UNIVERSIDADE ESTADUAL DE LONDRINA

ALEXSANDRO KOIKE

AVALIAÇÃO DAS VARIANTES rs231775 E rs231779 DO GENE *CTLA4* EM PACIENTES COM CARCINOMA UROTELIAL DE BEXIGA

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Orientador: Prof. Dr. Marcell Alysson Batisti Lozovoy
Co-orientadora: Profa. Dra. Andréa Name Colado Simão

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RESUMO

INTRODUÇÃO: O câncer de bexiga é o 10º tumor mais incidente e o 13º mais letal no mundo. O carcinoma urotelial de bexiga (CUB) é o tipo histológico mais freqüente, correspondendo a 90% dos casos. A quantidade de mutações somáticas de uma determinada sequência genômica de um tumor (TMB) comparada ao tecido normal varia em cada tipo de tumor. Os tumores com maiores TMB são o melanoma, seguido do carcinoma de não pequenas células do pulmão e o CUB. Esses tumores tendem a responder melhor ao tratamento imunoterápico. São exemplos de imunoterápicos, as drogas inibidoras de proteínas de ponto de checagem: a anti-proteína de morte programada-1(anti-PD-1); o anti-ligante da proteína de morte programada-1(PD-L1) e o anti-antígeno 4 do linfócito T-citotóxico (anti-CTLA-4). Esses imunoterápicos revolucionaram o tratamento do câncer nos últimos 10 anos. A maior ou menor expressão dessas proteínas na célula tumoral e nos linfócitos estão relacionadas ao prognóstico e resposta ao tratamento, sendo utilizados, também, como biomarcadores de resposta terapêutica. Entretanto, no CUB, parte dos biomarcadores disponíveis muitas vezes falha ao aferir o prognóstico e a eficácia do tratamento a ser realizado. O melhor entendimento dos efeitos das variantes genéticas de citocinas e da expressão de proteínas de ponto de checagem poderia servir como biomarcador do diagnóstico, prognóstico e/ou da decisão do tratamento.

OBJETIVO: Buscar um melhor entendimento das diferentes variantes genéticas de citocinas relacionadas ao CUB, avaliar a frequência de 2 variantes genéticas do *CTLA4* rs231775(+49AG) e rs231779(+1822CT) e relacionar com suscetibilidade e fatores prognósticos como o estadiamento da doença e a presença de metástases.

SUJEITOS E MÉTODOS: Foi realizada uma revisão da literatura de variantes genéticas de citocinas relacionadas ao CUB seguindo as normas para revisões sistemáticas e meta-análises (PRISMA). Também foi realizado um estudo caso-controle, no qual foram selecionados 140 pacientes com diagnóstico de CUB submetidos a cirurgia, recrutados pelo Ambulatório de Urologia do Hospital do Câncer de Londrina, além de 145 indivíduos controle, provenientes de doadores de sangue do Hemocentro Regional de Londrina (CAAE: 17084619.2.0000.5231). Os dados clínicos foram obtidos por questionário aplicado a todos os participantes. Foram avaliadas as variantes genéticas do gene *CTLA4*(rs231775 e rs231779) por metodologia de reação em cadeia de polimerase em tempo real com sondas específicas tipo TaqMan®.

RESULTADOS: Na revisão da literatura, encontramos 36 estudos caso-controle de variantes de citocinas relacionadas ao CUB. As variantes genéticas de *IL27*(rs153109), *IL6*(rs1800795), *IFNG*(rs62559044), *IL2*(rs2069762) e *IL12*(rs3212227), cujas expressões dessas variantes tendem a diminuir a produção dessas citocinas, mostraram aumento de risco e recorrência ao CUB. As variantes genéticas de *TNFA*(rs1800629, rs1799724 e rs1799964), *IL22*(rs2227485), *IL31*(rs4758680), *IL32*(rs12934561 e rs28372698), *TGFB*(rs1800470 e rs1800471), *TGFBR1*(rs868), *IL4*(rs2243248 e rs2243250), *IL8*(rs4073), *IL10*(rs1800896 e rs1800871), *IL17*(rs2275913, rs763780), *IL23R*(rs10889677 e rs188444), *IL18*(rs187283 e rs1946518) e *IL13*(rs1800925), cujas expressões dessas variantes tendem a elevar a produção dessas citocinas, mostraram aumento ao risco, da recorrência e da progressão tumoral ao CUB. As variantes genéticas do *IL1*(rs1143634 e rs16944), estudadas em 2 artigos, mostraram resultados divergentes em relação ao risco ao CUB. Em relação ao nosso estudo caso-controle realizado, verificamos que os genótipos heterozigotos no modelo overdominante do *CTLA4* rs231775(+49AG) e rs231779(+1822CT) apresentaram diferenças significativas como fator protetor ao CUB. O genótipo codominante heterozigoto rs231779(+1822CT) também se mostrou como fator protetor ao CUB. Não identificamos diferenças estatísticas em relação a invasividade da camada muscular, da presença de metástases, do grupo de risco, da falha ao tratamento com o Bacilo de Calmette-Guérin (BCG) e da recorrência pós primeiro tratamento cirúrgico. O modelo de regressão logística utilizado na comparação entre indivíduos controle e CUB demonstrou que os genótipos homozigóticos das duas variantes, associados a idade e o tabagismo foram preditores da doença, explicando em 89,1% a presença do CUB.

CONCLUSÃO: Os estudos das diversas variantes genéticas de citocinas demonstram que sua expressão no CUB apresenta uma ampla gama de resultados, nem sempre intuitiva, possivelmente justificada pelo pleiotropismo da ação das citocinas no sistema imune e no momento a qual suas ações interferem na carcinogênese do CUB. No estudo caso-controle realizado, propomos que os modelos polimórficos overdominantes do *CTLA4* (rs231775 e rs231779), em conjunto com idade e tabagismo, podem servir como potenciais biomarcadores de suscetibilidade ao risco do CUB. As variantes genéticas do *CTLA4* no CUB são pouco compreendidas e sua relação ainda há de ser esclarecida, necessitando de mais estudos para sua melhor compreensão.

PALAVRAS CHAVES: Carcinoma urotelial de bexiga, citocinas, variante de nucleotídeo único, CTLA-4, rs231775, rs231779.

Koike, Alexsandro**. Evaluation of *CTLA4* gene rs231775 and rs231779 variants in patients with urothelial bladder cancer**. 2023. 123f. Thesis (Doctoral in Clinical and Laboratory Pathophysiology) – Londrina State University, Londrina 2023.

ABSTRACT

INTRODUCTION: Bladder cancer is the 10th most common tumor and the 13th most lethal in the world. Urothelial bladder carcinoma (UBC) is the most frequent histological type, accounting for 90% of cases. The amount of somatic mutations of a given genomic sequence in a tumor (TMB) compared to normal tissue varies in each type of tumor. The tumors with the highest TMB are Melanoma, followed by non-small cell lung carcinoma and UBC. These tumors tend to respond better to immunotherapy treatment. Examples of immunotherapies are drugs that inhibit checkpoint proteins: anti-programmed death protein-1 (anti-PD-1); anti-programmed death protein-1 ligand (PD-L1) and anti-cytotoxic T-lymphocyte antigen 4 (anti-CTLA-4). These immunotherapies have revolutionized cancer treatment in the last 10 years. The greater or lesser expression of these proteins in tumor cells and lymphocytes is related to prognosis and response to treatment, and they are also used as biomarkers of therapeutic response. However, in UBC, some of the biomarkers available often fail to gauge the prognosis and effectiveness of the treatment to be carried out. A better understanding of the effects in cytokines genetic variants and the expression of checkpoint proteins could serve as biomarkers for diagnosis, prognosis and/or treatment decisions.

OBJECTIVE: To search a better understanding of the different cytokines genetic variants related to CUB, to evaluate the frequency of 2 genetic variants of *CTLA4* rs231775 (+49AG) and rs231779 (+1822CT) and to relate them to susceptibility and prognostic factors such as the stage of the disease and the presence of metastases.

SUBJECTS AND METHODS: A literature review of genetic variants of cytokines related to UBC was carried out following the guidelines for systematic reviews and meta-analyses (PRISMA). A case-control study was also carried out, in which 140 patients diagnosed with UBC who had undergone surgery were selected, recruited by the Urology Clinic of the Londrina Cancer Hospital, as well as 145 control subjects, who were blood donors from the Regional Blood Bank of Londrina (CAAE: 17084619.2.0000.5231). Clinical data was obtained through a questionnaire administered to all participants. The genetic variants of the *CTLA4* gene (rs231775 and rs231779) were assessed by real-time polymerase chain reaction methodology, using specific TaqMan®-type probes.

RESULTS: In the literature review, we found 36 case-control studies of UBC-related cytokine variants. Genetic variants of *IL27* (rs153109), *IL6* (rs1800795), *INFG* (rs62559044), *IL2* (rs2069762) and *IL12* (rs3212227), whose expression tends to decrease the production of these cytokines, showed increased risk and recurrence of UBC. Genetic variants of *TNFA* (rs1800629, rs1799724 and rs1799964), *IL22* (rs2227485), *IL31* (rs4758680), *IL32* (rs12934561 and rs28372698), *TGFB* (rs1800470 and rs1800471), *TGFBR1* (rs868), *IL4* (rs2243248 and rs2243250), *IL8* (rs4073), *IL10* (rs1800896 and rs1800871), *IL17* (rs2275913, rs763780), *IL23R* (rs10889677 and rs188444), *IL18* (rs187283 and rs1946518), *IL13* (rs1800925), whose expressions of these variants tend to increase the production of these cytokines, showed an increase in risk, recurrence and tumor progression to UBC.The *IL1* genetic variants (rs1143634 and rs16944) studied in 2 articles showed divergent results in relation to the risk of UBC. In our case-control study, we found that the heterozygous genotypes in the *CTLA4* overdominant model rs231775 (+49AG) and rs231779 (+1822CT) showed significant differences as a protective factor for UBC. The heterozygous codominant genotype rs231779 (+1822CT) was also shown to be a protective factor against UBC. We found no statistical differences in invasiveness of the muscle layer, presence of metastasis, risk group, failure of BCG treatment and recurrence after the first surgical treatment. The logistic regression model used to compare control and UBC individuals showed that the homozygous genotipe of the two variants associated with age and smoking were predictors of the disease, explaining 89.1% of the presence of UBC.

CONCLUSION: Studies of the various genetic variants of cytokines show that their expression in UBC presents a wide range of results, not always intuitive, possibly justified by the pleiotropism of the action of cytokines on the immune system and the time at which their actions interfere in the carcinogenesis of UBC. In the case-control study carried out, we propose that the overdominant polymorphic models of *CTLA4* (rs231775 and rs231779), together with age and smoking, could serve as potential biomarkers of susceptibility to the risk of UBC. The genetic variants of *CTLA4* in UBC are poorly understood and their relationship has yet to be clarified, requiring further studies for a better understanding.

KEYWORDS: Urothelial bladder carcinoma, cytokines, single nucleotide variant, CTLA-4, rs231775, rs231779.

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LISTA DE ABREVIATURAS E SIGLAS

ADC	Anticorpo droga conjugada
Ag	Antígeno
AP-1	Fator de ativação da proteína 1 (*Activating Protein-1*)
APC	Célula apresentadora de antígeno
BCG	Bacilo de Calmette-Guerin
BTA stat	Antígeno tumoral da bexiga STAT (*Bladder Tumor Antigenstat*)
CaB	Câncer de bexiga
CD4+	Grupamento de diferenciação 4 (*Cluster* of Differentiation 4)
CD8+	Grupamento de diferenciação 8 (*Cluster* of Differentiation 8)
CDKN2A	Inibidor 2A da quinase ciclina dependente (*cyclin-Dependent kinase inhibitor 2A*)
ctDNA	DNA do tumor circulante (*Circulanting Tumor DNA*)
CTL	Linfócito T-citotóxico (*Cytotoxic T Lymphocyte*)
CTLA-4	Antígeno 4 do linfócito T-citotóxico (*Cytotoxic T-lymphocyte Antigen-4*)
CUB	Carcinoma urotelial de bexiga
DC	Célula dendrítica (*Dendritic Cell*)
EBRT	Radioterapia de feixe externo (*External Beam Radiation Therapy*)
FDA	Administração de Alimentos e Medicamentos (*Food and Drug Administration*)
flCTLA-4	Forma completa do CTLA-4 (*full length Cytotoxic T-lymphocyte Antigen-4)*
FGFR	Receptor do fator de crescimento fibroblástico (*Fibroblast Growth Factor Receptor*)
GM-CSF	Fator estimulatório de colônia granulocitário-macrofágico *(Granulocyte Macrophage Colony Stimulating Factor*)
HER	Receptor do fator de crescimento epidermal humano
IL	Interleucina
IMC	Índice de massa corpórea
IDO	Indoleamina 2,3-deoxigenase
INCA	Instituto Nacional do Câncer
IFN- γ	Interferon-gama
MDSCs	Células supressoras mieloderivadas
MAPK	*Mitogen Activated Protein Kinase*
MHC	Complexo maior de histocompatibilidade (*Major Histocompatibility Complex*)
NFAT	Fator nuclear de células T ativadas (*Nuclear Factor of Activated T*-Cells)

NF-κB	Fator nuclear Kappa B (*Nuclear Factor Kappa B*)
NKG2D	Receptor estimulatório membro D do grupo 2 de células Natural Killer
NMP22	Proteína de matriz nuclear número 22(*Nuclear Matrix Protein Number 22*)
PA	Pressão arterial
PD-1	Proteína de ponto de verificação “morte programada-1” (*Programmed death-1)*
PD-L1	Proteína ligante “morte programada-1” (*Programmed death-ligand 1)*
PGE-2	Prostaglandina E-2
QT	Quimioterapia
sCTLA-4	CTLA-4 solúvel (*soluble Cytotoxic T-lymphocyte Antigen-4)*
SLD	Sobrevida livre de doença
SG	Sobrevida global
SNP	Polimorfismo de nucleotídeo único (*Single Nucleotide Polymorphism*)
SNV	Variante de nucleotídeo único (*Single Nucleotide Variant*)
STAT	Sinal transdutor e ativador de transcrição (*Signal Transducer and Activator of Transcription*)
TAM	Macrófagos associado ao tumor (*Tumor Associated Macrophages*)
TERT	Transcritase reversa da telomerase (*Telomerase Reverse Transcriptase*)
TLS	Estrutura linfóide terciária (*Tertiary Lymphoid Structure*)
TMB	Carga mutacional tumoral (*Tumor Mutational Burden*)
TNF- **α**	Fator de necrose tumoral alfa **(***Tumor Necrosis Factor Alpha***)**
TP53	Proteína tumoral P53 (*Tumor Protein 53*) ou p53
TRAIL	Ligante indutor relacionada a apoptose do Fator de Necrose Tumoral (*TNF-related apoptosis-Inducing ligand*)
Treg	Célula T regulatória
TGF-β	Fator de transformação de crescimento beta
VEGF	Fator de crescimento vascular endotelial

SUMÁRIO

1. INTRODUÇÃO

1.1 Câncer de Bexiga

No Brasil, o câncer de bexiga é o 7º câncer mais incidente no homem e o 14º na mulher sendo que a maioria dos casos ocorre após a 5-6ª década de vida. Em 2017, foi responsável por 3.021 óbitos no Brasil (INCA, 2022). Ao nível mundial ocupa a 10ª posição na incidência de todos os tipos de câncer com uma estimativa para 2025 de 651.049 casos novos e 246.242 mortes, ocupando a 13ª posição no número de mortes (FERLAY *et al.*, 2018; SUNG *et al.*, 2021; TEMPO *et al.,* 2023; GLOBOCAN, 2023).

Histologicamente, o câncer de bexiga apresenta-se 90% das vezes como carcinoma urotelial de bexiga (CUB), 5% como carcinoma epidermóide e menos de 2% das vezes como adenocarcinoma ou outro tipo histológico (WALSH *et al.*, 2002; LENIS *et al.*,2020).

Os principais fatores de risco para CUB são o tabaco, idade, exposição a produtos químicos, história prévia de tratamento com radioterapia ou ciclofosfamida, história familial e sexo masculino (FREEDMAN *et al*, 2011; SIEGEL *et al*., 2019; MAIORANO *et al*., 2022). De modo geral, tabagistas têm 3 vezes mais chances de desenvolver CUB em relação aos não-fumantes (FREEDMAN *et al*., 2011).

Processos inflamatórios crônicos (como o uso de sonda vesical de demora, cálculos vesicais) e infecções, principalmente pelo *Schistossoma haematobium* (mais prevalente no Egito e Oriente Médio) estão relacionados ao carcinoma epidermóide da bexiga (ISHIDA *et al.*, 2018; CHUNG *et al.*, 2013; LANDSKRON *et al.*, 2014; VERMEULEN *et al.*, 2015).

O sintoma mais comum no CUB é a hematúria macroscópica assintomática. O diagnóstico da lesão tumoral vista pelo ultrassom é o método mais freqüente de diagnóstico. A cistoscopia armada com biópsia é diagnóstica e muitas vezes é o tratamento pela facilidade da realização da cirurgia em tumores em fases iniciais. Nos tumores uroteliais de trato urinário alto (rins, pelve renal e ureteres), a urotomografia ou ressonância nuclear magnética são exames de escolha. São indicadas quando há hematúria assintomática com ultrassom de bexiga normal e sem dilatação de ureter. A ureterorrenoscopia flexível é outro método diagnóstico

para a investigação da hematúria assintomática de causa não definida por outros meios diagnósticos (NCCN, 2023).

O CUB, quando diagnosticado e tratado precocemente, tem alta probabilidade de cura, sendo que 98% dos pacientes diagnosticados como tumores superficiais e de baixo grau (estádio Ta, vide tabela1) estarão vivos em 5 anos. Já nos pacientes metastáticos, menos de 5% estarão vivos em 5 anos (SIEGEL *et al.*, 2019; MARTINI *et al.*, 2020). O prognóstico mesmo nos melhores estudos tem mostrado sobrevida global média não alcançando mais que 14 a 16 meses nesses pacientes (BALAR *et al*., 2017; POWLES *et al*., 2021).

Apesar dos avanços alcançados nos últimos anos com as novas drogas imunoterápicas e anticorpo conjugada (ADC), 50% dos pacientes tratados experimentarão recorrência.

Tabela 1. Câncer de Bexiga – Estadiamento TNM.

T		Tumor Primário
Tx		Tumor primário não pode ser identificado
T0		Sem evidência de tumor primário
Ta		Carcinoma papilar não invasivo
Tis ou TC*is*		Carcinoma *in situ*(C*is*)
T1		Tumor infiltrando a lâmina própria
T2		Tumor infiltrando a camada muscular
	pT2a	Tumor infiltrando a camada muscular superficial (metade interna)
	pT2b	Tumor infiltrando a camada muscular profunda (metade externa)
T3		Tumor infiltrando tecido perivesical
	pT3a	Microscopicamente
	pT3b	Macroscopicamente
T4		Tumor infiltrando órgãos perivesicais: estroma prostático, vesículas seminais, útero, vagina, parede abdominal e pélvica
	pT4a	Invasão da próstata, vesicular seminal, útero e vagina
	pT4b	Invasão da pelve e parede abdominal
N		Envolvimento de linfonodos
Nx		Linfonodos não podem ser avaliados
N0		Sem metástases linfonodais
N1		Linfonodo único metastático na pelve (região perivesical, obturador, artéria ilíaca interna e externa, linfonodo sacral)
N2		Múltiplos linfonodos metatáticos na pelve (região perivesical, obturador, artéria ilíaca interna e externa, linfonodo sacral)
N3		Linfonodo metastático na artéria ilíaca comum
M		Metástase a distância
M0		Sem metástase a distância
M1		Metástase a distância
	M1a	Metástase a distância limitada a linfonodo além da ilíaca comum
	M1b	Metástase a distância não linfonodal

Fonte: Classificação de tumores malignos - TNM (BRIERLEY *et al.*, 2017).

1.2 Imunoterapia – Aspectos Históricos

Na década de 70, Morales *et al.* (1976) demonstraram o efeito da ação do Bacilo de Calmette-Guérin (BCG) no tratamento de tumores uroteliais superficiais de bexiga (MORALES *et al.*, 1976; LAMM *et al.*, 1980). Apesar do mecanismo de ação não ser totalmente compreendido, sugere-se que a ação do BCG ocorra pela hiperestimulação inespecífica da resposta imune local, muito provavelmente estagnada pelo microambiente tumoral (REDELMAN *et al.*, 2014; SALUJA *et al.*, 2018). Em meados da década de 80, vários trabalhos utilizando a interleucina 2 (IL-2), citocina relacionada a proliferação de linfócitos, em pacientes com câncer

metastático, mostraram resultados significativos em ganho de sobrevida global (FYFE *et al.*, 1996; ATKINS *et al.*, 1999; ROSENBERG *et al.*, 2014). A partir do melhor conhecimento dos mecanismos imunológicos e da ação das citocinas, a utilização de imunobiológicos (anticorpos produzidos *in vitro* para uso em humanos e que interferem em diferentes vias de sinalização imunológica) se tornaram uma ferramenta terapêutica importante contra o câncer. Outros imunoterápicos também foram sendo descobertos, culminando nos anos 2000 com a compreensão dos mecanismos de pontos de checagem das vias da proteína de morte programada 1/ligante morte programada 1 (PD-1/PDL-1) e do antígeno 4 do linfócito T citotóxico (CTLA-4) relacionadas com a sobrevida de linfócitos T (ISHIDA *et al.*, 1992; KRUMEL and ALLISON, 1995).

A descoberta das vias de sinalização do PD-1/PD-L1 e do CTLA-4 abriram um novo segmento no tratamento do câncer. Anteriormente, a imunoterapia era representada apenas pelo BCG e algumas outras citocinas, como a IL-2, o Interferon Gama (IFN-γ) e inibidores de Fator de Necrose Tumoral Alfa (TNF-α) (FYFE *et al.*, 1996; ATKINS *et al.*, 1999). Na época, o tratamento padrão dos diversos tipos de câncer baseava-se na cirurgia, radioterapia ou quimioterapia. Em 2011, o órgão norte-americano, *Food Drug and Administration* (FDA), liberou a utilização do Ipilimumabe (droga anti-CTLA-4) em pacientes com melanoma irressecável ou metastático. Na época, a sobrevida global média desses pacientes não superava os 6 meses e, com o tratamento com o Ipilimumabe, a sobrevida passou para cerca de 11 meses (HOOS *et al.*, 2010; HODI *et al.*, 2010; ROBERT *et al.*, 2011). Posteriormente, com a descoberta de outro agente, o Nivolumabe (anti PD-1) e, com a associação do Ipilimumabe, elevou-se a sobrevida do Melanoma metastático para 52% de pacientes vivos em 5 anos (LARKIN *et al.*, 2019). Devido a melhor compreensão desses mecanismos e aos avanços obtidos, o Prêmio Nobel de Medicina de 2018 foi concedido aos cientistas James P. Allison e Tasuku Honjo (KRUMEL and ALLISON, 1995; ISHIDA *et al.*, 1992; SMITH *et al.*, 2018).

