Dr. João Carlos Prolla (*in memorian*), com enfoque em citologia ginecológica e câncer de colo de útero.

- **AV dos Santos,** GT dos Santos, RL Brackmann, JC Prolla, CG Bica. Follow-up of women with cervical cytological abnormalities: progression and regression events.Asian Pacific journal of cancer prevention: APJCP 20 (4), 1019.

Concomitantemente tive a oportunidade de colaborar com o estudo POP Brasil de análise da prevalência de infecção por HPV (genital, anal e oral) na população brasileira, incluindo a organização e logística do laboratório de epidemiologia clínica, padronização de técnicas, recebimento e processamento das amostras.

- Wendland, E. M. ; Villa, L. L. ; Unger, E. R. ; Domingues, C. M. ; Benzaken, A. S. ; Maranhao, A. G. K. ; Kops, N. L. ; Bessel, M. ; Caierao, J. ; Hohenberger, G. F. ; Horvath, J. D. ; Santos, G.T. ; Mello, B. P. ; **Santos, A. V. ;** Dal Berto, M. ; Bica, C. G. ; Pereira, G. F. M. ; Moreno, F. C. . Prevalence of HPV infection among sexually active adolescents and young adults in Brazil: The POP-Brazil Study. Scientific Reports, v. 10, p. 4920, 2020.

Neste período, fui autora e colaborei com projetos nos três eixos universitários: ensino, pesquisa e extensão. Fui coautora de capítulos de livros, materiais educativos, e ministrei aulas, palestras e cursos de citologia, bioética, biossegurança, epidemiologia, pesquisa científica em câncer de colo uterino e saúde da mulher. Supervisionei alunos de iniciação científica dos cursos de graduação em medicina, em farmácia e em biomedicina da UFCSPA. E no eixo extensão, fui membro da equipe organizadora do Projeto Mulheres em Ação, realizando atividades no Rio Grande do Sul e em Manaus com populações ribeirinhas, voltadas para prevenção de câncer de colo de útero e câncer de mama, bem como prevenção e diagnóstico de infecções sexualmente transmissíveis.

Em 2017 iniciei meu doutorado, sob orientação da Prof. Claudia Bica, voltado em um primeiro momento para análise de câncer de colo uterino e infecção por HPV em populações de risco (gestantes de alto risco e em mulheres imunossupressas). Em paralelo, iniciei projetos de pesquisa sobre fatores de risco associados ao câncer de esôfago, tanto carcinomas quanto adenocarcinomas esofágicos, sob coorientação do médico e oncologista Dr. Rafael José Vargas.

Porém, em 2018, devido a algumas limitações orçamentárias, foi necessária a modificação do projeto de doutorado, que foi substituído pelo estudo atual sobre biomarcadores prognósticos em câncer de esôfago, na linha de pesquisa de marcadores tumorais, fortalecendo as pesquisas realizadas anteriormente no Complexo Hospitalar Santa Casa.

Nesse mesmo ano, iniciei minha participação no Grupo de Pesquisa Translacional em Oncologia. Iniciamos novos estudos sobre câncer de esôfago, que além desta tese resultarão em trabalhos de conclusão de residência multidisciplinar em oncologia, e de conclusão de curso em biomedicina. Também foram produzidos resumos e posters, apresentados em eventos científicos, tais como *Next Frontiers To Cure Cancer* (A.C. Camargo), XXI Congresso Brasileiro de Oncologia Clínica, Congresso UFCSPA: conectando saúde e sociedade, e Jornadas Acadêmicas do PPG Patologia.

Ao longo da pós-graduação, tive a oportunidade de colaborar com outros pesquisadores em projetos nas temáticas de câncer de colo uterino, câncer de esôfago, câncer de mama, HPV, SOD2, e outros marcadores tumorais, utilizando diferentes metodologias de análise, como citologia, imunohistoquímica e biologia molecular. Alguns ainda não publicados, mas que corroboraram para a minha trajetória e crescimento profissional.