Devido ao sucesso desses resultados, outros tumores como o carcinoma de não pequenas células do pulmão, câncer de rim e câncer de colon/reto também obtiveram resultados em aumento da sobrevida global (CAMACHO *et al.*, 2009;

HODI *et al.*, 2010; MOTZER*et al.*, 2018; HELLMAN *et al.*, 2019; LARKIN *et al.*, 2019; ANDRE *et al.*, 2022).

Desde então, novos inibidores de ponto de checagem vêm sendo descobertos. No caso do CUB, em 2016 foi introduzido o Atezolizumabe (anti PDL-1) para os casos de carcinoma urotelial avançado ou metastático que progrediu após quimioterapia contendo platina ou, após a progressão da doença pós quimioterapia neoadjuvante ou adjuvante contendo platina (FDA, 2016).

1.3 Biomarcadores nos Tumores Uroteliais

No CUB, o tratamento em estadios precoces influi de maneira dramática na sobrevida do paciente. Enquanto pacientes com tumores superficiais de baixo grau (Ta) tratados têm sobrevida de mais de 98% em 5 anos, somente 36% e 5% dos pacientes, respectivamente, tratados em estádio T2 e metastáticos estarão vivos em 5 anos (LENIS *et al.*, 2020). Considerando esses fatos, inúmeros marcadores e meios diagnósticos vêm sendo empregados na tentativa de se diagnosticar precocemente e prognosticar melhor os resultados dos tratamentos a serem realizados. Várias metodologias são empregadas na identificação de biomarcadores, sejam por métodos de imagem, identificação direta do tumor via cistoscopia, análise do tecido tumoral (seja pelos aspectos histopatológicos, expressão de antígenos, metabólitos, receptores de membrana), testes genéticos (como análise de mutações somáticas ou germinativas), biópsias líquidas, entre outras.

1.3.1 Exames de Imagem

O advento da urografia excretora, da ultrassonografia e posteriormente, da tomografia com achados incidentais, trouxe um benefício inquestionável no diagnóstico precoce do CUB. Outros métodos associados à cistoscopia, como a utilização de fontes de luz de comprimentos de onda modulada ou a utilização de materiais fluorescentes (ácido hexaminolevulínico e hexaminolevulinato) injetados

intravesicalmente, podem ajudar no diagnóstico de lesões intravesicais não visíveis (KRIEGMAIR *et al.*, 1996; JICHLINSKI *et al.*, 1997; KOENIG *et al.*, 1999; BRYAN *et al*., 2008; BOCHENEK *et al*., 2019).

1.3.2 Análises de Urina

Exame de urina, como a citologia oncótica tem sido utilizado para o acompanhamento da recidiva após a ressecção da lesão. Apesar da alta especificidade da citologia oncótica para tumores de alto grau, ela apresenta baixa sensibilidade nos casos de tumores de baixo grau (GREGOIRE *et al.*,1997; LOTAN *et al.*, 2003; KARAKIEWICZ *et al.*, 2006). Outras análises de antígenos na urina têm sido pesquisadas, como a pesquisa de proteínas do aparelho mitótico nuclear número 22(NMP22®); o Antígeno Tumoral da Bexiga STAT (BTA Stat®) e pesquisas de aneuploidias cromossomais. Entretanto esses testes são utilizados em escala menor pela dificuldade de disponibilidade e pela falta de padronização dos resultados obtidos (SOLOWAY *et al.*, 1996; CHENG *et al.,* 2005; GREENE *et al.*, 2006; HAJDINJAK *et al.*, 2008; MOWATT *et al.*, 2010).

1.3.3 Análise do Tecido Tumoral

A carcinogênese envolve processos de seleção de clones tumorais que escaparam da eliminação imune. Um dos mecanismos propostos ao escape tumoral seria a produção de citocinas, que levam a um microambiente tumoral imunossuprimido, ou seja, apesar da atividade imunológica, esse ambiente criaria condições para que a célula tumoral perpetue. Foi observado que, tumores que apresentavam à histologia estruturas linfoides terciárias (TLS-*tertiary lymphoid structures,* definidas como zonas de tecido ricas em células T, B e dendríticas) têm respostas mais pronunciadas aos imunoterápicos e poderiam ser um preditor terapêutico. Além disso, sua presença poderia ser um marcador histológico de bom

prognóstico a resposta ao tratamento (CHOUEIRI *et al*., 2022; MASUDA *et al.*, 2022).

Outro marcador, o fator transcritor de linfócito B (POU2F2) presente no tecido tumoral tem sido relacionado a pior resposta terapêutica, portanto seria um marcador de mau prognóstico e de má resposta ao tratamento imune (POWLES *et al.*, 2021).

Outros marcadores, como a Transcriptase Reversa da Telomerase (TERT), Proteína tumoral P53 (TP53), Inibidor 2A da Quinase Ciclina Dependente (CDKN2A) e o Inibidor 2 da Quinase Ciclina Dependente (CDKN2), parecem ser úteis para a escolha de drogas anticorpo conjugado, como o Efortumabe Vedotin. Apesar de levarem a taxas de resposta promissoras, ainda necessitam de mais estudos (JINDAL *et al.*, 2022; KLAASSEN, 2022).

1.3.4 Análise de Aspectos Moleculares no Tecido Tumoral

Estudos da expressão gênica no tecido tumoral urotelial têm permitido uma classificação molecular que poderia determinar seu comportamento carcinogênico (McCONKEY*et al.*, 2015). A análise de subtipos moleculares como um possível biomarcador tumoral no CUB para a escolha do tratamento tem mostrado resultados promissores (CHOI *et al.*, 2014; ROBERTSON *et al.*, 2023). O CUB partilha expressão de marcadores de padrões luminal, mesenquimal e outras mais indiferenciadas (LAUSS *et al.*, 2000; TCGA Network, 2014). Várias entidades têm proposto diferentes taxonomias para a classificação molecular dos tumores uroteliais, mas, de uma maneira geral, 2 subtipos moleculares podem ser consideradas principais, o basal e o luminal (CHOI *et al.*, 2014; LERNER *et al.*, 2016). Como exemplo de biomarcador molecular terapêutico, o subtipo luminal infiltrado parece responder melhor à imunoterapia e o subtipo basal seria mais responsivo a quimioterapia a base de Platina (NECCHI *et al.*, 2021). Entretanto, devido a diversidade da expressão molecular no CUB, há a sobreposição dessa classificação molecular com resultados terapêuticos variados (ROBERTSON *et al.*, 2023).

1.3.5 Análises Genéticas

Em relação a marcadores prognósticos, análises de mutações genômicas, como a do receptor do fator de crescimento fibroblástico 3 (FGFR3) ou fusões de FGFR2/3 (presentes em 28% dos pacientes com CUB), têm sido importantes na condução da escolha do tratamento com inibidores do FGFR. A utilização do Erdafintinibe (um inibidor de FGFR) em pacientes com CUB metastático e refratário a outras linhas de tratamento mostrou ganho de sobrevida global média de 13,8 meses e tem sido uma opção nos pacientes com essa mutação em que a quimioterapia a base de platina não obteve sucesso (LORIOT *et al.*, 2019).

A mutação do receptor do fator de crescimento epidérmico humano-2 (HER2) é utilizada como fator prognóstico e de decisão no tratamento do câncer de mama e gástrico (WOLFF *et al.*, 2006; BANG *et al.*, 2010). Entretanto no CUB, estudos têm mostrado divergência na expressão dessa proteína com o resultado no tratamento não correspondendo ao mesmo sucesso alcançado nos cânceres anteriormente citados (ALBARRAN *et al.*, 2022; SANGUEDOLCE *et al.*, 2023). Novas drogas anti-HER2 (RC48, disitamabe vedotin) associado ao toripalimabe, tem mostrado resultados promissores no CUB em estudos de fase 2 (SHENG *et al.*, 2022).

A quantidade de mutações somáticas de uma determinada sequência genômica de um tumor (TMB) comparada ao tecido normal, varia em cada tipo de tumor. Tumores que apresentam TMB alto, como o melanoma e o carcinoma de não pequenas células do pulmão, respondem bem aos inibidores de PD-1/PD-L1 e do CTLA-4, e têm sido utilizados como biomarcadores tumorais (SHA *et al*., 2020).

A análise da expressão do PD-L1 no tecido tumoral no CUB também tem sido utilizada na escolha para o uso de imunoterápicos, como é o caso do uso do avelumabe. Powles *et al.*, (2021) mostraram ganho de sobrevida global em pacientes que apresentavam expressão de PD-L1 presente ou TMB elevado em pacientes que fizeram uso de avelumabe (anti-PD-L1) pós quimioterapia. Entretanto, sabe-se que, apesar da negatividade da expressão do PD-L1 na célula tumoral, muitos desses pacientes com CUB respondem ao tratamento imunoterápico com inibidores da PD-1/PD-L1, levando a considerar que outros fatores além do TMB e

expressão do PD-L1 estariam envolvidos nesses casos (MOTZER *et al.*, 2020; POWLES *et al.*, 2021; KLAASSEN, 2022).

A biópsia liquida, método que utiliza a análise da presença de DNA tumoral circulante (ctDNA) na corrente sanguínea, vêm sendo cada vez mais pesquisada na prática clínica. CHRISTENSEN *et al.*, (2019) verificaram que a presença de ctDNA em pacientes pós cistectomia radical apresenta taxa de sobrevida global menor que quando ausente. O mesmo valeria para a presença do ctDNA pós quimioterapia, mostrando a utilidade para a decisão terapêutica e prognóstica desse marcador. Além disso, é um marcador de baixa invasividade e de fácil reprodutibilidade.

Outros biomarcadores tumorais vêm sendo pesquisados no CUB como em alterações epigenéticas, variantes genéticas em microssatélites, microRNAs, vesículas extracelulares (ex.: corpos apoptóticos, microvesículas e exossomas) além de estudos em metabolômica e proteômica (FRIGERIO *et al.*, 2007; ISSAQ *et al.*, 2008; ESTELLER *et al.*, 2009; SNOWDON *et al.*, 2013; OEYEN *et al.*, 2019).

Sabendo-se que a atividade imunológica exerce um papel preponderante no controle tumoral, expressão de genes de citocinas, como encontrado em variantes genéticas de nucleotídeo único (SNV) também tem sido estudas. Inúmeros trabalhos demonstram a associação das expressões de determinadas citocinas com o risco de CUB, bem como o prognóstico e taxa de resposta ao tratamento com BCG (JAISWAL *et al.*, 2013; LIMA *et al.*, 2015).

Apesar de todo o conhecimento até aqui adquirido, os biomarcadores disponíveis para o CUB, mostram-se ainda com resultados nem sempre previsíveis. Portanto, existe a necessidade da busca de biomarcadores com melhor assertividade para o diagnóstico e que permita uma melhor escolha a quimioterápicos ou imunoterápicos.

1.4 Carcinogênese e Imunologia

A homeostase tecidual consiste num equilíbrio do conjunto celular em exercer a sua função tecidual específica, corrigir disfunções decorrentes de injúrias metabólicas e mutacionais, e, manter o equilíbrio proliferativo das células tronco

desse tecido. Eventos que levam a disfunções da vigilância imunológica e a mutações podem permitir que clones celulares anormais ao tecido venham a proliferar levando ao processo carcinogênico. Inúmeros mecanismos celulares participam dessa maquinaria que regula a inibição e ação dos protoncogenes e genes supressores tumorais. Mutações nesses genes que levam a disfunções aumentam a probabilidade carcinogênica. Hanaham & Weinberg, (2000a, 2011b e 2022c) sistematizaram características inerentes e necessárias da célula tumoral para a progressão carcinogênica (figura 1). Há uma complexa interação entre células tumorais, células do sistema imune e o microambiente tumoral onde a pressão de seleção irá favorecer ou eliminar a célula cancerosa.

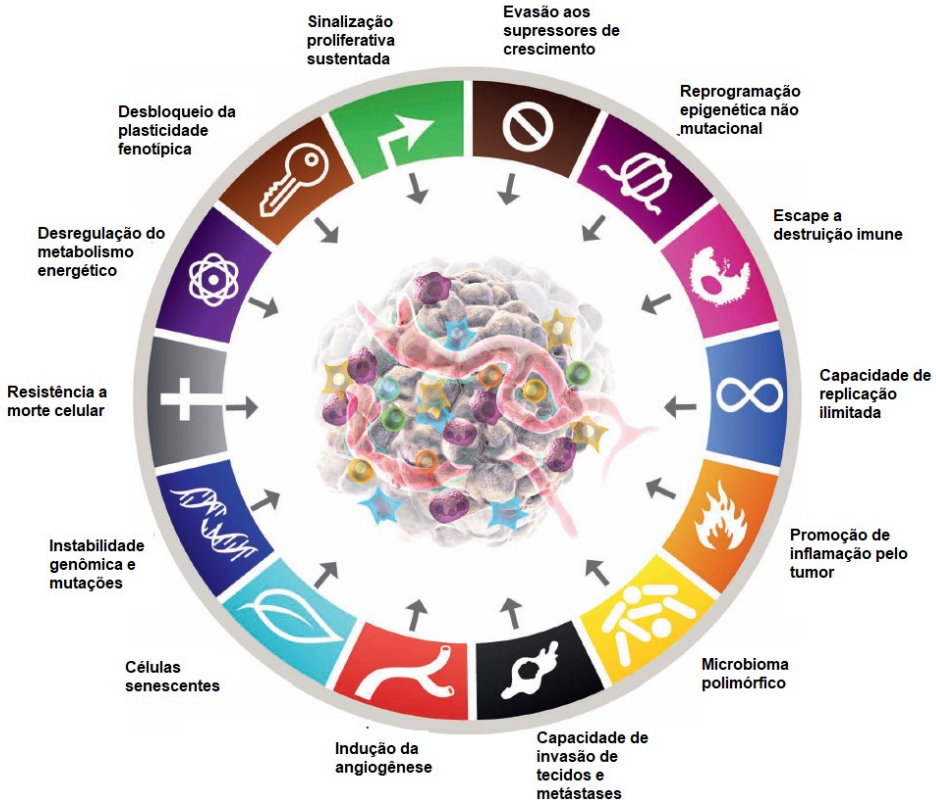


Figura1 - **"*The Hallmarks of Cancer*"**, adaptado de Hanahan & Weinberg (2022). Características inerentes e adquiridas para o sucesso proliferativo do tecido tumoral.

Schreiber *et al.,*(2011) sistematizaram 3 fases nas quais os tumores progridem e se consolidem (figuras 2, 3 e 4):

1- **Eliminação:** Uma vez que a célula neoplásica é formada, mecanismos da imunidade inata buscam a eliminação da célula tumoral pelo reconhecimento de antígenos tumorais expressos. Esses antígenos podem ser provenientes:

a) da expressão de genes mutados; b) da hiperexpressão de antígenos próprios; c) de antígenos normais que deixaram de ser expressos (como antígenos fetais) e; d) de antígenos derivados de oncovírus. As principais citocinas envolvidas nesta fase são as interleucinas 1, 6, 12 (IL-1, IL-6 e IL-12), interferon gama (IFN-γ) e fator de necrose tumoral alfa (TNF-α). As principais células da imunidade inata, atuantes nesta fase, são os macrófagos, células dendríticas e as células *natural killer* (NK). Paralelamente, essa atividade também leva a resposta imune adaptativa com células TCD8+ e resposta celular tipocélulas T *helper* 1 (Th1).

2- **Equilíbrio**: Caso o sistema imune não tenha sucesso em eliminar as células tumorais, uma fase de equilíbrio se instala. Esse período é variável, podendo durar anos e se caracteriza por uma fase em que clones tumorais estão em constante multiplicação e mutação sendo contrabalanceados pela eliminação insuficiente do sistema imune. Nesta fase, um microambiente pró-tumoral é formado. Nesta fase, células do sistema imune e células tumorais produzem citocinas, fatores de crescimento mesenquimais e angiogênicos. Caso o sistema imune não consiga superar as células tumorais, o microambiente formado torna-se favorável a célula tumoral. A presença de macrófagos polarizados para a resposta M2, células mielóides supressoras derivadas (MDSCs), presença de células T reguladoras (Treg), citocinas como fator transformador do crescimento tipo beta (TGF-β), e interleucinas 10 e 4 (IL-10, IL-4) favorecem a manutenção de um microambiente estável, com neovascularização e promoção da transição epitélio mesenquimal.

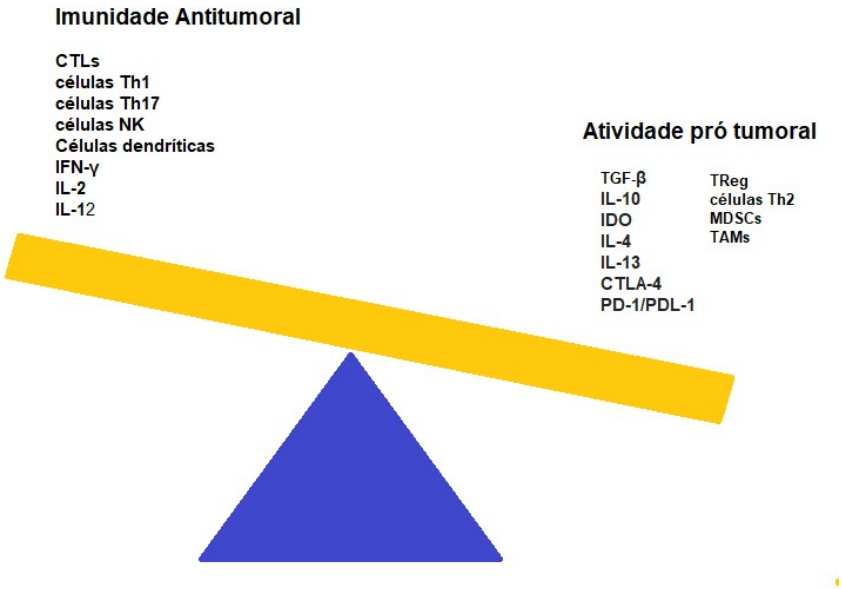


Figura 2 – **Equilíbrio do sistema imune na carcinogênese**. O desequilíbrio da ação de células do sistema imune, citocinas e expressão de receptores influenciam na atividade imunidade anti-tumoral. CTLs: Linfócito T-citotóxico; Th1: células T helper 1; Th17: T helper 17; NK: Natural Killer; IFN-γ: Interferon gama; IL-2: Interleucina 2; IL-12: Interleucina 12; TGF-β: Fator de transformação de crescimento beta; IL-10: Interleucina 10; IDO: Indoleamina 2,3-Dioxigenase; IL-4: Interleucina 4; IL-13: Interleucina 13; CTLA-4: Antígeno 4 do linfócito T-citotóxico; PD-1: Proteína de ponto de verificação morte programada-1; PD-L1: Proteína ligante morte programada-1; TReg: célula T regulatória; Th2: células T helper 2; MDSCs: células mieloderivas supressoras; TAMs: Macrófagos associados ao tumor. Fonte: adaptado de PITT *et al*., 2016.

3- **Escape:** Caso o sistema imune não consiga eliminar o tecido neoplásico ou que clones tumorais imunorresistentes prevaleçam, uma fase de escape se instala. Nesta fase, os clones tumorais imunoadaptados escapam da vigilância imune, a proliferação celular tumoral impera e eventos mestastáticos podem ocorrer. Participação de mecanismos de escape como a expressão de PD-L1, CTLA-4; amplificação da ação das células Treg, macrófagos M2 e citocinas (como a IL10, IL-6, TGF-β) favorecem a incapacidade do sistema imune em eliminar os clones tumorais.

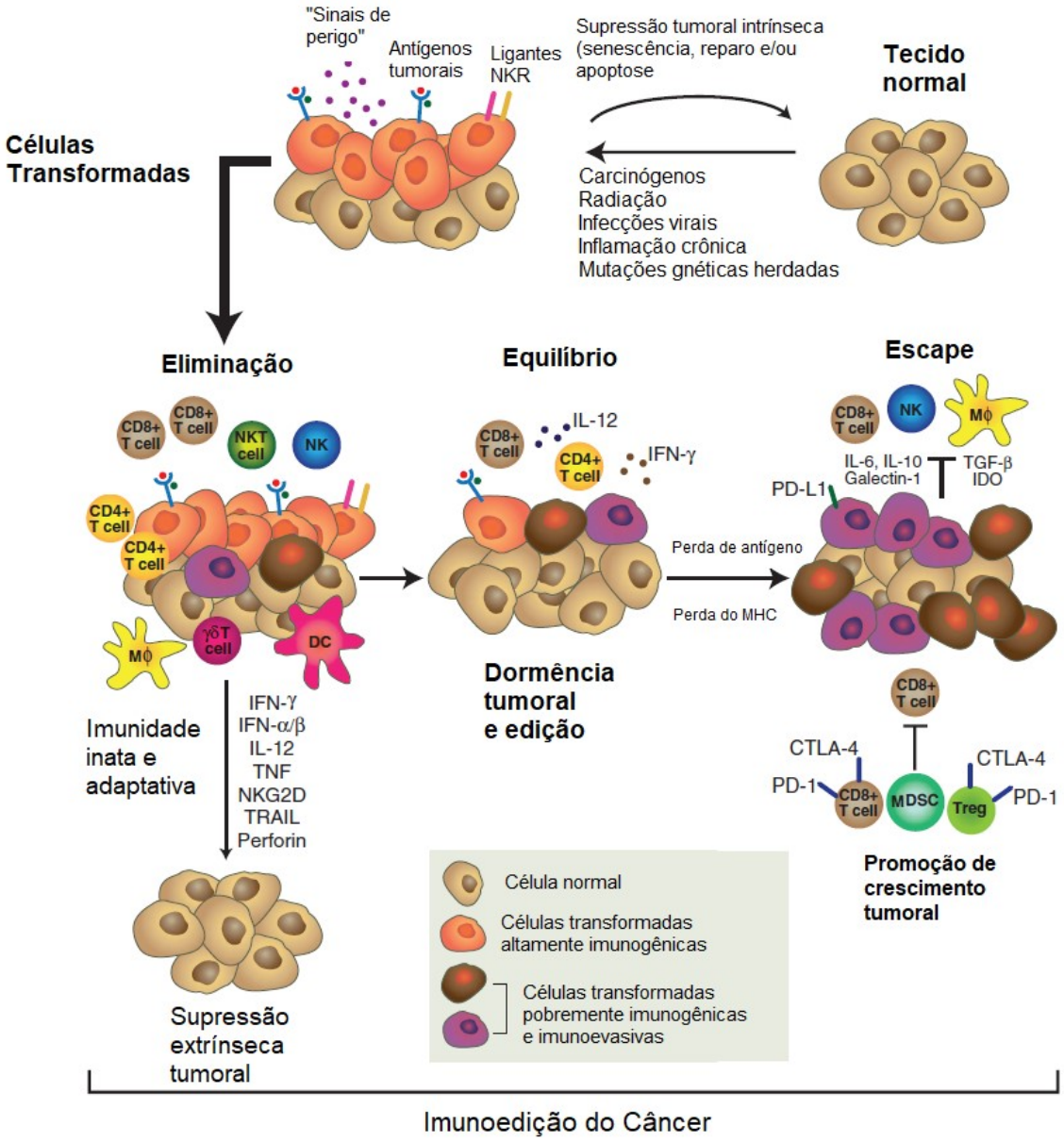


Figura 3 – **Imunoedição do câncer**. Ilustração esquemática das 3 fases da carcinogênese sugerida por Schereiber*et al.*, 2011. A displasia inicialmente ocorre pela promoção de fatores intrínsecos e extrínsecos à célula normal. Uma vez ocorrida a mutação, uma fase de eliminação inicia onde células da imunidade inata e adaptativa e citocinas inflamatórias agem na tentativa de erradicar as células tumorais. Caso as células tumorais persistam, uma segunda fase de equilíbrio se instala, onde existe uma dinâmica entre as células tumorais contra o sistema imune e que se mantém em equilíbrio. Através da pressão de seleção, clones tumorais sobreviventes se sobressaem e uma terceira fase de escape se instala e a progressão carcinogênica se torna descontroladapodendo levar a metástases. As durações destas fases são variáveis e tampouco sucessivas. TCD8+cell: células T CD8; TCD4+ cell: células T CD4; NKT cell: célula Natural Killer alvo; NK cell: células Natural Killer; IL-12: Interleucina 12; MØ: macrófago; IFN-γ: Interferon gama; IFN α/β: Interferon alfa/beta; NKG2D: Membro D do grupo 2 do Natural Killer; TRAIL: TNF-Ligante relacionado a indução

de apoptose do TNF; MHC: Complexo Maior de Histocompatibilidade; PD-1: Proteína de ponto de verificação morte programada-1; PD-L1: Proteína ligante morte programada-1; CTLA-4: Antígeno 4 do linfócito T-citotóxico; MDSCs: células mieloderivadas supressoras; IDO: Indoleamina 2,3-Dioxigenase. Fonte: adaptado de SCHEREIBER *et al*., 2011.

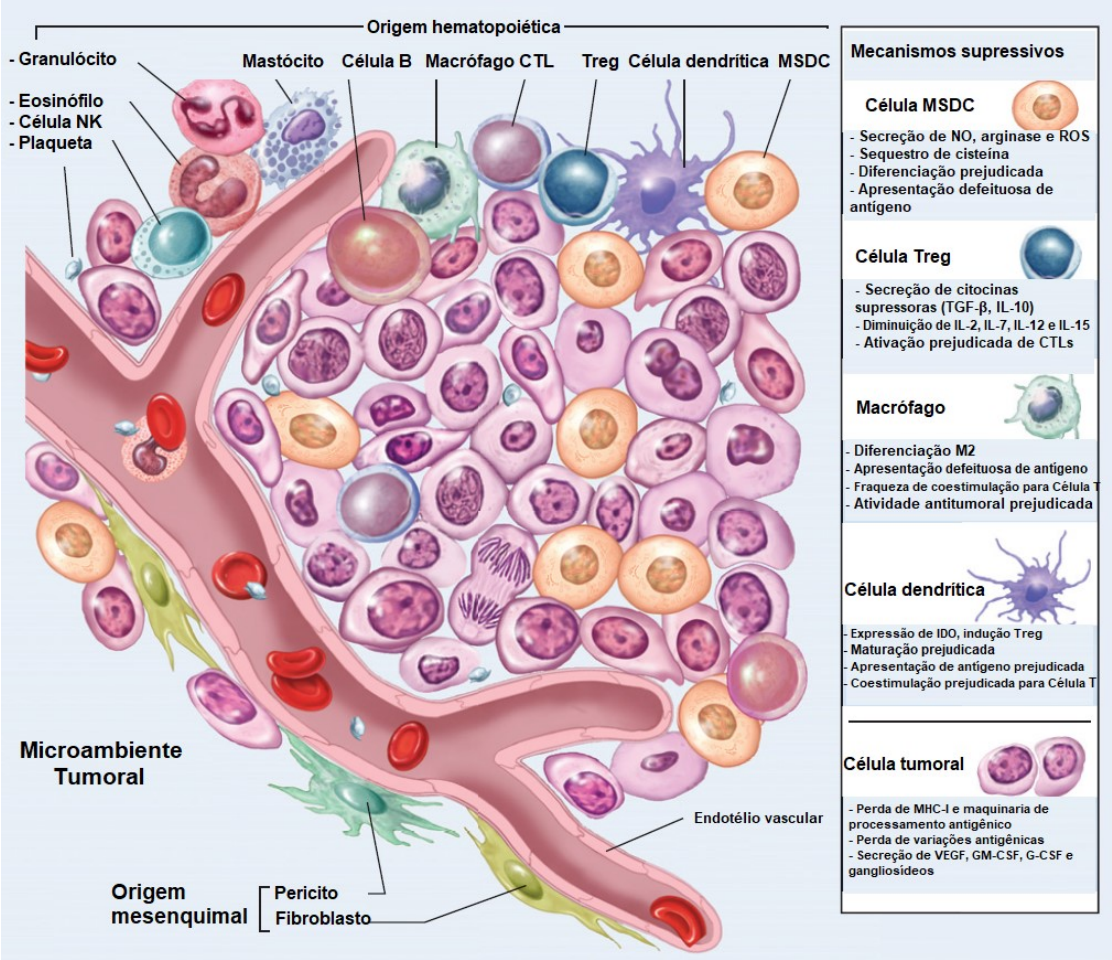


Figura 4 – **Microambiente tumoral**. A não eliminação das células neoplásicas associadas a liberação de citocinas antiinflamatórias e fatores de crescimento vascular e mesenquimal, polarização fenotípica do marcófago M2, atração de células MDSC, inativação de células NK e dendríticas, levam a criação de um microambiente imunossuprimido propício a progressão e de proteção tumoral. NK cell: células natural killer; CTL: células T citotóxicas; MDSC: células mieloderivadas supressoras; NO: óxido nítrico; ROS: espécies reativas de oxigênio; Treg cell: célula T regulatória; IDO: Indoleamina 2,3-Dioxigenase; TGF-β: Fator de transformação de crescimento beta; IL: interleucina; VEGF: Fator de crescimento vascular endotelial; GM-CSF: Fator estimulatório de colônia granulocitário-macrofágico; G-CSF: Fator estimulatório de colônia granulocitário; glangiosides: glangliosideo. Fonte: KERKAR *et al*., 2012.

1.5 CTLA-4

O CTLA-4 ou CD152, consiste numa glicoproteína de membrana expressa nas células T ativadas e que interage com 2 proteínas ligantes da familia B7 (B7-1 ou CD-80 e B7-2 ou CD-86). O gene *CTLA4* está localizado no cromossomo 2q33 e é composto de 4 exons. Os exons 1 e 2 codificam o domínio extracelular, o exon 3 codifica o domínio transmembrana e o exon 4 codifica o domínio cistoplasmático. Por um mecanismo de *Splicing*, o exon 3 é excluído e traduzida na forma plasmática, o CTLA-4 solúvel (sCTLA-4) (MAGISTRELLI *et al.*, 1999).

A forma flCTLA-4 (*full length* CTLA-4) é a forma ativa como receptor de membrana (WARD *et al.*, 2013, GU *et al.*, 2018). Em células T não ativadas, o flCTLA-4 está internalizado e não está expresso na membrana celular. A translocação para a membrana ocorre durante o processo de apresentação do antígeno à célula T a partir da resposta ao 2º sinal do CD28 com seu ligante B7. Logo, a expressão do CTLA-4 na sua forma transmembrana é o início de uma resposta de controle contra a hiperativação imune (Figura 5) (LINSLEY *et al.*, 1992; FREEMAN *et al.*, 1992).

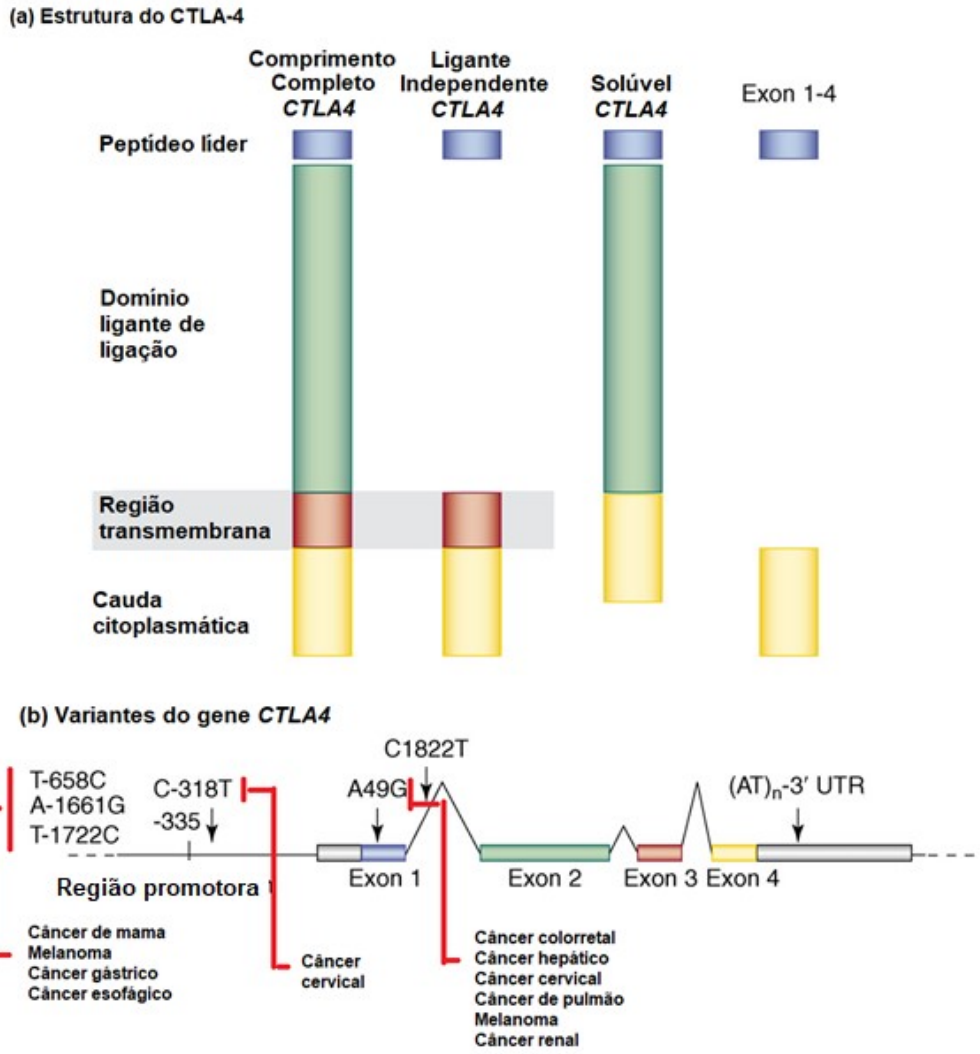


Figura 5 - **Estrutura do gene *CTLA4*, variantes do gene *CTLA4* e tumores associados.** a) o gene *CTLA4* é composto de 4 exons e 3 introns. O CTLA-4 presente na membrana das células T está na sua forma completa enquanto o CTLA-4 presente no plasma perde, no processo de *splicing*, o 3º exon que é responsável pela sua fixação na região transmembrana; b) as variantes rs231775 (+49AG) e rs231779 (+1822CT) se situam próximas a região promotora e do sítio de ligação, o que poderia justificar as características de maior produção e maior avidez de ligação característica dessas variantes. UTR: região não traduzida. Fonte: adaptado de VALK *et al*., 2008; Zhao *et al*., 2018.

O CTLA-4 e o PD-1 são considerados os principais mediadores envolvidos no controle negativo para ativação de células TCD4+. Num processo normal de ativação da célula TCD4^{+}, a APC apresenta um antígeno via MHC de classe 2 à célula T *naive* (1º sinal). Nesse momento, ocorre também a ligação do B7(CD80 ou CD86) ao CD28 (2º sinal). Esses dois sinais levam a expressão do CD40L que se liga ao CD40 da APC. Assim, esse sinal leva a produção de citocinas pela APC

referente ao antígeno apresentado, originando as repostas dessa célula $CD4^+$ ativada (Th1, Th2, Th17, e Treg) (ABBAS, 2019). Essa célula $TCD4^+$ ativada migra para o local onde o processo imunológico está instalado e atua de acordo com o tipo de resposta definido (no caso da resposta anti-carcinogênica, a resposta Th1 é a mais efetiva).

Sabe-se que, no microambiente tumoral, existe um estado imunossuprimido instalado patologicamente. Nesse microambiente, as proteínas receptoras B7 estão subexpressas nas APCs. Com a maior avidez do CTLA-4 pela B7 do que o CD28 pela B7, no momento em que a APC (com as proteínas B7 subexpressas) apresentam o antígeno tumoral à célula TCD4+ *naive*, ocorre uma maior ligação do B7 ao CTLA-4 em detrimento do CD28. Isso leva a falha na ativação da célula $TCD4^+$ e ocorre o processo de anergia celular (KRUMMEL *et al.*, 1995, PERKINS *et al.*, 1996). Assim, a resposta antitumoral torna-se prejudicada. Além disso, ocorre maior ativação das células Treg que expressam o CTLA-4 constitutivamente (SCHWARTZ *et al.*, 2001). Essa ativação das células Treg é amplificada pelo seu maior consumo de IL-2. Como as células Treg não produzem IL-2 e expressam constitutivamente o receptor da cadeia alfa da IL-2 (forma ativa do receptor), a pouca IL-2 disponível é consumida pelas células Treg em detrimento de outras células T no microambiente tumoral (YAGI *et al.*, 2004; deLA ROSA *et al.*, 2004).

O CTLA-4 solúvel (sCTLA-4), é produzido principalmente pelas células Treg, monócitos e células dendríticas imaturas, sem a necessidade de ativação (WARD *et al.*, 2013). A ação do sCTLA-4, em estados não patológicos, é o de manter o equilíbrio da resposta imune através da ação sistêmica (MAGISTRELLI *et al.,* 1999). O sCTLA-4 se liga a proteína B7 da APC prejudicando a ligação com o CD28 e consequentemente prejudicando a ativação da célula $TCD4^+$*naive*. Portanto, num ambiente sem estímulo da ativação linfocitária, a produção basal de sCTLA-4, mantém uma condição de imunossupressão plasmática dos linfócitos circulantes.

No microambiente tumoral, tem sido encontrado níveis de sCTLA-4 aumentados e estão relacionados a maior inativação da resposta imune contra o tumor (WARD *et al.*, 2013). Erfani *et al*., (2010) encontraram níveis séricos de sCTLA-4 elevados em pacientes com câncer de mama, sugerindo que o sCTLA-4 poderia agir como supressor da ativação da célula T *naive*.

Interessantemente, níveis séricos elevados de sCTLA-4 têm sido relacionado a melhor resposta do ipilimumab (anti-CTLA-4) em pacientes com Melanoma (LEUNG *et al.*, 2014). Variantes do *CTLA4*, como o rs3087243 (CT60G>A) tem sido relacionado a maior expressão do sCTLA-4 e poderia ser um biomarcador de escolha para o tratamento imunoterápico (HAMMRICH *et al*., 2018).

Por um mecanismo ainda não muito bem explicado, o sCTLA-4 também pode levar ao estímulo de ativação das células $TCD4^{+}$ (OAKS *et al*., 2000; PERES-GARCIA *et al.*, 2007). Portadores do alelo A, da variante rs3087243 (CT60G>A), têm níveis séricos maiores de sCTLA-4 que os de alelo G. Perez-Garcia *et al.,*(2007) avaliaram um grupo de doadores de medula óssea que eram portadores ou não da variante rs3087243 (CT60G>A). Constataram que, receptores de transplante de medula (que estavam em tratamento por tumores hematopoiéticos) e que receberam de doadores com o genótipo GG (ou seja, menores níveis de sCTLA-4) tinham prognóstico pior daqueles receptores que receberam de doadores com genótipo AA (níveis maiores de sCTLA-4). Isso poderia corroborar com a hipótese de maior ativação de células T pelo sCTLA-4.

Os imunoterápicos anti-CTLA-4 disponíveis comercialmente no momento são o ipilimumabe e o tremelimumabe. Sua utilização em associação com outros inibidores de PD-1/PD-L1 nos cânceres de pulmão, rim, colon/retal e melanoma tem mostrado resultados significativos na melhora da sobrevida global (MOTZER *et al.*, 2018, HELLMAN *et al*., 2019; LARKIN *et al.*, 2019; HODI *et al.*, 2010; CAMACHO *et al.*, 2009; ANDRE *et al*., 2022). Entretanto, no CUB a imunoterapia com os anti-CTLA-4 não tem tido o mesmo sucesso do que tem se observado nos outros tumores (GALSKI *et al.*, 2018). Apesar de apresentarem alguns resultados com boa tolerabilidade não são superiores a reposta a quimioterapia tradicional (SHARMA *et al.*, 2020).

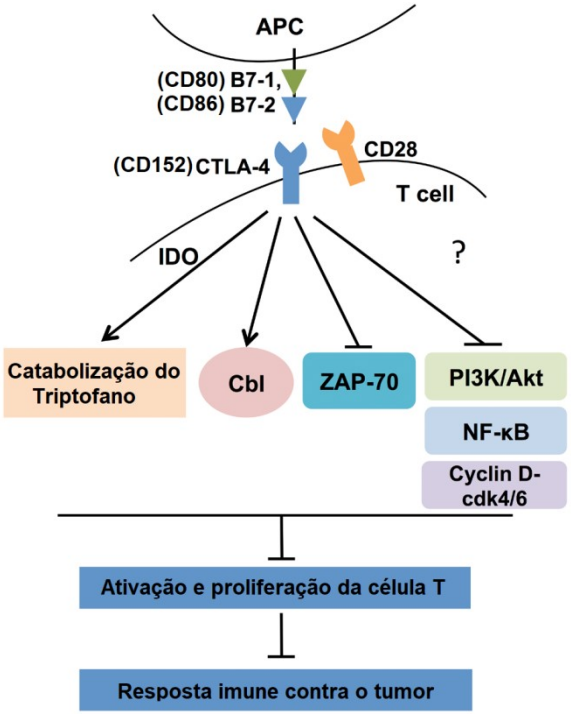


Figura 6 – **Mecanismo de ação do CTLA-4 no câncer.** No órgão linfonodal, a apresentação do antígeno tumoral junto com o 2º sinal (representado pela ligação B7-CD28 pela APC) leva a ativação das células TCD4+. Concomitantemente, o CTLA-4 que está internalizado é expresso na membrana celular assim que o linfócito é ativado. APCs provenientes do microambiente tumoral podem expressar inadequadamente a proteína B7 e sua escassez leva a inibição do sinal de ativação pela ligação com o CTLA-4 (que possui mais avidez ao B7 do que o CD28). Essa ligação CTLA-4/B7 é internalizada na célula TCD4+, a proteína B7 é degradada nos lisossomos (diminuindo ainda mais a disponibilidade do B7) e o CTLA-4 é reciclado e re-expresso novamente na membrana celular. O sinal desencadeado pelo CTLA-4 induz: a via da Indoleamina-2,3-deoxigense (IDO) que cataboliza o triptofano; promove a expressão da proteína Linfoma de Linhagem B de Casitas (Cbl); suprime a formação da proteína Zeta associada de 70KDa (ZAP-70) e induz a inibição das vias do PIK3/Akt,ciclina D-cdk4/6 e NF-kB. Como resultado final, há a desativação e a inibição da proliferação da célula TCD4+, prejudicando a resposta imune antitumoral. As drogas anti-CTLA-4 atuam bloqueando a ação do CTLA-4 e, consequentemente, leva a ativação das células TCD4+. Fonte: adaptado de Valk *et al.,* 2008; Zhao *et al.,* 2018; Abbas, 2019. APC: Célula Apresentadora de Antígeno.

Inúmeras variantes genéticas do *CTLA4* têm sido identificadas, sendo muitas relacionadas a doenças autoimunes e ao câncer (KAILASHIYA *et al.,* 2022; SUN *et al., 2009*). Das variantes genéticas do *CTLA4,* a mais estudada é o rs231775(+49A/G). Nessa variante missense ocorre uma troca da Adenina (A) pela Guanina (G) o que leva a troca de tradução do aminoácido Treonina (T) pela Alanina

(A). Essa variante do *CTLA4* formado tem maior afinidade pela ligação ao B7.1 que o CTLA-4 selvagem. Consequentemente espera-se que ocorra maior inibição da ação das células $TCD4^+$ e $CD8^+$ e aumento da atividade das células Treg. Isso é confirmado em estudos onde a variante rs231775 está relacionado ao risco do CUB e cânceres de rins, mama, fígado e pulmão (GUO *et al*., 2015; ISITMANGIL *et al*., 2016; WEI *et al.,* 2021; FANG *et al*., 2018; WAN *et al.*,2022, WANG *et al.*,2013; MAO *et al*., 2020; JAISWAL *et al*., 2014).

Outra variante que investigamos neste trabalho, a rs231779, é pouco estudada em relação aos cânceres e tem sido relacionada ao câncer colorretal (Figura 5) (GE *et al.,* 2015; SUN, *et al.*, 2008).

As variantes avaliadas neste trabalho (rs231775 e rs231779) tendem a levar a maior expressão do *CTLA4*, podendo estar relacionadas a carcinogênese.

2. **JUSTIFICATIVA**

A sobrevida decorrente do CUB metastático pouco tem se alterado nos últimos 20 anos apesar de todos os avanços em novos quimioterápicos, imunoterápicos e drogas anticorpo conjugadas (BELLMUNT *et al*., 2017). Como exemplo, países do norte da Europa onde existe um amplo acesso da população ao tratamento médico, a sobrevida global em 5 anos de pacientes diagnosticados com CUB era de 73% no período de 1991-1995 e de 78% no período de 2016-2020 (NORDCAN, 2023).