- L Olias. Estudo do perfil das pacientes que utilizam o serviço público de saúde para rastreamento do câncer de colo uterino e a associação com os fatores de risco. [Dissertação de Mestrado]. Orientadora: Claudia Bica. Coorientador: **Aniúsca Vieira dos Santos**. UFCSPA, 2019.

- Simone Valvassori, Giovana Tavares dos Santos, Aniúsca Vieira dos Santos, Claudia Giuliano Bica. Percepção de Adolescentes sobre fatores de risco associados ao HPV e outras infecções sexualmente transmissíveis. [Artigo não publicado - Dissertação de Mestrado].

- **Aniúsca Vieira dos Santos**, Giovana Tavares dos Santos, Antonella Jacobsen Kaul, Luiz Henrique Santana de Araújo, Barbara Pereira Mello, Luisa Lina Villa, Eliana Marcia Wendland, Claudia Giuliano Bica. Prevalence of genital HPV infection and associated factors among riverside women of the Brazilian Amazon. [Artigo ainda não publicado].

- LG Machado, **AV dos Santos**, GT dos Santos, CG Bica.Rastreamento do câncer do colo uterino em mulheres indígenas Mbyá-guarani. SANARE-Revista de Políticas Públicas 19 (2), 2020.

- C Silva , A. S. e, Bica, C. G., **Vieira, A.**, & Fantin. Molecular detection of oncogenic subtypes of human papillomavirus (HPV) in a group of women in the Amazon region of Brazil. Acta Scientiarum. Health Sciences 42 (1), 8, 2020.

- Maiquidieli Dal Berto, **Aniúsca Vieira dos Santos**, Giovana Tavares dos Santos, Gabriela Krüger da Costa, Pettala Rigon, Rafael José Vargas Alves, Claudia Giuliano Bica. Val16ala-sod2 polymorphism may be a predictor of recurrence risk of breast cancer in patients treated with adjuvant tamoxifen. [Artigo não publicado, Tese de Doutorado]

- Dal Berto, M., dos Santos, G.T., **dos Santos, A.V**. et al. Molecular markers associated with the outcome of tamoxifen treatment in estrogen receptor-positive breast cancer patients: scoping review and in silico analysis. Discov Onc 12, 37 (2021). https://doi.org/10.1007/s12672-021-00432-7

8. APÊNDICES (Opcional)

8.1. Protocolo de Revisão Sistemática - Estudo piloto

NIHR | National Institute for Health Research

PROSPERO
International prospective register of systematic reviews

Citation

Aniusca Vieira dos Santos, Giovana Tavares dos Santos, Maiquidieli Dal Berto, Ivana Beatrice Mânica da Cruz, Rafael Vargas Alves, Claudia Giuliano Bica. The impact of SOD2 on the prognosis of esophageal cancer: systematic review of the literature. PROSPERO 2019 CRD42019129554 Available from: https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42019129554

Review question

The expression of SOD 2 has prognostic impact on esophagus cancer?

Searches

We will search the following databases MEDLINE, SciELO and EMBASE, studies in any language, published in the period 01/01/2000 until 11/30/2018, and in humans. Moreover, we will search grey literature databases and other literature sources. Search terms are built around key words (and synonyms) "Esophageal Neoplasms", "Superoxide Dismutase 2". Additional details of the search strategy can be found in the attached PDF document.

Search strategy

https://www.crd.york.ac.uk/PROSPEROFILES/129554_STRATEGY_20190521.pdf

Types of study to be included

This systematic review will include observational studies.

Condition or domain being studied

Many biomarkers were proposed as predictors of adverse neoplastic events in esophagus. So far, no systematic review has been conducted to verify the difference in the prognosis of esophageal cancer according to the expression of SOD2. In times of gene therapy and functional imitation of antioxidant enzymes, understanding the impact of polymorphisms and expression of SOD2 on patient survival may be useful tool in the application of new therapies for esophageal cancer and change of prognosis.Therefore, this review will focus on the impact of SOD2 on the prognosis of esophageal cancer.

Participants/population

INCLUSION:

Studies with female or male patients with esophageal cancer, without age restriction.