Dados epidemiológicos do Reino Unido mostram resultados semelhantes, onde pacientes com CUB em estadio T1 tratados, 80% permanecem vivos após 5 anos, enquanto pacientes M1 somente 10% permanecem vivos (CANCER RESEARCH UK, 2020). Nos últimos estudos em pacientes em estadio M1 e tratados, os melhores resultados mostram sobrevida média de 18 meses (BALAR *et al.*, 2017; POWLES *et al.*, 2021). Ou seja, a importância de se diagnosticar precocemente qual paciente irá progredir para estadios superiores traz uma diferença altamente significativa na sobrevida. Para isso, biomarcadores que possam predizer quais pacientes evoluirão ou quais terão melhor resposta ao

tratamento são essenciais no tratamento do CUB. Até o momento, não encontramos trabalhos relacionando as variantes genéticas estudadas por nós no CUB na população brasileira. Além disso, trabalhos relacionando o rs231775 são contraditórios em relação ao CUB necessitando de outros estudos para melhor definir a ação desse polimorfismo (WANG *et al,* 2014; MAO *et al,* 2014).

3. OBJETIVOS

3.1 Objetivo Geral

- Avaliar a associação entre as variantes genéticas rs231775 e rs231779 do *CTLA4* estão associadas com a suscetibilidade e o prognóstico do Carcinoma Urotelial de Bexiga (CUB).

3.2 Objetivos Específicos

- Realizar uma revisão sistemática de diferentes variantes de citocinas envolvidas no CUB;

- Determinar a frequência da variante do *CTLA4,* rs231775 e rs231779, em pacientes com CUB e indivíduos controle;

- Verificar a associação entre as variantes genéticas, a suscetibilidade e o prognóstico dos pacientes com CUB.

4. SUJEITOS E MÉTODOS

Este trabalho consistiu em dois artigos, sendo um de revisão de literatura e outro artigo de estudo caso-controle.

4.1 Revisão de Literatura

A revisão de literatura seguiu as regras do *Guideline* de relatos para revisões sistemáticas e meta análises – PRISMA. A revisão buscou artigos de estudos clínicos, meta-análises, estudos observacionais e de Coorte. O principal objetivo foi revisar a influência das citocinas no câncer e buscar na literatura, variantes de nucleotídeo único de citocinas relacionadas ao CUB. Os dados foram buscados em sites de dados da PUBMED, Lilacs, *Scientific Electronic Library Online* (SCIELO) e *Science Direct* em inglês, espanhol ou português. O período definido de busca na literatura foi de 01/01/1990 a 31/12/2022.

4.2 Delineamento do Estudo Caso-Controle

Este é um estudo caso controle onde foram selecionados 140 pacientes com diagnóstico de CUB confirmado por biópsia, no período de janeiro de 2020 a abril de 2022 e recrutados pelo ambulatório de urologia do Hospital do Câncer de Londrina.

Foram incluídos 145 indivíduos controles, selecionados entre indivíduos doadores de sangue saudáveis do Hemocentro Regional de Londrina. Os dados clínicos foram obtidos por questionário aplicado (APÊNDICE 11.1). Os critérios (clínicos e laboratoriais) de inclusão e exclusão dos indivíduos do grupo controle seguiram o protocolo definido para aptidão de doação de sangue e definidos pela Portaria nº 158 de 04/02/2016 do Ministério da Saúde do Brasil (Ministério da Saúde, 2016).

Todos os pacientes do grupo com CUB deste estudo foram selecionados respeitando-se os seguintes critérios:

a) Critérios de inclusão:

- idade entre 18 e 99 anos;
- ambos os sexos;
- os pacientes com diagnóstico de CUB deveriam ter confirmação do câncer pela biópsia.

b) Critérios de exclusão:

- uso de medicamentos anti-inflamatórios;
- uso de suplementos antioxidantes;
- presença de doenças inflamatórias agudas e/ou crônicas não relacionadas ao CUB;
- presença de doenças infecciosas agudas e/ou crônicas;
- história prévia de outros cânceres e;
- doença renal crônica.

Os pacientes com CUB após terem sido operados e, de acordo com o resultado da peça do anátomo-patológico, foram classificados como tumor de baixo ou alto grau e estadiados conforme o sistema de estadiamento TNM da União Internacional de Controle do Câncer (CHANG *et al., 2016;* BRIERLEY *et al.,* 2017).

Posteriormente, esses pacientes foram estratificados da seguinte maneira:

1) De acordo com o *Guideline* da Associação Americana de Urologia para o câncer de bexiga, os pacientes foram classificados em (CHANG *et al.,* 2016). O “p” significa análise decorrente da avaliação anátomo patológica da peça cirúrgica:
    a. **Baixo risco**. Tumores de baixo grau e lesão pTa e com lesão única e menor que 3cm de diâmetro. Neoplasia papilar urotelial com baixo potencial maligno também foi enquadrado neste ítem;
    b. **Risco intermediário**. As que enquadram numa das seguintes condições:
        i. Tumores de baixo grau: lesão pT1 ou maior que 3cm ou múltiplos ou que recorreram dentro de 1 ano após o primeiro tratamento;
        ii. Tumores de alto grau: lesão pTa e lesão menor que 3 cm e solitário;
    c. **Alto risco**. As que enquadram numa das seguintes condições:
        i. Tumores de alto grau: presença de lesão p*Cis* ou lesão pT1 ou lesão maior que 3 cm ou multifocal;
        ii. Qualquer das seguintes situações: paciente que tiveram falha ao tratamento com BCG ou outras variações histológicas ou

presença de invasão linfovascular ou invasão de uretra prostática.

2) Recorrência após primeiro tratamento. Foram considerados aqueles pacientes que não tinham história prévia de CUB, tinham cistoscopia ou Re-Ressecção Endoscópica de Bexiga sem câncer e que, durante o seguimento, foi diagnosticada nova lesão confirmada por biópsia;

3) Invasão de camada muscular. De acordo com o estadiamento TNM da peça cirúrgica foi classificado como:
    a. Músculo invasivo: maior ou igual ao estadiamento pT2;
    b. Não músculo invasivo: menor que o estadiamento pT2.

4) Presença ou ausência de metástases. As metástases foram identificadas por métodos de imagem (Tomografia Computadorizada, Ressonância Nuclear Magnética ou Cintilografia). Dois pacientes, um com adenomegalia ilíaca bilateral e massa pélvica extra-vesical e outro com massa pélvica, também foram considerados metastáticos. Os outros 4 pacientes metastáticos apresentavam metástases abdominais e/ou torácicas.

5) Falha ao tratamento com BCG. O esquema de administração do BCG, seguiu a conduta pré definida pelo setor de quimioterapia do Hospital do Câncer de Londrina: 6 ciclos de administração intravesical semanal, seguido de 1 administração mensal por 12 a 24 meses. Caso, durante o tratamento com BCG, houvesse recidiva tumoral diagnosticada por método de imagem e confirmado por biópsia via cistoscopia ou ressecção transuretral da bexiga, esses pacientes eram enquadrados como portadores de falha ao tratamento ao BCG.

O presente estudo foi submetido e aprovado pelo Comitê de Ética em Pesquisa Envolvendo Seres Humanos da Universidade Estadual de Londrina

(CAAE: 170846619.2.0000.5231), conforme ANEXO I (9.1). Todos os procedimentos realizados no estudo envolveram participantes humanos estavam de acordo com as normas éticas do comitê de investigação institucional e com a declaração de Helsinque de 1964 e suas alterações posteriores. Todos os pacientes assinaram o termo de consentimento livre e esclarecido (TCLE) (anexo 10.2).

4.2.1 Obtenção da Amostra

Foram coletados cerca de 20 mL de sangue dos pacientes e indivíduos controles, sendo, 12 mL em tubo de soro (sem anticoagulante) e 8 ml em tubo de EDTA (com anticoagulante). Todos os pacientes com CUB, na data da colheita dos exames, tinham o diagnóstico do câncer há pelo menos 60 dias (prazo médio entre o encaminhamento do serviço à data do tratamento oncológico onde foi feito a biópsia do diagnóstico).

As amostras de sangue foram centrifugadas à 3.500 rpm por 15 minutos e o plasma, soro e *buff coat* foram aliquotados e congelado à -80ºC para posterior análise laboratorial.

4.2.2 Análises das Variantes de Nucleotídeo Único do *CTLA4*

O DNA genômico foi extraído de leucócitos de sangue periférico (*buff coat)* utilizando um procedimento de coluna de resina (Biopur, Biometrix Diagnostika, Curitiba, Brasil), seguindo as recomendações do fabricante. A concentração de DNA foi medida com um espectrofotômetro Nano Drop 2000c (Thermo-Scientific, Waltman, Massachusetts, EUA) a 260 nm e a pureza foi avaliada através da medição da razão 260/280 nm. Para identificar as variantes *CTLA4* rs231775(+49 A>G) e SNV *CTLA4* rs231779(+1822 C>T) foi utilizada a reação em cadeia da polimerase em tempo real (qPCR) com o método TaqMan® (Thermo Fisher Scientific, Waltham, Massachusetts, EUA). A qPCR foi realizada em termociclador

(Applied BiosystemsQuantStudio 5Thermal Cycler, Thermo Fisher Scientific, Waltham, Massachusetts, EUA) com um controle negativo (sem amostra de DNA).

4.2.3 Análise Estatística

O tamanho da amostra foi calculado para um poder estatístico de 80% e significância estatística com p< 0,05, e com o objetivo de identificar uma diferença estatística nas variáveis entre os grupos de pelo menos 10%. Foi utilizado o programa estatístico G Power Windows^{R}. Os dados categóricos foram avaliados pelo teste do qui-quadrado ($\chi 2$) e expressos em número absoluto (n) e percentagem (%). Foram calculados o *odds ratio* (OR) e o intervalo de confiança a 95% (IC 95%). Os dados contínuos foram avaliados pelo teste de Mann-Whitney e expressos como mediana e intervalo de percentil (25%-75%). O valor de p foi ajustado para múltiplas variáveis (idade, sexo, etnia e tabagismo) pelo teste de regressão logística binária ou multinomial, quando apropriado. Todas as análises estatísticas foram realizadas com o SPSS para Windows, versão 22.0 (SPSS 31 Inc., CHIGADO, IL, EUA).

5. RESULTADOS

Os resultados desta tese estão apresentados em dois artigos.

O artigo 1 é uma revisão bibliográfica intitulada como “Variantes Genéticas de Citocinas Associadas ao Câncer: Uma Interface com o Câncer Urotelial de Bexiga”. O intuito dessa revisão foi buscar artigos relacionando as variantes genéticas de citocinas com seus mecanismos imunológicos para o melhor entendimento dos mecanismos imunes relacionados ao CUB.

O artigo 2 foi obtido a partir da análise dos dados coletados, sendo intitulado “Variantes Genéticas do *CTLA4* Associadas a Suscetibilidade do Câncer Urotelial de Bexiga”.

Esses artigos serão submetidos posteriormente a periódicos científicos com QUALIS A1 ou A2.

5.1 ARTIGO 1

Genetic Variants of Cytokines Associated with Cancer: An Interface in Urothelial Bladder Cancer

Keywords: bladder cancer, single nucleotide variants, cytokines.

Summary

Bladder cancer is the 7th most prevalent tumor in the world, affects more men than women and tobacco is the main risk factor. Urothelial Bladder Carcinoma (UBC) is the most prevalent histological type, accounting for about 90% of cases. A characteristic of tumor tissue in UBC is that it has a large number of mutations. It is theorized that tumors with these characteristics are more responsive to immunotherapy treatment due to the higher expression of neoantigens. With greater knowledge of tumor escape mechanisms via Programmed Death-1 (PD-1) and Cytotoxic T Lymphocyte Associated Protein-4 (CTLA-4), several immunotherapy drugs have shown promising results. However, other factors that unbalance the immune response, such as cytokine expression in the tumor microenvironment, can lead to immune deficiency in tumor elimination. Single nucleotide variants (SNVs), related to cytokine expression genes, have been related to UBC, either by its overexpression or not.

The objective of this review is to evaluate the role of cytokines and their genetic variants found in the literature that are related to UBC.

Introduction

Bladder cancer is the 7th most prevalent tumor and the 10th most incident in the world. It affects more men than women, and 90% of those affected are older than 55 years[1]. Smoking is the main risk factor, but exposure to aromatic amines, family history, previous pelvic radiation, previous chemotherapy with cyclophosphamide, diabetes mellitus and genetic syndromes (such as Lynch Syndrome) are also predisposing factors to bladder cancer[2,3].

UBC is the most frequent histological type and corresponds to 90% of cases. Another histological type, squamous cell carcinoma corresponds to 3 to 7% of bladder tumors and may reach up to 80% of cases in certain regions of the Mediterranean due to infection by *Schistossoma haematobium*. This is due to chronic inflammation caused by the parasite eggs deposited in the bladder mucosa with consequent metaplasia and carcinomatous differentiation[4,5]. Individuals with the presence of intravesical catheter of chronic use, by the same process, also have an increased incidence of bladder cancer[5]. Due to this, squamous metaplasia is found

in up to 80% of paraplegics with indwelling probing and with a 5% incidence of squamous cell carcinoma in this group[3]. Other causes related to this dysplastic process are the presence of bladder stones and recurrent urinary tract infection[5,6,7]. Another histological type is adenocarcinoma, which accounts for less than 2% of cases of bladder tumors. It may be a primary cause due to the persistence of urachus or bladder exotrophy[8].

The main clinical symptom of UBC is painless macroscopic hematuria. In carcinoma *in situ*, the tumor invasion of the lamina propria and the muscular layer of the bladder, irritative sensory stimuli of the detrusor muscle may occur, manifesting as dysuria and voiding urgency[3,9]. Ultrasonographic diagnosis is the most common incidental imaging finding. For the investigation of hematuria due to urothelial tumors of the upper tract and kidney, tomography of the urinary tract is the indicated examination. Cystoscopy with biopsy is the standard test in the diagnosis of UBC. Urine oncotic cytology can be an auxiliary test in the investigation and has a sensitivity of 41% for the diagnosis, especially for high-grade tumors [3].

About 70% of the UBC diagnosed cases are localized disease (pathological stage: C*is*, Ta and T1) and are classified as superficial tumors. 25% invades the deep musculature of the urothelium (T2 and T3) and about 5% are locally advanced or metastatic (T4 and/or M1). The UBC can also be classified in relation to histological aggressiveness, being divided into low-grade and high-grade tumors. Regarding the risk of progression, we can divide it into low-risk tumors (Ta, single lesion and smaller than 3 cm), moderate risk (low-grade tumors, which may be larger than 3 cm and/or multiple in the absence of C*is*) and high risk (presence of at least 2 factors: T1, high-grade tumors, multiple and recurrent low-grade Ta, presence of C*is*, lesions larger than 3 cm)[10].

Regarding mortality, low-risk, low-stage tumors below T1 have a 5-year cancer-specific survival of 98%, while metastatic tumors have a survival rate of no more than 10%[10].

The treatment of bladder cancer is generally stage-based, and superficial tumors are treated with endoscopic resection followed or not with intravesical *Bacillus Calmette-Guerin* (BCG). Invasive tumors (higher than T2) are usually treated with cystectomy and chemotherapy or maximal endoscopic resection followed by chemoradiotherapy [12,13].

Intravesical immunotherapy with BCG has been used in the treatment of UBC for more than 40 years, being an established therapy in the adjuvant treatment of endoscopic resection of superficial tumors[12,14]. The mechanism of action of BCG occurs by nonspecific immunomodulation of the urothelium. The bacillus after infecting the urothelium, leads to activation of the immune system, release of cytokines, activation of the response with Natural Killer (NK) cells and T helper-1 (Th1) response and, consequently, leading to neoplastic cell death through this response[15]. In studies by Morales *et al*, 1976, the reduction of recurrence in superficial tumors by the introduction of BCG was 55-65% and has been

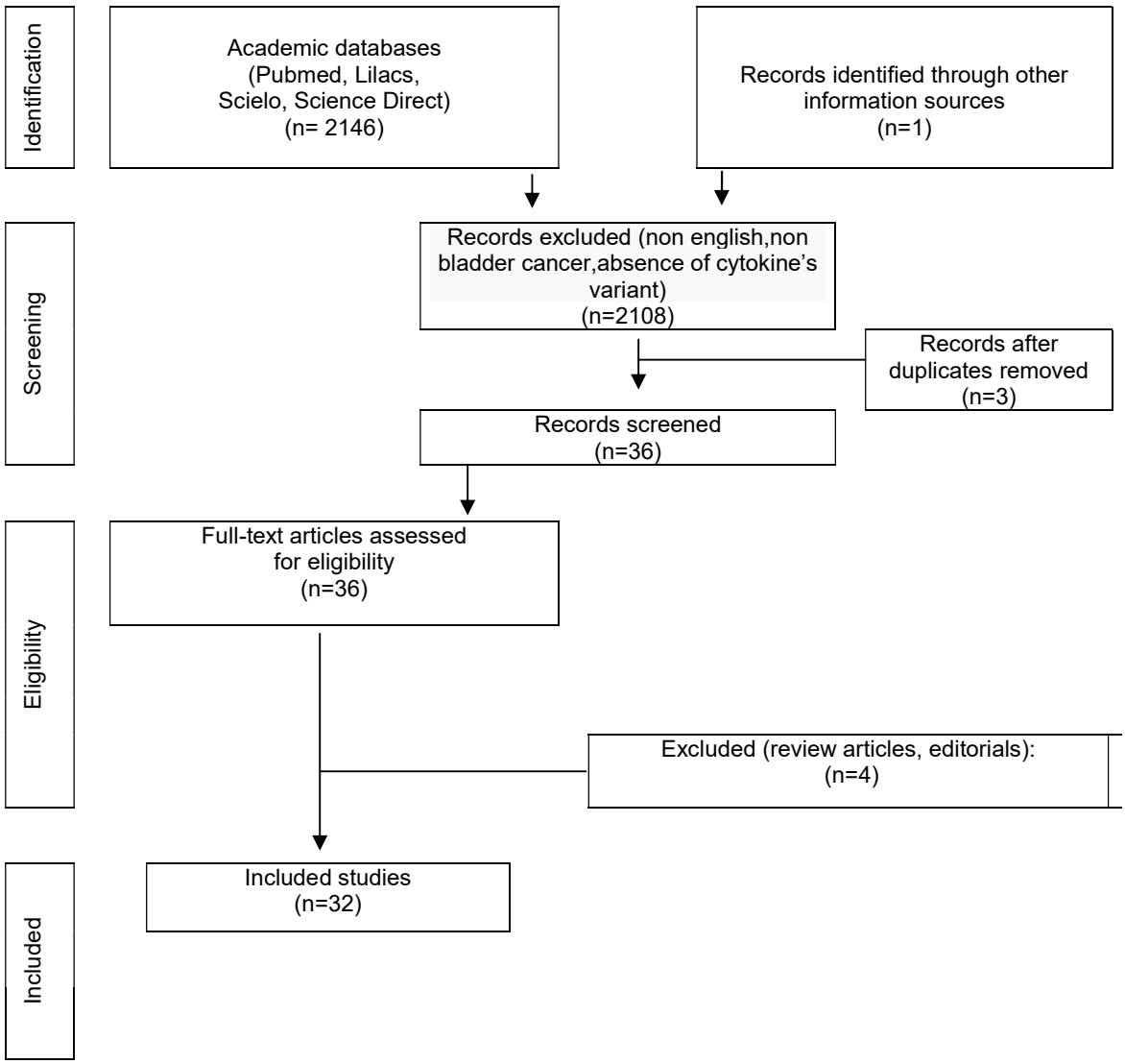
corroborated and well established in guidelines of UBC treatment[12]. However, despite the treatment performed, about 50% of high-grade tumors recur whitin 2 years, of which 25% progress to invasive tumors (higher than T2)[3]. In these cases, neoadjuvant chemotherapy followed radical cystectomy are the standard treatment with an improvement in overall survival of about 5%[16]. However, 15% of these cases evolve to metastases regardless of the treatment performed[10]. Several tumor biomarkers for UBC have been proposed[17,18,19], but none definitively to predict which tumors will respond or evolve aggressively and which will require other additional therapies (e.g., immunotherapy or chemotherapy)[20,21]. A challenging example of a biomarker would be to predict which tumor variants would respond to neoadjuvant chemotherapy and which surgery should be indicated early[22,23,24]. It is known that the time of delay from diagnosis to treatment is related to the progression of the disease[25].

Therefore, considering that the immune response through cytokines is a factor associated with the development and progression of the disease and that genetics variants could lead to modifications in the behavior of the immune response to UBC, this study proposes to carry out a bibliographic survey for a better understanding of the immunological mechanisms involved in UBC. In addition, the search for a possible biomarker could lead to an improvement in the follow-up and decision-making of the treatment to be performed in these patients with UBC.

Materials and Methods

This review is in accordance with the rules of the Reporting Guideline for Systematic Reviews and Meta-Analyses – PRISMA. The review sought articles from clinical trials, meta-analyses, observational and cohort studies. Data were searched from PUBMED, Lilacs, Scientific Electronic Library Online (SCIELO), and Science Direct data sites in English, Spanish, or Portuguese. The terms used were: ("bladder cancer" OR "urinary bladder carcinoma" OR "urinary bladder cancer" OR "urothelial cancer" OR "urothelial carcinoma") AND ("single nucleotide polymorphisms" OR SNP OR SNV OR "single nucleotide variants”).

Figure 1 – Flow diagram for selection of studies



Immune Surveillance and Carcinogenesis

Since the article published by Hanahan and Weinberg, 2000, regarding the conditions that lead to the carcinogenic mechanism, much has been discovered about the interaction of the immune system in the tumor microenvironment [26].

Once the cell begins its dysplastic process, mechanisms of the innate immune system come into play. The recognition of the tumor cell by the immune system may occur by the presentation of neoantigens resulting from mutated proteins, overexpression of own antigens, antigens previously no longer expressed (such as fetal antigens) or antigens derived from oncoviruses[27]. The main cells active in this initial phase are Natural Killers (NK) cells, macrophages and dendritic cells (DC). The cytokines related to this phase, Interleukin 1 (IL-1), Interleukin 6 (IL-6), Interferon

gamma (IFN-γ), Interleukin 12 (IL-12), Tumor Necrosis Factor-alfa (TNF-α), act by activating the adaptive immune system. TCD8+ cells, activated mainly by the Th1 response, together with macrophages and NK cells, act by eliminating neoplastic cells by direct cytotoxicity. In the same way that the initial inflammatory activity can lead to the interruption of carcinogenesis, in a second moment, it can also be a perpetuating factor of the neoplasm[28,29,30]. Studies that correlate the distribution of lymphocytes in the tumor environment and the presence of IFN-γ and TNF-α concentrations have been related to better tumor control and prognosis[31,32]. Once there is no elimination of the neoplastic cell, an equilibrium phase sets in. Thus, a tumor microenvironment is created, with the presence of macrophages polarized to M2, action of T regulatory (Treg) cells with expression of cytokines with anti-inflammatory, neovascularization and fibroblastic action. In this phase, which can last for years, the constant attack of the cells of the immune system against the neoplastic cells generate immunoadapted clones. Subsequently, a phase of tumor escape occurs, where the immunological mechanisms become inefficient and tumor growth by immunoadapted clones prevails[27,33]. It is interesting to observe that, in the established microenvironment, there is the action of Treg cells and the response of M2 macrophages. The cytokines related to this microenvironment lead to neovascularization, expression of growth factors and immunosuppression, resulting in tumor growth[27,34]. Studies in colorectal and breast cancer show that the relationship between TCD8+/Treg cells are critical for prognosis[35,36,37]. Certain SNVs related to hyper- or underexpression of cytokines, which can lead to an imbalance in immune cell populations, are largely related to carcinogenesis[38,39]. Therefore, cytokines related to the development of this state have become the key in understanding tumor escape[28,40,41].

We will discuss below several cytokines related to tumor development with their genetic variants related to some degree of carcinogenesis of genitourinary tumors.

Results and Discussion

Tumor Necrosis Factor alfa and beta (TNF-α and TNF-β)

TNF-α is a cytokine mediator of acute inflammatory response and is produced primarily by macrophages, DC and NK cells. TNF-β (also known as lymphotoxin) is molecularly similar to TNF-α and shares the same biological functions[43,44]. Along with IL-1 and IL-6, they are the main cytokines of acute inflammatory activity of the innate immune system. Its direct action on tumor cells leads to cellular apoptosis and is related to the direct cytotoxic action of NK cells[45,46]. However, in the tumor microenvironment, its action can be carcinogenic and the tumor cells themselves can produce it[47]. TNF-α intracellular signaling pathways lead to 2 outcomes: 1- apoptosis via Caspase activation 8 or; 2- inflammatory process and cell proliferation

via Activating Protein-1 (AP1), Mitogen Activated Protein Kinase (MAPK) and Nuclear Factor Kappa (NF-kB)[48,49]. During the chronic inflammatory process, the action of TNF-α can lead to the production of angiogenic factors (such as Vascular Endothelial Growth Factor or VEGF) and proteases, which leads to neoplastic invasion and metastatic predisposition[32,47]. High serum concentrations of TNF-α have been found in bladder cancer[50,51] and its action in this carcinogenic environment is related to stage and prognosis[52]. The most related *TNFA* gene variants are in promoter regions and related to higher expression of this cytokine: -308G/A; -488G/A; -859C/T and -1031T/C [53-57]. These studies demonstrated that these variants are related to the increased risk of UBC and when diagnosed, are related to the multiplicity of lesions and recurrence of the disease. Following this line, the use of anti-TNF drugs has been tested along with checkpoint inhibitors in treatment of Melanoma. Infliximab or Certolizumab therapy plus Nivolumab and Ipilimumab seem to lead an increased response rate to immunotherapy, most likely due to the effect of TNF-α in promoting resistance to anti-PD-1 therapy [58,59]. But, in UBC, the anti- and pro-carcinogenic action of TNF-α seems to depend on other related actors and has not been used for its treatment[45].

Interferon-gamma (IFN-γ)

IFN-γ is produced by NK cells and T cells (CD4+ and CD8+). Together with IL-12, they carry the Th1 response, which is the most active against carcinogenesis. Dysfunctions that lead to decreased expression of IFN-γ can lead to an M2 macrophage response and activation of Treg cells[29,60]. Ahirwar*et al*, 2009, in a study with the variants *IFNG* +874T/A (rs62559044), whose variants tends to decrease the expression of this cytokine, showed increased risk for UBC. In this study, it was observed that patients with this variants and submitted to surgery followed by treatment with BCG, tend to recur more frequently than the control group[61].

Interleukin 1 (IL-1)

IL-1 has a similar action to TNF-α, participating in the acute inflammatory response[62]. It has a similar origin to TNF-α and can be produced by macrophages, neutrophils, epithelial and endothelial cells. The members of the IL-1 family are composed of 11 types of cytokines, three of which are the classic forms: IL-1 (IL-1α, IL-1β and the IL-1 receptor antagonist or IL-1RA); IL-18 and IL-33[62,63]. IL-1β is the most active form and its production, along with IL-18, is conditioned to signaling via NF-kB, inflammasome activation and cleavage by Caspase 1[63,64]. IL-1RA is biologically inactive and competes for the same IL-1 receptors, causing a decrease in IL-1 action[63]. The participation of IL-1β in carcinogenesis may be antagonistic[65]. In an initial phase, the action of acute phase inflammatory cytokines (IL-1β, TNF-α and IL-6), cells of innate immunity (NK, macrophages and DC) and adaptive

(activated T cells) are the main agents of tumor cell destruction[65,66,67]. However, when tumor escape occurs, the continued release of IL-1 may promote the production of vascular and fibroblastic growth factors, induction of myeloid derived suppressor cells (MDSC) and activation of the M2 macrophage response, thus creating a favorable tumor microenvironment[68,69].

It is hypothesized that, in the chronic inflammatory process, the increase in the levels of IL-1β, IL-6 and TNF leads to continuous signaling of the NF-kB pathway and mitogenic activity of protein kinase (MAPK), favoring inflammation itself, increased cell survival, differentiation and cell proliferation[65,70,71]. Several tumors have their carcinogenesis related to IL-1 and the chronic inflammatory process[69,72,73,74], including UBC[75].

Similarly, variants that decrease IL-1RA expression (and increase IL-1 action are related to UBC[76,77]. In cultures of neoplastic cells Scheneider*et al*, 2021, identified that low expression of IL-1RA correlates with greater capacity of invasion and migration of neoplastic cells [79]. Substances such as tobacco can decrease the expression of IL1-RA and thus correlate with worse prognosis and increased tumor aggressiveness[80].

In the 2nd intron of the gene *IL1RA* there is a sequence of tandem repeats of 86 base pairs[81]. The variations in the number of these repetitions are called variable number of tandem repeats (VNTR) and the number of repeats of these base pair sequences affects IL1-RA production. There are 5 variants for the *IL1RN* gene (*IL1RN*1, IL1RN*2, IL1RN*3, IL1RN*4* and *IL1RN*5*) which influence the number of VNTRs and consequently the production of IL1-RA. For exemple, the *IL1RN*2* allele is related to a higher inflammatory response than the *IL1RN*1* allele [82].

We found 4 studies relating *IL1* and *IL1RA* variants with conflicting results among them. Many of these contradictions maybe due to the pleiotropic action of IL-1 in the carcinogenic process[61,76,77,78].

Transforming Growth Factor beta (TGF-β)

TGF-β is a cytokine that participates in cell control and differentiation and has an important role in tumor escape[83]. In the tumor microenvironment, TGF-β is associated with low expression of MHC-I in tumor cell and in the presence of IL-2, participates in the differentiation of TCD4+ cells to induced Treg[84]. In addition, the expression of IL-10 and TGF-β induce immunosuppression of NK cells, dendritic cells, macrophages and TCD8+ cells [85,86]. Its action stimulates collagen synthesis and neovascularization. It contributes to the formation of tumor sites with immune exclusion. TGF-β also leads to decreased expression of cellular cytotoxicity genes (perforins, granzymes, Fas ligand, and IFN-γ) of NK cells and TCD8+ cells[87,88]. Interestingly, the interaction of TGF-β with IL-6 and IL-1 is related to the Th17 response, which is notably inflammatory. Carcinogenesis could be related, in this case, to the chronic inflammatory process[89]. The Th1 response, which is the best

anti-tumor immune response, can also be antagonized by the presence of TGF-β. In an experiment with mice with the inactivated *TGFB1* gene, a lethal multi-organ immune disease was observed due to exacerbation of a Th1 and Th2 response [90]. Increased *TGFB* gene expression in patients with UBC is related to cancer risk, progression and mortality[91,92].

Interleukin 2

IL-2 is produced primarily by TCD4+ cells activated through the stimulation of antigen-presenting cells (APC). Through its autocrine action, IL-2 is responsible for the signal of proliferation of specific antigen TCD4+ cells, as well as B cells and NK cells[93]. IL-2 also acts on Treg cells (which have receptors with high affinity for IL-2) and are important components to regulate the immune response acting negatively (by IL-2 competition) in the proliferation of TCD4+ cells[94,95]. In addition, IL-2 along with TGF-β lead to greater differentiation for the production of Treg cells[96]. Variants that decrease the expression of IL-2 and, consequently, favor the proliferation of Treg cells in the tumor microenvironment, could predispose to carcinogenesis [97,98]. However, few studies have related the variant of *IL2* with UBC. We found only 1 case/control study, covering 365 cases of UBC and 390 controls where it was found a negative correlation of the *IL2* -330T/G variant (rs2069762) to UBC[99].

Interleukin 6

IL-6, along with IL-1β and TNF, are the main cytokines of the acute inflammatory response. It has pleiotropic action with local and systemic effects, antagonistic and related to the activation of various cytokines[100]. Its effects lead to the production of acute phase proteins, stimulates neutrophil proliferation, differentiation of IL-17-producing T cells auxiliary and antibody-producing B cells [101,102]. IL-6 is synthesized by lymphocytes, macrophages, DC, fibroblasts, endothelial cells and, eventually, by tumor cells[102,103].

IL-6 binds to its receptor (IL-6R), forms a complex with the gp130 protein and activates signaling pathways such as Janus kinase/Signal Transducer and Activator of Transcription-3 (JAK/STAT3), MAPK, Phosphatidylinositol 3-kinase/AKT (PI3K/AKT) and NF-kB[104,105]. When activated, it triggers processes related to angiogenesis, cell proliferation, migration, and cell resistance to apoptosis. High levels of IL-6 have been found in diseases related to chronic inflammatory processes (such as rheumatoid arthritis), inflammatory bowel diseases and also hematopoietic and solid tumors[104,105,106]. When chronically expressed by tumor cells, it is related to tumor progression and metastasis[107]. In addition, the IL-6/JAK/STAT3 signaling pathway can positively regulate the production of Myeloid-Derived Suppressor Cells (MDSC), thus favoring the maintenance of the tumor cell in its microenvironment[108,109]. In addition to activating the Th17 response, IL-6 also

promotes the Th2 response by inducing IL-4 expression and, consequently, may inhibit the Th1 response[103].

In vitro culture, Okamoto *et al*, 1997, demonstrated that IL-6 promoted growth of bladder tumor cells, but not of normal urothelial cells[110]. In addition, increased serum and urinary levels of IL-6 have been found in patients with UBC[111,112]. Studies have shown that individuals with cancer and increased serum IL-6 levels have a worse prognosis and survival[109,113,114].

IL6 SNVs have been related to several neoplasms[115], with genotypes (-174G>C,-592G>C,-597G>A) being the most studied[116]. Associated to UBC, the most studied variant is -174G>C.The expression of this cytokine in UBC is not well established, but in a study by Fishman et al., 1998, in patients with juvenile chronic arthritis, it was shown that the CC genotype is related to lower levels of IL-6 (117). Studies of this variant have shown conflicting results, some as a protective factor[118] and others (conversely) related to higher risk for UBC[28,119,120,121].

An important fact is that serum IL-6 levels do not necessarily correspond to their tissue levels[122]. Leibovic*et al*., 2005, suggest that there could be some mechanism that leads to the adaptive advantage of the neoplastic cell exposed to low levels of IL-6 resulting from this SNV[28]. In addition, multiple effects of IL-6 on the balance of the Th1/Th2 response and its chronic action on the tumor microenvironment in the different stages of carcinogenesis could justify the discrepancy of these findings [103,123,124].

Interleukin 4

IL-4 is produced by activated TCD4+ cells, Th2 response lymphocytes, and mast cells. IL-4 acts in the differentiation and activation of B cells and, in the presence of IL-2 and absence of IL-12, promotes the Th2 response in addition to inhibiting the Th1 response. In the tumor microenvironment it acts as the main activating agent of the M2 phenotype of the macrophage. This can lead to cell proliferation, angiogenesis, resistance to apoptosis and tumor invasion[125]. Studies with mice with IL-4 deficiency showed a decrease in the incidence of carcinogen-induced tumors, which could demonstrate the pro tumorigenic action of this cytokine[126]. In a 2016 meta-analysis, Jia *et al*, 2017, with 43 studies, 16,739 individuals with cancer and 22,540 control individuals, and evaluating *IL4* variant that lead to increased IL-4 cytokine (rs2243250, rs2070874 and rs79071878), found the association with many cancers, including UBC[56,77,78,127,128]. In the study by Bozdogan*et al*, in 2013[77], it is suggested that smoking associated with *IL4* variant could increase the risk for high-grade UBC.

Interleukin 8

IL-8, also called CXCL8, is a chemotactic cytokine. It is produced mainly by macrophages and also by lymphocytes and epithelial cells. Its main function is the recruitment of neutrophils in the initial phase of inflammation [130]. Tumor cells can produce IL-8 and, with this, lead to MDSC recruitment, neovascularization, promotion of the epithelial-mesenchymal transition [131,132] and, consequently, tumor progression and metastasis [133].

Regarding UBC, elevated neutrophil levels are seen in the urine soon after instillation of intravesical BCG[134,135]. Neutrophils act as essential antitumor agents in the mechanism of action of intravesical BCG and may have local action predicting response[136]. In addition, elevated urinary IL-8 levels have been found in patients with UBC compared to controls [137,138,139], in addition to being related to recurrence [140,141] and tumor grade [137,138]. Tallman *et al*, 2000, suggested that elevated levels of urinary IL-18 and IL-8 after BCG could predict outcome of UBC treatment[142].

Wang *et al.*, 2014, in a meta-analysis of 47 studies, involving 12,917 cases of different types of cancer and 17,689 controls, analyzed the variant of *IL8* -251A>T (whose result leads to an increase in IL-8) and found a significant association with the risk of various types of cancer, including in relation to UBC[143].

Interleukin 10

IL-10 has a pleiotropic action, acting as a brake on the inflammatory process[144,145]. They are produced by macrophages, activated LT cells and mainly Tregs cells[145]. Its effects are the inhibition of the expression of IL-12, IL-1, TNF in macrophages and dendritic cells. Because they are produced by the same cells that activate the inflammatory process, their actions are an example of self-regulation. The rs3024505 (C>T) SNV located immediately adjacent to the IL-10 gene and leading to decreased IL-10 expression, has been related to ulcerative colitis [146].

High levels of IL-10 in the urine have been found in high-grade UBC and is related to tumor recurrence and could be explained by its immunosuppressive response[52].

Basturk *et al*, 2006, evaluated SNVs of patients undergoing tumor resection followed by BCG. It has been observed that patients who had the variant *IL10* -1082G/A rs1800896 and *IL10* GCC/GCC haplotype (-1082G/A, rs1800896; -819C/T, rs1800871; -592C/A, rs1800872) have a higher risk for disease progression due to an increase serum levels of IL-10[128].

Interleukin 12

IL-12 is produced mainly by activated DCs and macrophages. It stimulates the production of IFN-γ by Innate Lymphoid Type 1 Cells (ILC1), NK cells and TCD8+ cells. One of the actions on NK cells and TCD8+ cells is to increase their cytotoxicity.

In addition, together with IFN-γ, it stimulates the differentiation of LT naïve cells for the Th1 response[149]. Therefore, the action of IL-12 generates potent antitumor effects[150]. Greiner *et al*, 2021, using a targeted IL-12 agonist drug (NHS-IL12), demonstrated significant reduction of induced bladder tumor in mice[151].

Regarding *IL12* variants, Zheng *et al,* 2017, in a meta-analysis evaluating IL-12 SNVs (rs568408, rs2243115, rs3212227), included 33 studies in a total of 12040 cancer cases and 12040 controls, found a relationship between these SNVs and cervical, cerebral, hepatocellular and nasopharyngeal cancers[152]. For the UBC, we found 2 studies involving the SNV rs3212227 (whose effect leads to decreased expression of IL-12) to bladder cancer[112,113]. These studies related this variant to the increased risk of UBC as well as the relationship with increased aggressiveness, progression and recurrence after BCG treatment[120,121].

Interleukin 17

The IL-17 family is composed by 6 types of interleukins (IL-17A~F). IL-17A and IL-17F are the most similar structurally and in function[153]. IL-17, together with TNF-α and the cytokines of the IL-1 family, are the main activators of the NF-kB transcription pathway and are essential agents in binding the inflammatory process from innate to adaptive immunity. It plays an important role in infections by extracellular bacteria and fungi, and the cytokine is characteristic of the Th17 response[154,155]. They promote the recruitment of neutrophils, participate in the process of acute rejection after organ transplantation and autoimmune diseases, such as rheumatoid arthritis[156,157]. IL-17 also stimulates the release of IL-6 and IL-8 by fibroblasts, endothelial and epithelial cells, osteoblasts, monocytes and macrophages[158,159]. In experiments with cell culture, it was demonstrated that IL-17 is involved in cell proliferation by stimulating angiogenic, fibroblastic and VEFG factors[160]. In the tumor microenvironment, IL-17, Interleukin 23 (IL-23) and the IL-23 receptor (IL-23R) are closely related to the carcinogenic process[161]. In addition, IL-17, TGF-β and VEFG present in this environment can lead to the recruitment of macrophages associated with the tumor, promote increased neovascularization, tumor invasiveness and stimulate the production of Treg cells [86,162-165]. Elevated levels of IL-17 are associated with a worse prognosis in colon cancer[166], pancreatic cancer [167,168] and better prognosis in ovarian tumors [162]. Regarding *IL17* variants, the most frequently associated with UBC are: *IL17A* -197G/A (rs2275913) and *IL-17F* 7488T/C (rs763780) being related to increased IL-17 expression [57,163,169,170,171].

Interleukin 23

IL-23 is produced primarily by macrophages and DC. It is the main cytokine that stimulates the Th17 response. However, this activation requires the presence of other cytokines already mentioned (IL-6, IL-1 and TGF-β)[90]. The signaling of these

cytokines induces the activation of the transcription factors STAT3 and Retinoic Acid-Related Orphan Receptor (RORγt) leading to greater expression and activation of the IL-23 receptor (IL-23R) and also amplifying the expression of IL-17, IL-22, IL-23 forming a positive amplification pathway maintaining the Th17 response [172,173]. Studies have related elevated levels of IL-17, IL-23 and the activation of the IL-6/STAT3 signaling pathway in melanoma growth in mice[173]. The increase in IL-23 and IL-17 promotes carcinogenic status through suppression of NK response, stimulation of VEGF production and activation of macrophages associated with the tumor[164,165,172]. Variants related to the IL-23 receptor (IL-23R), mainly rs6682925 and rs10889677, are the most related to carcinogenesis of this IL-23/IL23R pathway and Th17 response[174]. Liu *et al*, 2018, found elevated serum levels of IL-17 and increased expression of IL-23R in patients with UBC compared to healthy individuals. In addition, IL-23R expression in UBC tumor tissue was increased relative to normal adjacent tissue of UBC patients [175]. El Gedamy*et al*, 2021, studying the rs10889677 variant, identified elevated IL-23R expression and increased serum levels of IL-23 and IL-17 in patients with UBC[176]. The same author, studying the rs1884444 variant, found the opposite, decreased serum levels of IL-23 and IL-17 with these carriers having a decreased risk for UBC [177].

Interleukin 18

IL-18 is structurally similar to IL-1 and, similarly to IL-12, intensifies NKs activation and Th1 response[62,179]. IL-18 can also lead to activation of the Th2 response[64]. It participates in innate and acquired immunity and, like IL-1β, its production is mostly dependent on the activation of the inflammasome followed by cleavage by Caspase 1[180]. Disorders in IL-18 production can lead to autoimmune [179] and allergic [181] inflammatory diseases. Regarding carcinogenesis, chronically expressed IL-18 leads to chronic inflammation and is one of the explanations for tumor escape [29,30]. *IL18* variants (rs187283 and rs1946518) that increase IL-18 expression have been linked to gastric, colon, liver[182,183] and bladder cancer[39,148].

Interleukin 13

IL-13 is produced by CD4+(Th2) cells, NK cells, innate lymphoid type 2 cells (ILCs2), and mast cells. Along with IL-4 and IL-5, they are the characteristic cytokines of the Th2 response and play an important role in the defense against helminths and allergic conditions. It acts on B cells by stimulating the exchange to the IgE isotype[184]. In the epithelial cells of the airways and intestinal increases mucus production and intestinal peristalsis[185]. In addition, it induces macrophage differentiation to the M2 response. IL-4 and IL-13 share the same receptor (IL-4R) which explains part of the same responses[186]. In tumor cells, by activating Th2 inflammatory response, the promotion of carcinogenesis could be justified by the

maintenance of the tumor microenvironment leading to cell proliferation, angiogenesis, remodeling of the extracellular matrix, stimulation of tumor invasion, recruitment of Treg cells and inhibition of proliferation of CD8+T cells[187,188]. Malekzadeh *et al*, 2009, in blood samples from patients with UBC observed elevated levels of IL-13 in relation to healthy individuals[189]. A variant of *IL13*, SNV -1055C/T (whose SNV leads to increased IL-13 expression), was related to increased risk of UBC in smokers, but when assessed in the total sample of individuals, it was not significant for increased risk[190]. Cultures of tracheal and bronchial cells have shown that cigarette smoke can alter IL-13 expression, suggesting that IL-13 overexpression alone is not sufficient to increase the risk of UBC[191].

Interleukin 22

IL-22 is a member of the IL-10 family and, along with IL-17, are the characteristic cytokines of the Th17 response. In addition to the Th17 cell, NK cells and innate lymphoid cells (ILCs) can produce IL-22. It is related to the epithelial integrity of the skin and intestinal tract, liver, lung and kidneys. It acts on mechanisms that promote cell repair, activation of the local inflammatory response, induction in the production of mucins, cathelicidins and defensins[192]. IL-22 in the tumor microenvironment is related to decreased apoptosis, cell proliferation, and metastases through activation of the JAK2/STAT3 pathway[193,194,195].

IL22 variants have been linked to thyroid, lung, and colon cancer; inflammatory bowel diseases, hepatitis[196,197,198,199,200] and UBC is related to *IL22* -429C/T variant [201].

Interleukin 27

IL-27 is a member of the IL-12 family and is produced primarily by monocytes, macrophages, and DC[202]. In certain situations it can induce Th1 response, stimulate the action of TCD8+ cells and inhibit the Th17 response[203-207]. IL-27 may also activate NK cells and stimulate the release of INF-γ[208,202]. It may also suppress the formation of induced Treg cells[209]. Due to these effects, the antitumor action of IL-27 has been observed in experimental studies in head and neck cancers[208,210]. Regarding *IL27* variants, the *IL27* -964A/G variant (rs153109) has been related to several types of cancer, including UBC[211,212].

Interleukin 31

IL-31 belongs to the IL-6 family and is related to epithelial diseases (e.g., Atopic Dermatitis-AD) triggering pruritic processes and neuropoiesis[213,214,215]. Its production occurs in eosinophils, DC, CD4+CLA+ lymphocytes (cutaneous lymphocyte antigen+), basophils, monocytes, mast cells and mainly, the lymphocytes of the Th2 response[213,216,217]. One of the hypotheses of the pruritic effect caused by IL-31 in AD may be associated with the stimulation of IL-31 in the growth

of sensory nerve fibers of the skin[215]. In addition, elevated serum levels of IL-31 have been related to AD severity[218].