EXCLUSION:
Studies with SOD2 expression / polymorphism unclassified by molecular or immunohistochemical analysis, and without analysis of correlation for patients prognosis (survival).

Intervention(s), exposure(s)

Not applicable.

Comparator(s)/control

Not applicable.

Context

Studies evaluating the prognosis of an SOD2 expression / polymorphism.

Main outcome(s)

Evaluation of SOD2 expression / polymorphism by molecular or immunohistochemical analysis and correlation with prognosis (death or disease recurrence)

Measures of effect

Relative and absolute frequency; Disease-free survival; Overall survival.

Additional outcome(s)
Not applicable.

Measures of effect

Not applicable.

Data extraction (selection and coding)
Two authors will perform the data extraction independently using standardized forms. In case of disagreement, a third researcher will be consulted. The following data will be collected: publication title, authors, publication year, study design, characteristics of the population (number of participants, age and gender), esophageal neoplasms (adenocarcinoma or carcinoma), SOD2 (expression or polymorphism), methodology for analyzed SOD2 (i.e. polymerase chain reaction, immunohistochemistry) and Follow-up. If the study is reported in duplicate, the study published earlier or the one that provided more detailed information will be included.

Risk of bias (quality) assessment
The included studies will be assessed for quality using and adapted version of the NIH 'Quality Assessment Tool for Observational Cohort and Cross-Sectional Studies'

Strategy for data synthesis
We will provide a narrative synthesis of the findings from the included studies, structured around the prognosis of esophageal cancer in relation to a SOD2.

Analysis of subgroups or subsets
We will perform subgroup analysis for histological type of esophageal neoplasm and whenever possible in relation to SOD2 expression and polymorphisms.

Contact details for further information
Aniúsca Vieira dos Santos
aniusca.vieira@gmail.com

Organisational affiliation of the review
Federal University of Health Sciences of Porto Alegre

Review team members and their organisational affiliations
Mrs Aniusca Vieira dos Santos. Federal University of Health Sciences of Porto Alegre, Porto Alegre - Brazil
Dr Giovana Tavares dos Santos. Institute for Education and Research Hospital Moinhos de Vento, Porto Alegre, Brazil
Ms Maiquidieli Dal Berto. Federal University of Health Sciences of Porto Alegre – Porto Alegre, Brazil
Dr Ivana Beatrice Mânica da Cruz. Federal University of Santa Maria – Santa Maria, Brazil
Dr Rafael Vargas Alves. Department of Clinical Oncology, Santa Casa Hospital Complex, Porto Alegre,Brazil
Dr Claudia Giuliano Bica. Federal University of Health Sciences of Porto Alegre, Porto Alegre - Brazil

Type and method of review
Prognostic, Systematic review

Anticipated or actual start date
02 March 2019

Anticipated completion date
30 April 2020

Funding sources/sponsors
This study will be financed in part by the Coordination of Improvement of Higher Education Personnel - Brazil (CAPES) - Finance Code 001.

Conflicts of interest

Language
English

Country
Brazil

Stage of review
Review Ongoing

Subject index terms status
Subject indexing assigned by CRD

Subject index terms
Esophageal Neoplasms; Humans; Prognosis; Superoxide Dismutase

Date of registration in PROSPERO
16 July 2019

Date of first submission
22 March 2019

Stage of review at time of this submission

Stage	**Started**	**Completed**
Preliminary searches	Yes	Yes
Piloting of the study selection process	Yes	Yes
Formal screening of search results against eligibility criteria	Yes	Yes
Data extraction	Yes	Yes
Risk of bias (quality) assessment	No	No
Data analysis	No	No

Revision note
The anticipated completion date in the PROSPERO was uptaded from December 02th to April 30th. The reason is that the data extraction took longer than expected.

The record owner confirms that the information they have supplied for this submission is accurate and complete and they understand that deliberate provision of inaccurate information or omission of data may be construed as scientific misconduct.
The record owner confirms that they will update the status of the review when it is completed and will add publication details in due course.