Regarding carcinogenesis, elevated serum levels of IL-31 have been found in endometrial cancer, lymphomas and chronic myeloid leukemia[219,220]. The mechanism by which IL-31 would participate in carcinogenesis could be by the pro-inflammatory action of the Th2 response and increased expression of angiogenic factors such as EGF and VEGF[221]. In addition, IL-31 may activate the PIK3/AKT signaling pathway leading to proliferation and increased cell survival[222]. Regarding the variant of *IL31* in the UBC, we found only 1 case-control study with *IL31* C>A (rs4758680)[223].

Interleukin 32

IL-32 is a cytokine with pro- and anti-inflammatory actions, being initially identified in activated NK and LT cells[224,225]. Elevated serum levels have been found in diseases such as rheumatoid arthritis, inflammatory bowel diseases, and various cancers[225,226,227]. Studies show the synergistic action of IL-32 with pro-inflammatory cytokines such as IL-8 and TNF-α[225]. IL-32 is also related to tumors in which inflammatory activity is important in their carcinogenesis, such as gastric, colon and hepatocellular carcinoma tumors[227,228,229].

Paradoxically, IL-32 is also capable of inducing cellular apoptosis and amplifying the action of NK cells and cytotoxic T cells, also acting as an anti-tumor agent[230]. These different actions seem to be due to the various isoforms of IL-32 that can lead to antagonistic effects[229,230]. Seven isoforms of IL-32 arising from alternative splicing are known[231]. Studies show that these isoforms can act as: 1-Antitumor agents-inducing apoptosis in tumor cells by activating the response by cytotoxic T cells or; 2-Pro- or anti-tumor agents-regulating (positively or negatively) NFκB and STAT3 signaling pathways[230,232-235].

Regarding *IL32* variants, rs12934561 T/C and rs28372698 A/T are the most studied and are related to thyroid, lung, ovarian, colon and rectum, endometrium, and UBC[227,236-241].

Conclusion

Carcinogenesis is closely related to immunological processes, genetic susceptibility, environmental factors and senescence of the organism. The fine balance of action between cytokines, immune system, normal and tumor cells results in varying degrees of prognosis and aggressiveness. SNVs are essential, but not definitive, participants in this carcinogenic environment. The identification of these alterations, together with changes in environmental factors and clinical follow-up are essential in the early treatment of UBC, a disease in which early treatment dramatically alters the overall survival of the patient.

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Table 1. Genetic variants related to cytokines and urothelial bladder cancer.

Cytokine	rs	Effect	Country	Ethnicity	Cases	Result	Reference
TNF-α							
-308G>A[1]	rs1800629	↑ TNF-α	China	Asian	287	risk UBC	[53]
-488G>A[1] - 859C>T	rs1800629; rs1799724	↑ TNF-α	UK	Caucasian	196	↑ ↑ TG in inicial diagnosis	[54]
-1031T>C	rs1799964	↑ TNF-α	India	South Asian	220	↑ risk UBC	[55]
-308G>A	rs1800629	↑ TNF-α	Portugal	Caucasian	65UBC multiple lesions	↑ risk of multiple lesions	[56]
-1031T>C	rs1799964	↑ TNF-α	Portugal	Caucasian	70 UBC multiple recurrence	↑ risk for recurrence post BCG	[57]
INF-γ							
+874T>A	rs62559044	↓ INF-γ	India	South Asian	213	↑ risk UBC ↑ risk for recurrence post BCG	[61]
IL-1							
+3954C>T; -511C>T	rs1143634; rs16944	↓ IL-1 ↑ IL-1	Taiwan	Asian	138	NS	[78]
-511C>T; +3954C>T	rs16944; rs1143634;	↑ IL-1 ↓ IL-1	India	South Asian	120	NS ↑ risk UBC	[76]
VNTR Variant IL-1RN*2 gene		↑ ação IL-1					
VNTR Variant IL-1RN*1 gene		↓ ação IL-1	Turkey	Caucasian	100	↓ risk UBC	[77]
VNTR Variant IL-1RN*2 gene		↑ ação IL-1	India	South Asian	213	↑ risk UBC	[61]
IL-2							
-330T>G	rs2069762	↓ IL-2	China	Asian	365	↑ risk UBC	[99]
TGF-β							
+29C>T	rs1800470	↑ TGF-β	India	South Asian	232	↑ risk UBC	[92]
+915G>C	rs1800471	↑ TGF-β	Turkey	Caucasian	14 UBC rec/prog	↑ progressionrisk UBC	[128]
TGF-βR1							
+69A>G	rs868	↑ TGF-β	Spain	Caucasian	1157	↑ risk UBC	[91]
IL-6							
-174G>C	rs1800795	↓ IL-6	USA	Mixedethnicity	519	↑ risk UBC	[28]
-174G>C	rs1800795	↓ IL-6	India	South Asian	136	↑ risk UBC	[119]
-174G>C	rs1800795	↓ IL-6	Iran	Caucasian	261	↑ risk UBC	[120]
-174G>C	rs1800795	↓ IL-6	China	Asian	248	↑ risk UBC	Lu [121]
-174G>C	rs1800795	↓ IL-6	India	South Asian	232	↓ risk UBC	[118]
IL-4							
-590C>T	rs2243250	↑ IL-4	Portugal	Caucasian	65 UBC multiple lesions e Cis+	↑ recurrence risk UBC in multiple lesions and Cis+	[56]
intron3 70pb VNTR		↑ IL-4	Turkey	Caucasian	100	↑ risk UBC	[77]
intron3 70pb VNTR		↑ IL-4	Taiwan	Asian	138	↑ risk UBC	[78]
-1098G>T	rs2243248	↑ IL-4	Turkey	Caucasian	14 UBC withrec/prog	↑ progressionrisk UBC	[128]
IL-8							
-251T>A	rs4073	↑ IL-8	Taiwan	Asian	287	↑ risk UBC	[53]
-251T>A	rs4073	↑ IL-8	India	South Asian	205	↑ risk UBC, ↓ risk for recurrence post-BCG	[129]

continuation: Table 1. Genetic variants related to cytokines and urothelial bladder cancer.

Cytokine	rs	Effect	Country	Ethnicity	Cases	Result	Reference
IL-10							
-1082A>G	rs1800896	↑ IL-10	Turkey	Caucasian	14 UBC withrec/prog	↑ progression risk UBC	Basturk[128]
-819C>T	rs1800871	↑ IL-10	India	South Asian	214	↑ risk UBC	Ahirwar [144]
IL-12							
1188A>C	rs3212227	↓ IL-12	China	Asian	248	↑ risk UBC	Lu [121]
1188A>C	rs3212227	↓ IL-12	Iran	Caucasian	261	↑ risk UBC	Ebadi [120]
IL-17							
-197G>A	rs2275913	↑ IL-17	Portugal	Caucasian	70 UBC relapsed	↑ risk for recurrence post-BCG	Lima [57]
-197G>A; 7488T>C	rs2275913; rs763780	↑ IL-17	China	Asian	301	↑ risk UBC and ↑ agressive cancer risk	Zhou [171]
IL-23R							
+2284A>C	rs10889677	↑ IL-23	Egypt	Caucasian	100	↑ risk UBC	El-Gedamy [176]
-23G>T	rs188444	↓ IL-23	Egypt	Caucasian	100	↓ risk UBC	El-Gedamy [177]
+2284A>C	rs10889677	↑ IL-23	China	Asian	226	↑ risk UBC	Tang [178]
IL-18							
-137G>C; -607C>A	rs187283; rs1946518	↑ IL-18	India	South Asian	200	↑ risk UBC and; ↑ agressive cancer risk and ↑ risk for recurrence post-BCG	Jaiswal [148]
-137G>C	rs187283;	↑ IL-18	Poland	Caucasian	175	↑ risk UBC and ↑ risk for recurrence and progression post-treatment	Krajewski [39]
IL-13							
-1055 C>T	rs1800925	↑ IL-13	China	Asian	143	↑ risk UBC	Chu [190]
IL-22							
-429 C>T	rs2227485	↑ IL-22	China	Asian	210	↑ risk UBC	Zhao [201]
IL-27							
-964A>G	rs153109	↓ IL-27	China	Asian	499	↑ risk UBC	Zhou [211]
IL-31							
C>A	rs4758680	↑ IL-31	China	Asian	294	↑ risk UBC	Li [223]
IL-32							
T>C; A>T	rs12934561 rs28372698	↑ IL-32	China	Asian	321	rs12934561- ↑ risk UBC rs28372698 – ↓ OS UBC in MIBC	Yang [241]

Abreviations: NS - not significant UBC- urothelial bladder cancer; TG- tumoral grade; rec/prog-recurrence/progression; IL-1RA= Interleukin 1 receptor antagonist; VNRT= Variable number tandem repeat; IL-1RN*1= allele 1 of the gene IL1-RN; IL-1RN*2= allele 2 of the gene IL1-RN; Cis – carcinoma *in situ*; IL-23R- IL-23 receptor; OS – overall survival; MIBC – muscle invasive bladder cancer; 1- The -308G/A SNV and +488G/A SNV are defined as rs1800629.

5.2 ARTIGO 2

CTLA4 genetic variants associated with urothelial bladder cancer susceptibility

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Short running title: *CTLA4*genetic variants and bladder cancer

ABSTRACT

Background: *CTLA4* rs231775(+49A>G) and rs231779(+1822C>T) genetic variants in urothelial bladder carcinoma (UBC) and their influence on tumor presence and progression is not fully understood. Moreover, anti-CTLA-4 immunotherapy drugs in UBC have shown promising results; however, they have not been as efficient in UBC as in other cancers Therefore, the aim of this study was to evaluate the relationship of the*CLTA4*rs231775 and rs231779 variants and the UBC susceptibility, as well as with the stage, prognosis and response to treatment.

Material and Methods:A case-control study enrolled 140 patients with UBC and 145 healthy controls. The patients were stratified into non muscle invasive bladder cancer (NMIBC) and muscle invasive bladder cancer (MICB), without and with metastasis, without and with recurrence, with low/moderate risk and high/very high risk. Demographic, anthropometric, epidemiological, and clinical data were obtained from all the individuals using a structured questionnaire. The *CTLA4* variants were determined using real-time polymerase chain reaction (qPCR). Associations with UBC susceptibility or different prognostic settings were tested in the allelic, codominant, dominant, recessive, and overdominant genetic models.

Results: The UBC patients were older and mostly smokers than controls ($p<0.001$), with greater waist circumference and systolic and diastolic arterial pressure ($p=0.005$, $p=0.006$ and $p<0.001$, respectively). Differences were found between the UBC susceptibility among the patients carrying the heterozygote genotypes of *CTLA4* rs231775 [odds ratio [odds ratio (OR=0.40; 95% confidence interval (IC): 0.16-0.98, $p=0.045$) and rs231779 (OR=0.35; 95% IC: 0.14-0.87, $p=0.024$) variants in the overdominant models, both showing a protective effect for the UBC. The regression model considering the overdominant*CTLA4* genetic models for the two variants evaluated (rs231775 and rs231779), age and smoking was able to explain 89.1% of UBC compared to only 50.7% if these parameters not present. Moreover, this model explained about 77.0% for the rs231775 and 77.2% for the rs231779 of the susceptibility to UBC.

Conclusion: The AG and CT heterozygous genotypes of the *CTLA4* rs231775 and rs231779 variants in the overdominant model together with age and smoking may be useful as potential biomarkers for the UBC susceptibility.

Keywords: Bladder Cancer, Urothelial Bladder Cancer, Single Nucleotide Variant, CTLA-4, overdominant.

INTRODUCTION

Primary bladder cancer is the most common neoplasm of the urinary tract, and the histological type of transitional cell carcinoma accounts for approximately 90 % of cases. Urothelial bladder carcinoma (UBC) is the most prevalent histological type, accounting for about 90% of cases and is the histological type for which immunotherapy is currently used. In bladder cancer, several immunological mechanisms of tumor escape have been studied to guide the treatment choice, with the *PD1* and *CTLA4* pathways studies being the most important [1-3].

The Programmed Cell Death ligand 1 (PD-L1) expression on tumor cells has been used to guide treatment and predict immunotherapy outcomes; however, the cytotoxic T lymphocyte associated 4 (CTLA-4 or CD152) as a biomarker has not been as successful [4]. The low expression of induced CTLA-4 on T cells and the widespread expression of the B7 receptor on antigen-presenting cells (APCs) difficult the evaluation of this regulation pathway in the tumor microenvironment [2].

CTLA-4 structure is similar to the CD28 receptor and it's present on $CD4^+$T cells, B cells, Natural Killer cells (NK), dendritic cells, macrophages, Tregulatory (Treg) cells and in some tumor types [5-7]. CTLA-4 and CD28 compete for binding with B7.1 (CD80)or B7.2 (CD86) receptor proteins [8, 9].Compared to CD28, CTLA-4 has 20 times more binding affinity to B7 [10-12].

In the tumor microenvironment, which the neoplastic cells have managed to evade of the immune system response, the cells are configured in an immunosuppressed environment which the expression of major histocompatibility complex (MHC) class I and B7 proteins tend to be decreased [13]. When dendritic cells or $CD8^+$T cells of this microenvironment present the tumor antigen to naïve $CD4^+$T cells, proper activation does not occur due to the greater binding of B7 to CTLA-4 than to CD28. As a result, the non-activated naïve T cells go into anergy or apoptosis, thus decreasing the antitumor response [2,13].

The CTLA-4 protein exists in two isoforms, full length CTLA-4 (flCTLA-4) presents in the cell membrane as a receptor and a soluble form presents in plasma(sCTLA-4) [3,14]. The flCTLA-4 form inhibits T cell activation leading to anergy by the aforementioned process of B7 binding. The *CTLA4*gene is located on chromosome 2 (2q33) and has four exons, with exon 2 responsible for transcription of the transmembrane part of the protein [15]. The other three exons encode the leader sequence, the extracellular domain and the intracellular domain [16, 17]. By a splicing mechanism, exon 2 of the *CTLA4* gene can be deleted and this product is the soluble form released to plasma (sCTLA-4). The sCTLA-4 is mainly produced by T cells (*naïve* and regulatory), monocytes and immature dendritic cells [14,15]. Its action, in non-pathological states, is to maintain the balance of the immune response through a systemic immunosuppressive action. The sCTLA-4 binds to B7 of APC with greater avidity than CTLA-4 of naive T cell [15]. High serum levels of sCTLA-4 have been related to better response of Ipilimumab inpatients with melanoma [18]. *CTLA4* variants, such as rs3087243 (CT60G>A) have been related to higher sCTLA-4 expression and may be a biomarker of choice for immunotherapy treatment [10].

The most studied *CTLA4* variant in UBC is rs231775 ((+49A>G) and is also related to kidney, breast, liver and lung cancer [5, 19-22]. In this variant there is an exchange of Adenine for Guanine at position 49 which leads to the exchange of the amino acid threonine for alanine in the CTLA-4 protein, which has a higher affinity for binding to B7.1 than CTLA-4 coded by the A allele. In addition, increased serum levels of sCTLA-4 have been found in the UBC patients compared to the control group [19]. Consequently, it is expected that there is a high inhibition of the action of $CD4^+$ and $CD8^+$ T cells and an increased activity of Treg cells. The other *CTLA4* variant, rs231779 (+1822C>T) with an exchange of Citosine for Timine at position 1822 is poorly studied in cancer [23] and is related to autoimmune diseases, such as Grave's disease [24]. The single nucleotide variant (SNV) rs231775 showed to be almost perfectly linked with the rs231779 in Chinese population study [24].

UBC is a molecular heterogeneous disease with known genetic distinctive signatures, host (sex and age) and environmental risk factors (tobacco) involved in the etiology. Exogenous factors such as tobacco exposure, obesity and treatment's history of other cancers by radiotherapy are some risk factors and may override the effects of genetic variants [25]. *CTLA4* rs231775 (+49A>G) and rs231779 (+1822C>T) genetic variants in UBC and their influence on tumor presence and progression is not fully understood and present conflicting

results. While a case-control study showed that the rs134775 variant is a protective factor for UBC [26], two other studies showed that this variant could increase the risk of UBC [27,28].Moreover, anti-CTLA-4 immunotherapy drugs in UBC have shown promising results; however, they have not been as efficient in UBC as in other cancers.Therefore, a better understanding of the immunological mechanisms involved in the UBC physiopathology is necessary to guide the treatment and prognostic evaluation of these tumors. In this context, the aim of this study was to evaluate the relationship between the *CLTA4*rs231775 (+49A>G) and rs231779 (+1822C>T) SNVs and the UBC susceptibility, the stage, prognosis and response to treatment.

MATERIALS AND METHODS

Study Patients and Design

From January 2020 to April 2022, 140 UBC patients initially submitted to surgery and followed up at the Outpatient Clinic of the Londrina Hospital Cancer were included in the study. As controls, 145 healthy individuals were selected from the blood donors of the Regional Blood Center of Londrina.All UBC cases were confirmed by histopathology and staged according to the TNM staging system of the Union InternationaleContre le Cancer [29]. Bladder tumors were graded using the World Health Organization classification [30].Patients were stratified into low, moderate and high risk according to the American urological Association (AUA) guideline for bladder cancer [31]. Recurrence after the first treatment was defined in those patients with no previous history of bladder cancer who, during follow-up after curative surgery, had tumor recurrence diagnosed by cystoscopy and biopsy. Patients with metastases were diagnosed by imaging methods (Computed Tomography, *Magnetic Resonance Imaging* or Scintigraphy). The UBC patients were categorized into two groups according to different prognostic settings; patients were stratified into non muscle invasive bladder cancer (NMIBC) and muscle invasive bladder cancer (MICB), without and with metastasis, without and with bacillus Calmette-Guérin (BCG) recurrence, with low/moderate risk and high/very high risk.Pelvic adenomegalies and pelvic masses being considered as metastasis. Recurrence after BCG therapy was defined as the appearance of a new lesion confirmed by cystoscopy and biopsy during BCG treatment.

The demographic, anthropometric, epidemiologic, and clinical data were obtained by interviewing each individual in cases and controls using a structured questionnaire that detailed their sex, age, smoking status, lifestyle, medical history, history of cancer in the family, physical activity, use of medications, associated diseases and other bladder cancer treatments. Body weight was measured to the nearest 0.1 kg using electronic scales, with individuals wearing light clothing, but no shoes, in the morning; height was measured to the nearest 0.1 cm by using a stadiometer. Body mass index (BMI) was calculated as weight (kg) divided by height (m) squared.

Inclusion criteria was age between 18 and 99 years old. The exclusion criteria were the presence of inflammatory, infectious, autoimmune and other neoplastic diseases. All participants gave written informed consent according to the Declaration of Helsinki and the study protocol was fully approved by the Institutional Research Ethics Committees of State University of Londrina, Paraná, Brazil (CAAE: 170846619.2.0000.5231).

Genomic DNA extraction and Polymerase Chain Reaction

Venous blood samples (10 mL) were obtained at the study admission with ethilenodiaminotetracetic (EDTA) anticoagulant (Vacutainer™ System tubes, Becton-Dickinson, New Jersey, U.S). Plasma, serum and buffy coat were stored at -80°C until analyzed. Genomic DNA was extracted from the buffy-coat using a resin column procedure (Biopur™, BiometrixDiagnostika, Curitiba, Brazil), following the manufacturer's recommendations. The DNA concentration was measured with a NanoDrop 2000c spectrophotometer (Thermo-Scientific, Waltman, MA, USA) at 260 nm and purity was assessed by measuring the 260/280 nm ratio. Real-time polymerase chain reaction (qPCR) with the TaqMan™(Thermo Fisher Scientific, Waltham, Massachusetts, EUA) method was used to genotype the *CTLA4* rs231775(+49A>G) and *CTLA4* rs231779(+1822C>T) variants. The level of fluorescence of the qPCR products was evaluated using the Quantum Studio V™ Thermal Cycler (Applied Biosystems, Thermo Fisher Scientific, Foster City, CA, USA). Negative control (without genom ic DNA) was included in all qPRT performed.

Statistical analysis

The categorical data were expressed as absolute number (n) and percentage (%) and evaluated by chi-square ($\chi 2$) test. Continuous data were expressed as median and interquartile range (IQR: 25%–75%) and evaluated by Mann–Whitney test. The odds ratio (OR) and 95% confidence interval (95% CI) were calculated. Associations between UBC susceptibility or different prognostic settings with the genetic variants were tested in five different genetic models: allelic, codominant, dominant, recessive and overdominant. The p value was adjusted for multiple variables (age, sex, ethnicity, and smoking) by binary logistic or multinomial regression test, when appropriate. Nagelkerke values were used as pseudo R^2 effect sizes and accuracy with sensitivity and specificity were computed in all regression analyses.

The sample size was calculated for a statistical power of 80% and statistical significance with $p< 0.05$, and with the aim of identifying a statistical difference in the variables between the groups of at least 10% (GPower software v.3.1.9.4).All statistical analyzes were performed with SPSS for Windows, version 22.0 (SPSS 31 Inc., CHICADO, IL, USA) and statistical significance was set at $p < 0.05$.

RESULTS

Characteristics of the subjects

Table 1 shows that UBC patients and controls did not differ regarding the sex (p=0.172), ethnicity (p=0.617), and body mass index (BMI) (p=0.852). However, UBC patients were older (p<0.001), mostly smokers (p<0.001), with greater waist circumference (p=0.005), as well as systolic and diastolic arterial pressure (p=0.006 and p<0.001, respectively). Most patients used antihypertensive drugs (p<0.001), but a minority of them used hypocholesterolemic and hypoglycemic drugs (both with p<0.001). Of the 140 patients with UBC, 91(65.0%) had a history of cancer in the family, 109(77.9%) did not practice physical activity, 123(87.9%) did not have depression, 110(78.6%) without diabetes and 77(55.0%) had systemic arterial hypertension. Other data were shown in Table 2.

Comparison between groups for different genetic models

Associations with UBC susceptibility or different prognostic settings were tested in five different genetic models for the two SNVs genotyped, such as allelic, codominant, dominant, recessive and overdominant. All the analyses were adjusted by age, sex, ethnicity, and smoking, and are presented in Table 3.Differences were found between the UBC susceptibility for the heterozygous genotypes of *CTLA4* rs231775(OR=0.40; p=0.045) and rs231779 (OR=0.35; p=0.024) variants in the overdominant models. Patients carrying the AG genotype of the rs23177 A>G and those carrying the CT genotype of the rs231779 C>T showed less chance of presenting UBC, considering the protective effect in the overdominant model observed in both SNVs. The other groups did not differ regarding the allelic and genotype frequency of *CTLA4* rs231775 and rs231779 variants (Table 3).

Regarding to different prognostic settings, such as NMIBC and MICB, without and with metastasis, without and with recurrence, with low/moderate risk and high/very high risk, no difference was observed between the genotype frequency of *CTLA4* rs231775 and rs231779 variants in all genetic models. Moreover, no difference was found between the frequency of *CTLA4* rs231775 and rs231779 genotypes in all genetic models when UBC patients who used BCG were compared with those who did not use BCG therapy (data not shown)

Model´s prediction to evaluation of the susceptibility

A regression model was build considering the overdominant*CTLA4*genetic models for the two SNVs variants evaluated (rs231775 and rs231779), age and smoking. This model was able to explain 89.1% of UBC compared to only 50.7% without these parameters (Table 4). Nagelkerke's R^2 shows that the build model explains about 77.0% (rs231775) and 77.2% (rs231779) of the susceptibility to UBC. About this, we can demonstrate that even with age and smoking showing greater influence in this model [Exp(B) of 1.27 and 6.30 respectively for rs231775 and 1.27 and 6,10 for rs231779]. Logistic regression model analysis was also performed in UBC patients with recurrence after first treatment versus without recurrence; however, no significant differences were identified in the models tested (data not shown).

DISCUSSION

The main finding of the present study was that the *CTLA4*rs231775 (A>G) and rs231779 (C>T) genetic variants, in the overdominant model, patients carrying the heterozygous genotype for both SNVs (AG and CT, respectively) showed lowered chance to present UBC compared with patients with other genotypes. These results remained significant after adjusting for sex, age, BMI and smoking. In other words, patients carrying the homozygous genotypes for the major and minor alleles for the two SNVs, rs 231775 (AA +GG) and for rs231779 (CC+TT) and showed higher chance of presenting UBC when compared to controls, being considered a possible risk factor for the presence of UBC. In addition, it was shown that the predictor model composed of the homozygous genotypes of the two *CTLA4* genetic variants, associated with age and smoking, can explain the disease in 89.1% of cases.However, at different prognostic conditions which UBC patients were stratified, no significant difference was found in any of the genetic models evaluated, possibly demonstrating that the role of the *CTLA4* variants (rs231775 and rs231779) would be more associated with the disease initiating process and not with the prognosis.

One of the most important defense mechanisms of the organism against tumor cells comes from the immune system. Two immune pathways that may be involved with UBC are those mediated by CTLA-4 and PD-1. T cells play this role by recognizing neoantigens from tumor cells and thereby generating their elimination.Several mechanisms of failure in this process have been studied; however, the CTLA-4 pathway is still not well understood in this carcinogenic process. CTLA-4 is considered the main participant of the immune system as the checkpoint inhibition pathways, since it prevents potentially autoreactive T cells at the early stage of naïve T cell activation, typically in lymph nodes [32-35]. The PD-1 pathway regulates activated T cells in the effector stages of an immune response, mainly in peripheral tissues [32]. Therefore, it is possible that the polymorphic genes studied here generate an increase in *CTLA4* expression and lead the patient to greater susceptibility to the disease and not to prognosis.Although our study showed an association with *CTLA4* variants, in previous studies with patients with UBC, the rs231775 variant showed divergent results. Wang and coworkers [26], in a case-control study showed that the rs134775 variant is a protective factor for UBC, while two other studies showed that the variant could increase the risk of UBC [27,28].

According to some authors, the involvement of *CTLA4* in cancer may follow two pathophysiological lines. One is preceding the development of cancer, when under conditions of increased expression of *CTLA4*it would act resulting in less anti-tumor effect. The other line is with the cancer already installed, when the excess of *CTLA4* expression would lead to less inflammation, and consequently worsen the patient's prognosis [36-38].

Another hypothesis about the antagonistic effects of CTLA-4 in activating or inhibiting theTCD4^{+}T cell activation response would be through sCTLA-4. As sCTLA-4 is also produced by naive T cells and flCTLA-4 is only expressed after its activation on T cells, at early stages, sCTLA-4 could compete with B7-CTLA-4 binding and thereby lead to activation and amplification of the CD4^{+}T cell response. This counter intuitive discrepancy was also found by Liu and coworkers [39]when observed increased levels of sCTLA-4 in patients with active systemic lupus erythematosus. This mechanism of activation of naïve T cells by sCTLA-4 is not yet well understood, and there may be other pathways of activation or the time at which the immune process began [40-41]. In this hypothesis, there would be an imbalance between sCTLA-4 and flCTLA-4, which could explain, at least in part, our findings of the protective effect of heterozygous genotypes and the high chance of overdominance in both SNVs analyzed.

Isitmangiland coworkers[19], studying *CTLA4* variants (rs231775 and rs5742909) in patients with breast cancer showed no differences in the genotypes of these variants between the patients and control groups. However, there were differences between serum levels of sCTLA-4 and sCD28 between the groups, which could explain the carcinogenic predisposition.

In our study, the results lead us to two hypotheses to explain the observed phenomena: The first, regarding the findings showed in Table 3, where UBC patients and controls were compared, *CTLA4* being more expressed in the microenvironment where cancer was installed, as evidenced by the presence of genetic variants of *CTLA4* rs231775 (A>G) and rs231779 (C>T), would be previously acting as a pro-tumor factor, and that this would lead to an environment more conducive to the development of cancer by leaving the site more vulnerable to adverse conditions, as can occur in an infectious process or other possible aggressions.As the homozygous genotypes of the overdominant model were associated with the presence of UBC, in both genetic variants, and heterozygous genotypes were related to protection, the expression of *CTLA4* could be compromised in these patients, leading to a

greater chance of cancer when homozygosis is present. A possible explanation for this would be that heterozygous individuals, for a given genetic variant, have a significantly greater effect (positive heterosis) or a significantly lower effect (negative heterosis) than homozygous individuals for each allele [42].Interactions between multimeric protein products are believed to be a common source of overdominance, which is predicted to be quite common. In addition, intermediate expression levels may be optimal for specific functions as they tend to address different signaling pathways [43]. In a study of 231775 and rs231779 SNVs, it was found that in heterozygosity of these two variants there were higher levels of sCTLA-4 in breast cancer patients, but in the homozygosity states did not [23].In addition, the tumor cell by expressing transforming growth factor (TGF)-β could stimulate the production of CTLA-4 via Treg cells. However, such mechanisms are not yet well understood [23,44].

The other hypothesis is that environmental factors, such as medication use and comorbidities, would be related to the development of UBC, and may interfere as epigenetic factors and thus override the participation of the SNVs in these patients.It is also noteworthy that in the control group of our study, the prevalence of diabetics using metformin was 40.7% versus 20.0% of patients with UBC. Metformin by mechanisms not yet well understood, activates the enzyme AMP-activated protein kinase (AMPK) that inhibits the expression of genes linked to hepatic gluconeogenesis, amplifies mTOR signaling, activates p53, favors autophagy and apoptosis and decreases the generation of reactive oxygen species (ROS). Thus, benefits related to anti-tumor action are reported as decreased growth, cell survival and metastasis of tumor cells [45,46]. Such facts could justify a protective effect to UBC in individuals of the control group.

After stratifying patients according to prognosis and different clinical contexts, we also no found associations between invasiveness, metastasis, recurrence after first treatment and risk grade with the two *CTLA4*SNVs analyzed. These data are in agreement with the concept that *CTLA4* would be acting predominantly in the lymphnode environment and not peripherally, leading to less influence on the already formed tumor [29-32].

Some limitations of the present study should be considered, such as the cross-sectional design, which does not allow to make causal inferences and the unavailable data of the sCTLA4 plasma measurents of the individuals enrolled in this study. However, it has some strengths, such as that possible confounding variables including sex, age and ethnicity were controlled by the robust statistical analysis.

Taken the results together, we demonstrated that the overdominant model of the *CTLA4* rs231775(+49AG) and rs231779(+1822CT) genetic variants could be used as possible predictors of UBC, especially if associated with age and smoking.

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Authors' contributions

AK: Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing.ANCS: Conceptualization, Validation. Formal analysis. TMA: Methodology (genetic and laboratory analysis). KC de M: Methodology, Validation, Investigation, Data curation. BRE: Methodology, Validation, Investigation, Data curation. RHGG: Methodology, Validation, Investigation, Data curation. DFPY: Methodology, Validation, Investigation, Data curation. GLT: Methodology, Validation, Investigation, Data curation. EMVR: Formal analysis, Writing – review & editing. MABL: Conceptualization, Methodology, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration

Availability of data and materials

All datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The protocol was approved by the University of Londrina's Institutional Study Ethics Committee (CAAE: 170846619.2.0000.5231) and all participants and their guardians were told in full about the research and provided signed informed permission.

Consent for publication

All the authors agree to publish this paper

Competing interests

The authors declare that they have no competing interests.

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Table 1. Clinical characteristics of the patients with urothelial bladder cancer and healthy controls

Characteristics	**Healthy Controls (n=145)**	**UBC Patients (n=140)**	**p value**
Sex Female/Male	56(38.6) / 89(61.4)	47(33.6) / 93(66.4)	0.172
Age (year)	47 (43-54)	71(65-78)	<0.001
Ethnicity Caucasian/Non-Caucasian	119(82.1) / 26(17.9)	118(84.3) / 22(15.7)	0.617
BMI (kg/m^2)	26(24-29)	26(24-29)	0.852
Abdominal circumference (cm)	95(88-102)	98(92-105)	0.005
Systemic arterial pressure (mmHg)	120(110-130)	127(120-140)	0.006
Diatolic arterial pressure (mmHg)	79(70-80)	80(80-80)	<0.001
Tobacco (No/Yes)	124(86.1) / 20(13.9)	51(36.4) / 89(63.6)	<0.001
Anti-hipertensive drugs (No/Yes)	108(74.5) / 37(25.5)	65(46.4) / 75(53.6)	<0.001
Hipocholesterolemic drugs (No/Yes)	86(59.3) / 59(40.7)	120(85.7) / 20(14.3)	<0.001
Hipoglicemiant drugs (No/Yes)	86(59.3) / 59(40.7)	112(80.0) / 28(20.0)	0.001

Continuous data were expressed as median and interquartile range (IQR): 25 and 75%; categorical variables were expressed as absolute number (n) and percentage (%). UBC: urothelial bladder cancer.

Table 2. Clinical characteristics of patients with urothelial bladder cancer patients.

Cancer-associated characteristics	**UBC Patients (n=140)**
Family history of cancer (No/Yes)	49(35.0) / 91(65.0)
Physicalactivity (No/Yes)	109(77.9) / 31(22.1)
Other disease/medication	
Depression (No/Yes)	123(87.9) / 17(12.1)
Anti-depressive drugs (No/Yes)	119(85.0) / 21(15.0)
Sistemic Arterial Hypertension (No/Yes)	63(45.0) / 77(55.0)
Anti-hipertensive drugs (No/Yes)	65(46.4) / 75(53.6)
Hipocholesterolemic drugs (No/Yes)	120(85.7) / 20(14.3)
Diabetes (No/Yes)	110(78.6) / 30(21.4)
Hypoglycemicdrugs(No/Yes)	112(80.0) / 28(20.0)
NonSteroidal anti-inflammatory drugs (No/Yes)	133(95.0) / 7(5.0)
Infection on the collection data (No/Yes)	129(92.1) / 11(7.9)
Other Comorbidities (No/Yes)	92(65.7) / 48(34.3)
Cancer Characteristics	
Metastasis (No/Yes)	134(95.7) / 6(4.3)
Chemotherapy (No/Yes)	113(80.7) / 27(19.3)
Radioterapy (No/Yes)	132(94.3) / 8(5.7)
BCG (No/Yes)	99(70.7) / 41(29.3)
Stage <or =pT1 or>pT1(No/Yes)	119(85.0) / 21(15.0)
Tumor Grade (Low/High)	65(46.4) / 75(53.6)
Risk (Low/Intemediary/High)	55(39.3) / 13(9.3) / 72(51.4)
Recurrence (No/Yes)	59(44.7) / 73(55.3)
Age at diagnosis (year)	68 (61-74)

Data were expressed as median and interquartile range (IQR): 25% and 75%. Categorical variables were expressed as absolute number (n) and percentage (%).

BCG: Bacillus de Calmette-Guérin; pT1: pathological tumor 1 stage.

Table 3. *CTLA4*allelic and genotypedistribution among patients with urothelial bladder cancer and healthy controls in different genetic models

HC (n= 145)		**UBC (n= 140)**	**p value ***	**OR (CI 95%)**	**HC (n= 145)**		**UBC (n= 140)**	**p value***	**OR (CI 95%)**
***CTLA4*A>G (rs231775)**					***CTLA4C*>T(rs231779)**				
Allelic					Allelic				
A	173 (59.6)	169 (60.7)			C	174 (60.0)	169 (60.7)		
G	117 (40.4)	111 (39.3)	-	-	T	116 (40.0)	111 (39.3)	-	-
Codominant					Codominant				
A A	47 (32.4)	54 (38.6)	Ref.	Ref.	CC	48 (33.1)	54 (38.6)	Ref.	Ref.
A G	79 (54.5)	62 (44.3)	0.061	0.40 (0.15-1.04)	CT	78 (53.8)	61 (43.6)	0.046	0.37 (0.14-0.98)
G G	19 (13.1)	24 (17.1)	0.947	0.95 (0.25-3.55)	TT	19 (13.1)	25 (17.9)	0.766	1.22 (0.32-4.66)
Dominant					Dominant				
AG+ GG	98 (67.6)	86 (61.4)	Ref.	Ref.	CT+TT	97 (66.9)	86 (61.4)	Ref.	Ref.
AA	47 (32.4)	54 (38.6)	0.124	2.00 (0.02-4.87)	CC	48 (33.1)	54 (38.6)	0.126	1.99 (0.82-4.85)
Recessive					Recessive				
AA+ AG	126 (86.9)	116 (82.9)	Ref.	Ref.	CC+CT	126 (86.9)	115 (82.1)	Ref.	Ref.
GG	19 (13.1)	24 (17.1)	0.456	1.57 (0.47-	TT	19 (131)	25 (17.9)	0.243	2.08 (0.60-7.11)

				5.24)					
Overdominant					Overdominant				
AA+ GG	66 (45.5)	78 (55.7)	Ref.	Ref.	CC+TT	67 (46.2)	79 (56.4)	Ref.	Ref.
AG	79 (54.5)	62 (44.3)	0.045	0.40 (0.16-0.98)	CT	78 (53.8)	61 (43.6)	0.024	0.35(0.14-0.87)

Categorical variables were expressed by absolute number (n) and percentage (%).*Adjusted by age, sex, ethnicity, and smoking. HC: healthy controls; UBC: urothelial bladder cancer; OR: Odds ratio; Ref: Reference; CI: Confidence Interval.

Table 4: Binary logistic regression model between *CTLA4* variants (rs2317775 and rs2317779) and lifestyle (smoking) in patients with urothelial bladder cancer and healthy controls

Model	**Healthy Control (n=145)**	**UBC Patients (n=140)**	**p value**	**Exp (B)**	**95% CI**	**p value**	**R^2Nagel kerke**
CTLA4							
rs2317775 Overdominant (GG+AA)	66 (45.5) / 79 (54.5)	78 (55.7) / 62 (44.3)	0.026	2.64	1.12-6.20	<0.001	0.770
Age (year)	47 (43-54)	71 (65-78)	<0.001	1.27	1.20-1.34		
Smoking (No/Yes)	124 (86.1) / 20 (13.9)	51 (36.4) / 89 (63.6)	<0.001	6.30	2.29-17.30		
CTLA4							
rs231779 Overdominant (CC+TT)	66 (45.5) / 79 (54.5)	78 (55.7) / 62 (44.3)	0.015	2.92	1.23-6.94	<0.001	0.772
Age (year)	47 (43-54)	71 (65-78)	<0.001	1.27	1.20-1.34		
Smoking (No/Yes)	124 (86.1) / 20 (13.9)	51 (36.4) / 89 (63.6)	<0.001	6.10	2.78-17,33		

Continuous variables were expressed as median and interquartile range (IQR) 25%-75%; absolute variables were expressed as number (n) and percentage (%).*Adjusted by age, sex, ethnicity, and smoking. HC: healthy controls; UBC: urothelial bladder cancer; OR: Odds ratio.; CI: Confidence Interval.

6. CONCLUSÕES

Artigo 1

A carcinogênese no câncer de bexiga se relaciona intimamente ao processo imunológico, fatores ambientais e genéticos. Variantes genéticas de nucleotídeo único de citocinas são participantes essenciais, mas não definitivos. A identificação dessas alterações pode fornecer dados importantes na prevenção, escolha de tratamento e prognóstico da doença.

Na revisão da literatura, encontramos 36 estudos caso-controle de variantes de citocinas relacionadas ao CUB. As variantes genéticas de *IL27*(rs153109), *IL6*(rs1800795), *IFNG*(rs62559044), *IL2*(rs2069762) e *IL12*(rs3212227), cujas expressões dessas variantes tendem a diminuir a produção dessas citocinas, mostraram aumento de risco e recorrência ao CUB. As variantes genéticas de *TNFA*(rs1800629, rs1799724 e rs1799964), *IL22*(rs2227485), *IL31*(rs4758680), *IL32*(rs12934561 e rs28372698), *TGFB*(rs1800470 e rs1800471), *TGFBR1*(rs868), *IL4*(rs2243248 e rs2243250), *IL8*(rs4073), *IL10*(rs1800896 e rs1800871), *IL17*(rs2275913, rs763780), *IL23R*(rs10889677 e rs188444), *IL18*(rs187283 e rs1946518) e *IL13*(rs1800925), cujas expressões dessas variantes tendem a elevar a produção dessas citocinas, mostraram aumento ao risco, da recorrência e da progressão tumoral ao CUB. As variantes genéticas do *IL1*(rs1143634 e rs16944), estudadas em 2 artigos, mostraram resultados divergentes em relação ao risco ao CUB.

Artigo 2

Não encontramos associação das variantes genéticas rs231775 e rs231779 do *CTLA4* com a invasividade, presença de metástases, recorrência pós cirúrgica ou grau tumoral. Porém, foi evidenciado que o modelo overdominante da variante do gene *CTLA4* (rs231775 e rs231779) associados à idade e à presença do tabagismopossa servir de marcador de aumento de suscetibilidade para o CUB.

7 CONSIDERAÇÕES FINAIS

Consideramos como pontos fortes do nosso trabalho o fato de ser o primeiro descrito na população brasileira que retrata as variantes genéticas rs231775 e

rs234779 relacionados com o CUB, além de propomos um modelo preditor para a suscetibilidade ao CUB. O fato do trabalho ser um estudo caso-controle e com isso caracterizar uma prevalência e não uma incidência é um fator limitador deste estudo.

O CUB é um tumor que, diagnosticado e tratado precocemente, tem excelente prognóstico. Entretanto, com o aumento da idade populacional no mundo, sua incidência vem aumentando principalmente em países em desenvolvimento onde as condições da saúde pública são precárias e o diagnóstico em fases mais tardias são comuns. Nesta fase, o custo do tratamento é elevado, de alta morbidade e, apesar dos avanços nos últimos anos, o aumento da taxa de sobrevida permanece um desafio. Neste aspecto o conhecimento de fatores genéticos que possam predizer o diagnóstico e prognosticar melhor a escolha de tipos de tratamentos são ferramentas cruciais não só pelos aspectos individuais para com os doentes, mas para políticas públicas que visem atuar nesses grupos específicos de pessoas predispostas e para os doentes em tratamento.

O campo do conhecimento da Imunologia e da Genética vem tendo um grande destaque nos últimos anos. O conhecimento gerado entre o melhor entendimento da fisiopatologia e da atuação do sistema imune no câncer, da melhora tecnológica e da acessibilidade de novas análises genéticas, leva a perspectiva de que possamos atuar melhor e especificamente nos indivíduos predispostos. Não só pelas mudanças comportamentais comprovadamente sabidas, como o tabagismo e outros fatores ambientais e carcinogênicos, mas atuar nos grupos geneticamente suscetíveis. Além disso, dilemas éticos deverão ser cada vez mais discutidos dada a possibilidade de manipulaçãoes genéticas em mutações gene específicas.

Em nosso tabalho, mostramos que os aspectos genéticos podem ter relação com a suscetibilidade ao CUB e que a avaliação de biomarcadores genéticos pode auxiliar na conduta clínica. Neste caso, a interação com fatores ambientais, como a exposição ao tabagismo e a idade, em associação com estes biomarcadores genéticos, se mostrou interessante para associar com a suscetibilidade ao CUB. Mais estudos, em diferentes desenhos de pesquisa, podem resultar em entendimento mais claro destes desfechos, e devem ser explorados para melhor compreensão da fisiopatologia e da participação de SNVs no CUB.