Versions
16 July 2019
07 October 2020

8.2. Protocolo de Revisão Sistemática Artigo I

NIHR | National Institute for Health Research

PROSPERO
International prospective register of systematic reviews

To enable PROSPERO to focus on COVID-19 registrations during the 2020 pandemic, this registration record was automatically published exactly as submitted. The PROSPERO team has not checked eligibility.

Citation

Aniúsca Vieira dos Santos, Claudia Giuliano Bica. SOD2 (rs4880) POLYMORPHISM AS A PROGNOSTIC MARKER FOR CANCER: A SYSTEMATIC REVIEW. PROSPERO 2021 CRD42021254468 Available from: https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42021254468

Review question

What are the associations between the Superoxide dismutase 2 (SOD2) Val16Ala polymorphism and the prognosis of cancer patients?

Searches

We will search the following databases MEDLINE, Web of Science and Google Scholar (grey literature) studies in any language, published in the period 01/01/2010 until 01/01/2020, and in humans. Moreover, we will scan the reference lists of identified publications for additional studies. Search terms are built around keywords "neoplasm" and "superoxide dismutase 2", or their variations.

Types of study to be included

Inclusion criteria:

Cohort ou case-control studies, Human or Part with Human.
Abstract and full text available, any language.

Exclusion criteria:

Trial, Review, Totality cell culture, Animal model study, Dissertation, Conference proceeding, abstract or poster, or it was a duplication of a previous study.

Condition or domain being studied

Patients diagnosed with cancer and genotype analysis for the SOD2 Val16Ala polymorphism.

Participants/population

Female or Male, no age restriction; all cases were first diagnosed as cancer.

Intervention(s), exposure(s)

None.

Comparator(s)/control

None.

Main outcome(s)

Associations identified in the literature between the clinical outcome of cancer patients and the SOD2 Val16Ala polymorphism.

Measures of effect

Not applicable.

Additional outcome(s)

Information about possible interactions with treatment according to the type of cancer or dietary factors.

Measures of effect

Not applicable.

Data extraction (selection and coding)
Extracted data: first author, year of publication, the purpose of study, country of study, ethnicity of participants, sample number, study design (cohort or control case), selection of participants, type of cancer studied, time of follow-up, results, and frequency distribution of allele or genotype (according to availability in the article). The data will be extracted by 3 reviewers, consensus resolved divergences.

Risk of bias (quality) assessment
The included studies will be assessed for quality using the Newcastle-Ottawa Quality Assessment Scale (NOS) methodology for case-control studies and for cohort studies.

Strategy for data synthesis
We will make a narrative synthesis of the findings of the included studies, structured around the association between the SOD2 polymorphism (Val16Ala) and prognosis of cancer patients. Whenever possible, a meta-analysis of the data will be applied.

Analysis of subgroups or subsets
Not applicable.

Contact details for further information
Aniúsca Vieira dos Santos
aniusca.vieira@gmail.com

Organisational affiliation of the review
Federal University of Health Sciences of Porto Alegre

Review team members and their organisational affiliations
Dr Aniúsca Vieira dos Santos. Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA)
Professor Claudia Giuliano Bica. Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA)

Collaborators
Rafael José Alves Vargas. Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA) + Hospital Santa Rita-Complexo Hospitalar Santa Casa de Misericórdia de Porto Alegre.
Dr Gioavana Tavares dos Santos. Responsabilidade Social PROADI-SUS, Hospital Moinhos de Vento.
Antonella Jacobsen Kaul. Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA)
Camila de Paula Macedo. Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA)
Maiquidieli Dal Berto. Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA)

Type and method of review
Narrative synthesis, Prognostic, Systematic review

Anticipated or actual start date
06 May 2020

Anticipated completion date
28 June 2021

Funding sources/sponsors
No funding sources.

Conflicts of interest

Language
English

Country
Brazil

Stage of review
Review Ongoing

Subject index terms status
Subject indexing assigned by CRD

Subject index terms
MeSH headings have not been applied to this record

Date of registration in PROSPERO
11 June 2021

Date of first submission
11 May 2021

Stage of review at time of this submission

Stage	Started	Completed
Preliminary searches	Yes	No
Piloting of the study selection process	Yes	Yes
Formal screening of search results against eligibility criteria	Yes	Yes
Data extraction	No	No
Risk of bias (quality) assessment	No	No
Data analysis	No	No

The record owner confirms that the information they have supplied for this submission is accurate and complete and they understand that deliberate provision of inaccurate information or omission of data may be construed as scientific misconduct.
The record owner confirms that they will update the status of the review when it is completed and will add publication details in due course.

Versions
11 June 2021
11 June 2021

9. ANEXOS

9.1. Registro do Projeto na Comissão de Pesquisa UFCSPA

Nº de Registro de Projeto

2 mensagens

Aniúsca Vieira dos Santos <aniuscavieira@gmail.com> 19 de agosto de 2020 14:46
Para: compesq@ufcspa.edu.br
Cc: "claudia@ufcspa.edu.br" <claudia@ufcspa.edu.br>, Antonella Jacobsen <antonellajacobsen@gmail.com>

Olá,
Gostaria de solicitar o número do registro na COMPESQ do projeto " IDENTIFICAÇÃO E ASSOCIAÇÃO DOS FATORES DE RISCO PARA PACIENTES COM CÂNCER DE ESÔFAGO ATENDIDOS NO HOSPITAL SANTA RITA – PORTO ALEGRE/RS",

Aguardo retorno
Abraço

Aniúsca Vieira dos Santos

Biomédica (UNICRUZ)
Mestre em Patologia Geral e Experimental (UFCSPA)
Doutoranda no Programa de Pós Graduação em Patologia (UFCSPA)

Contato: (51) 3303-8715
Laboratório de Epidemiologia Clínica - Epiclin (UFCSPA)

Comissão de Pesquisa [UFCSPA] <compesq@ufcspa.edu.br> 20 de agosto de 2020 14:41
Para: Aniúsca Vieira dos Santos <aniuscavieira@gmail.com>
Cc: "claudia@ufcspa.edu.br" <claudia@ufcspa.edu.br>, Antonella Jacobsen <antonellajacobsen@gmail.com>

O número de registro deste projeto na ComPesq é 090/2018.

Atenciosamente,

Henrique Silveira
Assistente em Administração
ComPesq - UFCSPA
[Texto das mensagens anteriores oculto]

9.2. Parecer do Comitê de Ética da ISCMPA

IRMANDADE DA SANTA CASA DE MISERICORDIA DE PORTO ALEGRE - ISCMPA

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DA EMENDA

Título da Pesquisa: IDENTIFICAÇÃO E ASSOCIAÇÃO DOS FATORES DE RISCO PARA PACIENTES COM CÂNCER DE ESÔFAGO ATENDIDOS NO HOSPITAL SANTA RITA - PORTO ALEGRE/RS

Pesquisador: Claudia Giuliano Bica

Área Temática:

Versão: 3

CAAE: 68207117.0.0000.5335

Instituição Proponente: Irmandade da Santa Casa de Misericordia de Porto Alegre - ISCMPA

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 2.226.604

Apresentação do Projeto:

A avaliação anterior não se altera em razão da emenda.

Objetivo da Pesquisa:

Objetivo da Emenda:

Solicitar ajustes no TCLE afim de incluir no termo de consentimento a solicitação do acesso ao bloco de parafina arquivado na ISCMPA. A utilização do bloco permitirá a comparação da prevalência de genótipos do HPV em tecido esofágico e material proveniente da coleta com cepacol.

Avaliação dos Riscos e Benefícios:

Apresentados e adequados.

Comentários e Considerações sobre a Pesquisa:

A pesquisa encontra-se de acordo com a Norma vigente Resolução 466/12 para pesquisa em seres humanos.