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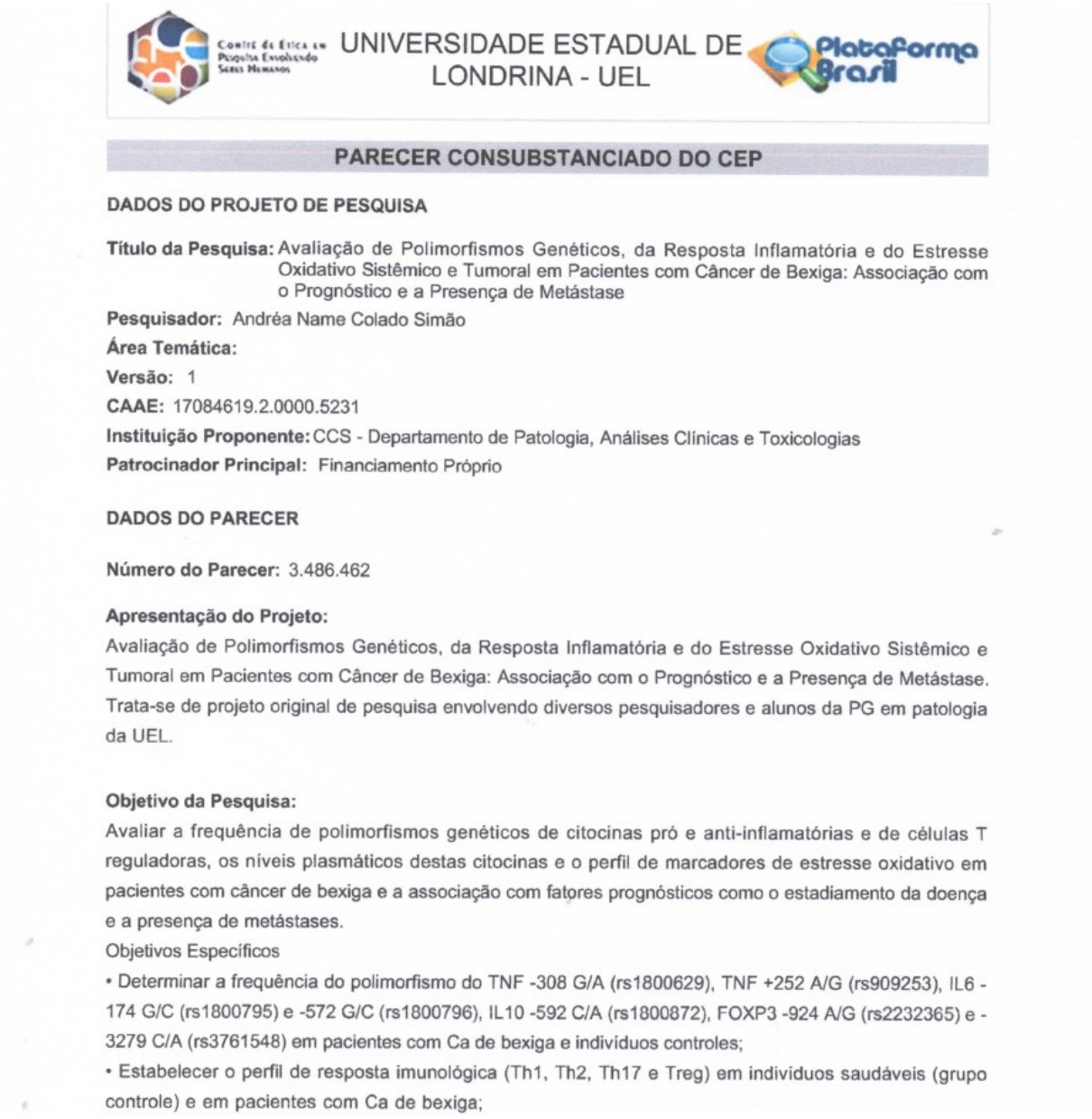
9. ANEXOS

9.1 Parecer Consubstanciado do CEP



UNIVERSIDADE ESTADUAL DE LONDRINA - UEL

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Avaliação de Polimorfismos Genéticos, da Resposta Inflamatória e do Estresse Oxidativo Sistêmico e Tumoral em Pacientes com Câncer de Bexiga: Associação com o Prognóstico e a Presença de Metástase

Pesquisador: Andréa Name Colado Simão

Área Temática:

Versão: 1

CAAE: 17084619.2.0000.5231

Instituição Proponente: CCS - Departamento de Patologia, Análises Clínicas e Toxicologias

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 3.486.462

Apresentação do Projeto:

Avaliação de Polimorfismos Genéticos, da Resposta Inflamatória e do Estresse Oxidativo Sistêmico e Tumoral em Pacientes com Câncer de Bexiga: Associação com o Prognóstico e a Presença de Metástase. Trata-se de projeto original de pesquisa envolvendo diversos pesquisadores e alunos da PG em patologia da UEL.

Objetivo da Pesquisa:

Avaliar a frequência de polimorfismos genéticos de citocinas pró e anti-inflamatórias e de células T reguladoras, os níveis plasmáticos destas citocinas e o perfil de marcadores de estresse oxidativo em pacientes com câncer de bexiga e a associação com fatores prognósticos como o estadiamento da doença e a presença de metástases.

Objetivos Específicos

• Determinar a frequência do polimorfismo do TNF -308 G/A (rs1800629), TNF +252 A/G (rs909253), IL6 -174 G/C (rs1800795) e -572 G/C (rs1800796), IL10 -592 C/A (rs1800872), FOXP3 -924 A/G (rs2232365) e -3279 C/A (rs3761548) em pacientes com Ca de bexiga e indivíduos controles;

• Estabelecer o perfil de resposta imunológica (Th1, Th2, Th17 e Treg) em indivíduos saudáveis (grupo controle) e em pacientes com Ca de bexiga;

• Avaliar a associação entre os polimorfismos genéticos de citocinas pró e anti-inflamatórias e os níveis plasmáticos e teciduais de citocinas, os marcadores inflamatórios (usPCR, VHS e ferritina) e de estresse oxidativo;
• Verificar se há associação entre a presença dos polimorfismos genéticos avaliados, dos níveis plasmáticos de citocinas e os marcadores de EO com o estadiamento da doença;
• Verificar se há associação entre a presença dos polimorfismos genéticos avaliados, dos níveis plasmáticos de citocinas e os marcadores de EO com a presença de metástases;
• Propor modelos preditores de diagnóstico e prognóstico utilizando a combinação de biomarcadores genéticos, imunológicos e de estresse oxidativo.

Avaliação dos Riscos e Benefícios:

Riscos:

A coleta de sangue pode ocasionar sinais decorrentes da punção venosa e consiste: dor no local da punção venosa ou pequeno hematoma e, muito raramente, vermelhidão ou infecção local. Mesmo sendo mínimos, caso ocorra algum tipo de desconforto o participante será prontamente atendido e amparado pelos farmacêuticos responsáveis pela coleta de sangue e um dos pesquisadores deste estudo.

Benefícios:

Este estudo contribuirá para o conhecimento das frequências de vários polimorfismos genéticos em pacientes com Ca de bexiga. Espera-se com este estudo contribuir para o entendimento dos mecanismos fisiopatológicos envolvidos no desenvolvimento e atividade da doença, assim como propor preditores de diagnóstico e progressão da doença.

Por fim, a avaliação do envolvimento de genes relacionados à citocinas pró e anti-inflamatórias, a identificação do perfil de citocinas plasmáticas e teciduais e do desequilíbrio redox em pacientes com Ca de bexiga, poderá contribuir para o melhor entendimento da fisiopatologia da doença, possibilitando a identificação de novos biomarcadores e alvos terapêuticos.

Comentários e Considerações sobre a Pesquisa:

Projeto construído corretamente. Nos riscos, porém, não está prevista a possibilidade do vazamento dos dados coletados, ou quais as medidas preventivas (ou paleativas) a serem tomadas neste caso.

No quesito orçamento, os pesquisadores estão responsáveis pelos gastos, ao passo que o material de consumo a ser utilizado já existe, a partir de projetos financiados prévios.

Considerações sobre os Termos de apresentação obrigatória:

O projeto apresenta folha de rosto, e TCLE de forma correta, a declaração da instituição co-participante - Hospital de Câncer de Londrina), os termos de confidencialidade e sigilo relativos aos participantes, e um termo de sigilo da guarda dos dados. Apresentou também Declaração de Manuseio Material Biológico / Biorepositório / Biobanco conforme as normas vigentes da UEL.

Recomendações:

aprovar

Conclusões ou Pendências e Lista de Inadequações:

aprovar

Considerações Finais a critério do CEP:

Prezado(a) Pesquisador(a),

Este é seu parecer final de aprovação, vinculado ao Comitê de Ética em Pesquisas Envolvendo Seres Humanos da Universidade Estadual de Londrina. É sua responsabilidade apresenta-Lo aos órgãos e/ou instituições pertinentes.

Ressaltamos, para início da pesquisa, as seguintes atribuições do pesquisador, conforme Resolução CNS 466/2012 e 510/2016:

A responsabilidade do pesquisador é indelegável e indeclinável e compreende os aspectos éticos e legais, cabendo-lhe:

- conduzir o processo de Consentimento e de Assentimento Livre e Esclarecido;
- apresentar dados solicitados pelo sistema CEP/CONEP a qualquer momento;
- desenvolver o projeto conforme delineado, justificando, quando ocorridas, a sua mudança ou interrupção;
- elaborar e apresentar os relatórios parciais e final;
- manter os dados da pesquisa em arquivo, físico ou digital, sob sua guarda e responsabilidade, por um período mínimo de 5 (cinco) anos após o término da pesquisa;
- encaminhar os resultados da pesquisa para publicação, com os devidos créditos aos pesquisadores e pessoal técnico integrante do projeto;
- justificar fundamentadamente, perante o sistema CEP/CONEP, interrupção do projeto ou a não publicação dos resultados.

Coordenação CEP/UEL.

UNIVERSIDADE ESTADUAL DE LONDRINA - UEL

Continuação do Parecer: 3.486.462

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1392498.pdf	05/07/2019 22:13:22		Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE.doc	05/07/2019 22:04:45	Andréa Name Colado Simão	Aceito
Projeto Detalhado / Brochura Investigador	Projeto.docx	05/07/2019 22:04:38	Andréa Name Colado Simão	Aceito
Declaração de Pesquisadores	confidencialidadesigilo.pdf	05/07/2019 22:01:33	Andréa Name Colado Simão	Aceito
Outros	sigilodados.pdf	05/07/2019 22:01:06	Andréa Name Colado Simão	Aceito
Declaração de Manuseio Material Biológico / Biorepositório / Biobanco	armazenamentomaterialbiologico.pdf	05/07/2019 22:00:38	Andréa Name Colado Simão	Aceito
Declaração de Instituição e Infraestrutura	HospitalCancerLondrina.pdf	05/07/2019 22:00:24	Andréa Name Colado Simão	Aceito
Folha de Rosto	folhaderosto.pdf	05/07/2019 21:58:10	Andréa Name Colado Simão	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

LONDRINA, 05 de Agosto de 2019

Assinado por:
Osvaldo Coelho Pereira Neto
(Coordenador(a))

9.2 Declaração da Instituição Colaboradora do Hospital do Câncer de Londrina

Hospital do Câncer de Londrina

DECLARAÇÃO

Declaro para os devidos fins, que o Hospital do Câncer de Londrina é colaborador no Projeto de pesquisa com o tema "***Avaliação de polimorfismos genéticos e do estresse oxidativo sistêmico e tumoral em pacientes com câncer de bexiga***" **que será conduzido pelo membro da equipe de Urologia HCL, Dr. Alexsandro Koike.**

Este estudo será caso-controle, no qual serão selecionados 200 pacientes com diagnóstico de Câncer de bexiga confirmado por biópsia, recrutados pelo ambulatório de Urologia do Hospital do Câncer de Londrina e 200 indivíduos controles selecionados entre pacientes que compareceram para o preventivo de rotina em consultório particular, pareados pelo sexo, idade e etnia.

O presente estudo será submetido ao Comitê de Ética em Pesquisa de Seres Humanos da Universidade Estadual de Londrina e será iniciado nesta Instituição somente após aprovação deste CEP.

Os **resultados do projeto devem ser encaminhados ao Hospital do Câncer de Londrina.**

O Hospital do Câncer de Londrina cumpre a resolução 466/2012 e demais Resoluções vigentes.

Sem mais nada a declarar, nos colocamos a disposição para quaisquer esclarecimentos que se fizerem necessários.

Londrina, 12 de junho de 2019.

Dr. Marcos Silveira Lapa
Diretor Clínico do HCL
CRM- PR: 17971
Dr. Marcos Silveira Lapa
Diretor Clínico - CRM/PR 17.971
Hospital do Câncer de Londrina

Enf. Elaine Cristina Baraldi Carmelo
Coordenadora da UPC HCL
COREN-PR 283 045
Elaine C. B. Carmelo
COREN-PR 283045
Diretoria Clínica Pesquisa Clínica e Residência Médica - Coordenação

Rua Lucilla Ballalai, 212 – Jd Petrópolis - CEP 86.015-520 – Londrina - PR
Fones: (43) 3379-2608
Home Page: **www.hcl.org.br**

10. APÊNDICE

10.1 Ficha de Coleta

<table>
<tr><td colspan="5">Data da coleta de dados: / / 20
Responsável pela coleta:</td></tr>
<tr><td colspan="5">1 – Nome do paciente:</td></tr>
<tr><td colspan="5">2 – Telefone:</td></tr>
<tr><td>3 – Registro ICL:</td><td>4 – DN:</td><td>5 – Idade:</td><td>6 – Peso:</td><td>7 – Atura:</td></tr>
<tr><td colspan="5">8 – Etnia: ( )Caucasiano ( )Não caucasiano ( )Asiático</td></tr>
<tr><td colspan="2">9 – Circunferência abdominal:</td><td colspan="2">10 – IMC:</td><td>11 - P.A.:</td></tr>
<tr><td colspan="5">12 – Histórico familiar de CA: ( )Sim, qual? Parentesco? ( )Não</td></tr>
<tr><td colspan="5">13 – Fez cirurgia nos últimos 3 meses? ( )Sim, quando? Que tipo? ( )Não</td></tr>
<tr><td colspan="5">17 – Depressão? ( ) Sim, diagnóstico por psiquiatra? ( )Não</td></tr>
<tr><td colspan="5">18 – Infecção na data da coleta? ( )Sim, qual? ( )Não</td></tr>
<tr><td colspan="5">19 – Diabetes? ( )Sim ( )Não</td></tr>
<tr><td colspan="5">20 – Hipertensão? ( )Sim ( )Não</td></tr>
<tr><td colspan="5">21 – Uso de medicamentos no momento da coleta?
Uso contínuo:_____
Não contínuo:_____</td></tr>
<tr><td colspan="5">22 – Tabagismo? ( )Sim ( )Não ( )Ex-fumante
Quanto tempo:
Período:</td></tr>
<tr><td colspan="5">23 – Atividade Física? ( )Sim, frequência? ( )Não
Quantos dias na semana?</td></tr>
<tr><td colspan="5">24 – Outras doenças:</td></tr>
<tr><td colspan="5">25 – Data do diagnóstico de CaB:</td></tr>
</table>

26 – Tratamento:

Cirurgia ( )Sim ( )Não

Recorrência: ( )Sim, data do primeiro diagnóstico. ( )Não

Radioterapia ( )Sim ( )Não

Qt ( )Sim ( )NãoData de início:Qual:

BCG ( )Sim ( )Não

Parâmetro	**Resultado**
HEMOGRAMA:	
Hemácia (HE)	
Hemoglobina (Hgb)	
Hematocrito (HT)	
RDW	
Leucócitos totais (WBC)	
NEUTROFILO	
LINFOCITO	
EOSINOFILO	
MONOCITO	
Plaquetas	
VELOCIDADE DE HEMOSSEDIMENTAÇÃO (VHS)	

10.2 Termo de Consentimento Livre e Esclarecido

Termo de Consentimento Livre e Esclarecido

"Avaliação de

morfismos Genéticos, da Resposta Inflamatória e do Estresse Oxidativo Sistêmico e Tumoral em Pacientes com Câncer de Bexiga: Associação com o Prognóstico e a Presença de Metástase"

Prezado(a) Senhor(a):

Gostaríamos de convidá-lo (a) para participar da pesquisa "Avaliação de Polimorfismos Genéticos, da Resposta Inflamatória e do Estresse Oxidativo Sistêmico e Tumoral em Pacientes com Câncer de Bexiga: Associação com o Prognóstico e a Presença de Metástase", a ser realizada no Ambulatório de Urologia do Hospital do Câncer de Londrina. O objetivo da pesquisa éestudar os mecanismos imunológicos, genético e de estresse oxidativo envolvidos no Câncer de bexiga. Sua participação é muito importante e ela se daria da seguinte forma realização de uma avaliação clínica pelo médico urologista e uma coleta de sangue. Esclarecemos que sua participação é totalmente voluntária, podendo você: recusar-se a participar, ou mesmo desistir a qualquer momento, sem que isto acarrete qualquer ônus ou prejuízo à sua pessoa. Esclarecemos, também, que suas informações serão utilizadas somente para os fins desta pesquisa e serão tratadas com o mais absoluto sigilo e confidencialidade, de modo a preservar a sua identidade. Todos os dados coletados, clínicos e laboratoriais, serão descartados após a publicação do estudo.

Esclarecemos ainda, que você não pagará e nem será remunerado(a) por sua participação. Garantimos, no entanto, que todas as despesas decorrentes da pesquisa serão ressarcidas, quando devidas e decorrentes especificamente de sua participação.

A sua participação neste estudo contribuíra para o melhor entendimento dos mecanismos fisiopatológicos envolvidos no desenvolvimento da doença. Quanto aos riscos,informamos que sua participação não acarretará qualquer risco à sua saúde nem alteração de qualquer um dos seus tratamentos. A coleta de sangue pode ocasionar sinais decorrentes da punção venosa e consiste: dor no local da punção venosa ou pequeno hematoma e, muito raramente, vermelhidão ou infecção local. Mesmo sendo mínimos, caso ocorra algum tipo de desconforto o participante será prontamente atendido e amparado pelos farmacêuticos responsáveis pela coleta de sangue e um dos pesquisadores deste estudo.

Caso você tenha dúvidas ou necessite de maiores esclarecimentos poderá nos contatar **(Andréa Name Colado Simão, Avenida Robert Koch 60, telefone: 3371-2321, 99627-8181, deianame@yahoo.com.br),** ou procurar o Comitê de Ética em Pesquisa Envolvendo Seres Humanos da Universidade Estadual de Londrina, situado junto ao prédio do LABESC – Laboratório Escola, no Campus Universitário, telefone 3371-5455, e-mail: cep268@uel.br.

Este termo deverá ser preenchido em duas vias de igual teor, sendo uma delas devidamente preenchida, assinada e entregue a você.

Londrina, ___ de _____de 20__.

Pesquisador Responsável

Profa Dra. Andréa Name Colado Simão

RG: 6.226.736-4

Tel: 3371-2321 / 99627-8181

Eu, _____ (colocarnome por extenso do participante da pesquisa), tendo sido devidamente esclarecido sobre os procedimentos da pesquisa, concordo em participar voluntariamente da pesquisa descrita acima.

Assinatura (ou impressão dactiloscópica):_____

Data:_____

*Termo de Consentimento Livre Esclarecido apresentado conforme normas da Resolução 466/2012 de 12 de dezembro de 2012.

10.3 Termo de confidencialidade e Sigilo

TERMO DE CONFIDENCIALIDADE E SIGILO

Eu, Andréa Name Colado Simão, **Brasileira, Casada, Bioquímica,** inscrito(a) no CPF/ MF sob o nº **014.336.329-85**, abaixo firmado, assumo o compromisso de manter confidencialidade e sigilo sobre todas as informações técnicas e outras relacionadas ao projeto de pesquisa intitulado **Avaliação de Polimorfismos Genéticos, da Resposta Inflamatória e do Estresse Oxidativo Sistêmico e Tumoral em Pacientes com Câncer de Bexiga: Associação com o Prognóstico e a Presença de Metástase**" a que tiver acesso nas dependências do Hospital Universitário De Londrina Da Universidade Estadual De Londrina.

Por este termo de confidencialidade e sigilo comprometo-me a:

1. não utilizar as informações confidenciais a que tiver acesso, para gerar benefício próprio exclusivo e/ou unilateral, presente ou futuro, ou para o uso de terceiros;

2. não efetuar nenhuma gravação ou cópia da documentação confidencial a que tiver acesso;

3. não me apropriar de material confidencial e/ou sigiloso que venha a ser disponível;

4. não repassar o conhecimento das informações confidenciais, responsabilizando-me por todas as pessoas que vierem a ter acesso às informações, por meu intermédio, e obrigando-me, assim, a ressarcir a ocorrência de qualquer dano e/ou prejuízo oriundo de uma eventual quebra de sigilo das informações fornecidas.

Neste Termo, as seguintes expressões serão assim definidas:

Informação Confidencial significará toda informação revelada ou cedida pelo participante da pesquisa, a respeito da pesquisa, ou associada à Avaliação de seus dados, sob a forma escrita, verbal ou por quaisquer outros meios. Avaliação significará todas e quaisquer discussões, conversações ou negociações entre, ou com as partes, de alguma forma relacionada ou associada com o desenvolvimento da pesquisa.

Informação Confidencial inclui, mas não se limita, à dados pessoais, informação relativa à operações, processos, planos ou intenções, informações sobre produção, instalações, equipamentos, segredos de negócio, segredo de fábrica, dados, habilidades especializadas, projetos, métodos e metodologia, fluxogramas, especializações, componentes, fórmulas, produtos, amostras, diagramas, desenhos de esquema industrial, patentes, oportunidades de mercado e questões relativas a negócios.

Pelo não cumprimento do presente Termo de Confidencialidade e Sigilo, fica o abaixo assinado ciente de que sanções judiciais poderão advir.

Londrina, 05 de julho de 2019.

Profa. Dra.Andréa Name Colado Simão

10.4 Declaração de Armazenamento de Material Biológico

CENTRO DE CIÊNCIAS DA SAÚDE, DEPARTAMENTO DE PATOLOGIA, ANÁLISES CLÍNICAS E TOXICOLÓGICAS

Ilmo. Sr.
PROF. DR. OSVALDO COELHO PEREIRA NETO
COORDENADOR DO COMITE DE ÉTICA EM PESQUISA ENVOLVENDO SERES HUMANOS - UEL

DECLARAÇÃO

Eu, professor professora Dra. Andréa Name Colado Simão docente adjunto, 40horas/semanais, com TIDE, lotada no departamento de Patologia, Análises Clínicas e Toxicológicas do Centro de Ciências da Saúde da Universidade Estadual de Londrina, declaro para o Comitê de Ética em Pesquisa Envolvendo Seres Humanos –CEP/UEL- que me comprometo a seguir todas as recomendações apresentadas na Resolução CEPE/CA nº052/2017 que regulamenta o armazenamento de material biológico humano em biorrepositório para finalidade de pesquisa, que coordenarei e que está em avaliação por este Comitê na presente data com o título "**Avaliação de Polimorfismos Genéticos, da Resposta Inflamatória e do Estresse Oxidativo Sistêmico e Tumoral em Pacientes com Câncer de Bexiga: Associação com o Prognóstico e a Presença de Metástase**".

Londrina, 05 de julho de 2019

Atenciosamente,

Profa. Dra. Andréa Name Colado Simão

10.5 Declaração de Armazenamento de Dados

UNIVERSIDADE ESTADUAL DE LONDRINA
CENTRO DE CIÊNCIAS DA SAÚDE, DEPARTAMENTO DE PATOLOGIA,
ANÁLISES CLÍNICAS E TOXICOLÓGICAS

Ilma. Sra.
Profa. Dr. OSVALDO COELHO PEREIRA NETO
COORDENADORA DO COMITE DE ÉTICA EM PESQUISA ENVOLVENDO SERES HUMANOS - UEL

D E C L A R A Ç Ã O

Eu, professora Dra Andréa Name Colado Simão, docente do Centro de Ciências da Saúde (CCS) da Universidade Estadual de Londrina (UEL), declaro para o Comitê de Ética em Pesquisa Envolvendo Seres Humanos – CEP/UEL- que me comprometo a conservar por tempo indeterminado, com o devido sigilo, os dados oriundos da coleta de dados com os participantes do presente protocolo de pesquisa que coordenarei, e que está em avaliação por este Comitê, intitulada **"Avaliação de Polimorfismos Genéticos, da Resposta Inflamatória e do Estresse Oxidativo Sistêmico e Tumoral em Pacientes com Câncer de Bexiga: Associação com o Prognóstico e a Presença de Metástase"**, em conformidade com a Resolução CNS 466/2012.

Declaro, ainda, que os referidos dados serão utilizados apenas e tão somente em futuras publicações decorrentes da pesquisa que ora tramita.

Londrina, 05 de julho de 2019.

Atenciosamente,

Profa Andréa Name Colado Simão