Considerações sobre os Termos de apresentação obrigatória:

Conforme justificava apresentada pelos pesquisadores:

Endereço: R. Profª Annes Dias,295 Hosp.Dom Vicente Scherer
Bairro: 6º andar - Centro **CEP:** 90.020-090
UF: RS **Município:** PORTO ALEGRE
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"...Se fez necessário realizar esta emenda para ajustes no TCLE do Projeto intitulado "IDENTIFICAÇÃO E ASSOCIAÇÃO DOS FATORES DE RISCO PARA PACIENTES COM CÂNCER DE ESÔFAGO ATENDIDOS NO HOSPITAL SANTA RITA - PORTO ALEGRE/RS" aprovado no comitê de ética em pesquisa da ISCMPA sob o parecer de nº 2.181.771/2017, afim de incluir no termo de consentimento a solicitação do acesso ao bloco de parafina arquivado na ISCMPA. A utilização do bloco permitirá a comparação da prevalência de genótipos do HPV em tecido esofágico e material proveniente da coleta com cepacol.
No TCLE versão 1.2, item 3 (Do procedimento para coleta de dados) foi incluída a frase "e caso tenha bloco de parafina resultante da biópsia de esôfago realizada/arquivada no Complexo Hospitalar Santa Casa também solicitamos o acesso a este material", e no item 4 (Da utilização, armazenamento e descarte das amostras) foi incluída a frase "Os blocos de parafina serão devolvidos ao arquivo hospitalar após o estudo", ambas as frases grifadas em amarelo..."

Conclusões ou Pendências e Lista de Inadequações:
Mediante as justificativas apresentadas não encontramos óbices.

Considerações Finais a critério do CEP:
Após avaliação das alterações efetuadas no estudo acima descrito, o presente Comitê não encontrou óbices quanto à implementação das mesmas.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_976033_E1.pdf	11/08/2017 16:26:03		Aceito
Outros	Carta_de_justificativa.pdf	11/08/2017 16:24:33	Aniúsca Vieira dos Santos	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_versao_1_2.pdf	09/08/2017 14:07:24	Aniúsca Vieira dos Santos	Aceito
Outros	Carta_ajuste_de_pendencias.pdf	12/07/2017 19:20:48	Aniúsca Vieira dos Santos	Aceito
Folha de Rosto	FOLHA_DE_ROSTO.pdf	09/05/2017 16:52:02	Jordan Boeira dos Santos	Aceito
Outros	FICHA_COLETA.pdf	09/05/2017 16:22:22	Jordan Boeira dos Santos	Aceito
Outros	QUESTIONARIO.pdf	09/05/2017 16:22:05	Jordan Boeira dos Santos	Aceito

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Outros	DECLARACAO_ISENCAO_ONUS.pdf	09/05/2017 16:21:21	Jordan Boeira dos Santos	Aceito
Declaração de Manuseio Material Biológico / Biorepositório / Biobanco	DECLARACAO_UTILIZACAO_MATERIAL_BIOLOGICO.pdf	09/05/2017 16:20:24	Jordan Boeira dos Santos	Aceito
Outros	DECLARACAO_UTILIZACAO_DADOS_PRONTUARIOS.pdf	09/05/2017 16:19:34	Jordan Boeira dos Santos	Aceito
Outros	DECLARACAO_CONFIDENCIALIDADE.pdf	09/05/2017 16:18:39	Jordan Boeira dos Santos	Aceito
Outros	FORMULARIO_INSCRICAO.pdf	09/05/2017 16:17:54	Jordan Boeira dos Santos	Aceito
Orçamento	ORCAMENTO.pdf	09/05/2017 16:15:42	Jordan Boeira dos Santos	Aceito
Declaração de Instituição e Infraestrutura	DECLARACAO_CHEFIA_PPV.pdf	09/05/2017 16:15:14	Jordan Boeira dos Santos	Aceito
Cronograma	CRONOGRAMA.pdf	09/05/2017 16:10:45	Jordan Boeira dos Santos	Aceito
Declaração de Instituição e Infraestrutura	DECLARACAO_CHEFIA_HSR.pdf	09/05/2017 16:09:21	Jordan Boeira dos Santos	Aceito
Projeto Detalhado / Brochura Investigador	PROJETO.pdf	09/05/2017 16:08:39	Jordan Boeira dos Santos	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

PORTO ALEGRE, 18 de Agosto de 2017

Assinado por:
ELIZETE KEITEL
(Coordenador)

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