UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de Botucatu

Instituto de Biociências de Botucatu - Unesp

CARCINOMA UROTELIAL: ESTUDO DE MODIFICAÇÕES PÓS-TRANSCRICIONAIS E DE PROTEÍNAS DE LIGAÇÃO AO RNA

ANDRÉ LUIZ VENTURA SÁVIO

BOTUCATU-SP
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“Faça o teu melhor, na condição que você tem, enquanto você não tem condições melhores, para fazer melhor ainda” Mario Sérgio Cortella

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"O pessimista vê dificuldades em todas as oportunidades. O otimista vê oportunidades em todas as dificuldades." Winston Churchill

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"Sem sonhos, a vida não tem brilho. Sem metas, os sonhos não têm alicerces. Sem prioridades, os sonhos não se tornam reais. Sonhe, trace metas, estabeleça prioridades e corra riscos para executar seus sonhos. Melhor é errar por tentar do que errar por se omitir" Augusto Cury

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1. RESUMO

O carcinoma urotelial representa um dos tipos mais comuns de neoplasias urinárias, apresentando altas taxas de recorrência, agressividade e progressão para doença músculo-invasiva. Devido à complexidade dos sistemas biológicos, pouco é conhecido sobre os mecanismos moleculares responsáveis pelos carcinomas uroteliais. Nos últimos anos, a introdução de novas ferramentas de bioinformática permitiu identificar novas moléculas e mecanismos implicados na carcinogenese. Neste estudo foram feitas duas abordagens com o objetivo de identificar novos potenciais biomarcadores para tumores uroteliais de baixo e alto graus. Inicialmente, a partir de dados de sequenciamento de RNA, foram avaliados os níveis de expressão gênica e o perfil de *splicing* do mRNA em amostras tumorais obtidas do biorrepositório da Faculdade de Medicina da USP (FMUSP). Os dados mostraram que os tumores de baixo e alto graus, comparados com tecido saudável de bexiga, apresentavam alteração na expressão em genes da via do *TP53,* e de splicing de mRNA de genes relacionados a ciclo celular, adesão, migração e processamento do RNA. Os tumores de alto grau, comparados aos de baixo grau, apresentavam aumento da expressão de genes relacionados à quimiotaxia (*GREM1, S100A12, NR4A1, IL6, CCL20, CXCL8, S100A9, CXCL10, CXCL11* e *CCL7)* e a funções neuronais *(EPHB2, CNTNAP2, KCNQ3, TENM2, RDH12, DPF1, SHISA9, SLC30A3, MME* e *MSI1).* Além disso, foram identificadas, exclusivamente nos tumores de alto grau, moficações de *splicing* em fatores de transcrição (*GAS5, RPL10, RPL13A* e *RPL37A*), com potencial impacto na produção proteica. Na segunda abordagem, foi realizado estudo funcional refrente ao papel das proteínas de ligação ao RNA (RBPs) na carcinogênese urotelial. Para isso, a partir de 405 casos de carcinomas uroteliais obtidos do *The Cancer Genome Atlas* - TCGA (384 alto e 21 baixo grau), foram mensurados os níveis de expressão de genes relacionados a 1.542 RBPs. No total, foram identificadas 236 RBPs com potencial atividade oncogênica nos tumores de alto grau, sendo 14 (*NOCT, CELF2, ENDOU, EXO1, EZH2, IFIT2, MOV10L1, MSI, PEG10, PTRF, TERT,*

TRIM71, WARS e *YBX2)* associadas a pior prognóstico, e selecionadas para o estudo funcional. A validação dos níveis de expressão dessas RBPs revelou que 8/14 (*EXO, EZH2, NOCT, TERT, MOV10L1, MSI1, WARS* e *YBX2*) também apresentavam níveis de expressão aumentados nas amostras tumorais obtidas do biorrepositório da FMUSP. A análise funcional em três linhagens celulares (UMUC3, T24 e J82) mostrou que o silenciamento de cada gene codificador das RBPs (total de 14) resultou na diminuição da proliferação e viabilidade celular, e no aumento das taxas de apoptose. Especialmente o silenciamento do gene *MSI1* (Musashi-1) mostrou forte impacto na viabilidade, proliferação, migração e invasão celular e apoptose, e também na diminuição da resistência celular a quimio e radioterapias (*in vitro).* Além disso, a análise de sequenciamento de mRNAs após o silenciamento do *MSI1*, evidenciou alterações na expressão de genes relacionados à diferenciação de células epiteliais, via Wnt e quimiotaxia. A análise, por CLIP *(Crooslinking Immunopreciptation)* dos alvos da musashi-1 mostrou que esta preferencialmente a se liga à região 3'UTR e a regiões intrônicas de mRNAs relacionados à tradução, processamento do RNA e às vias Wnt, TP53, PDGF e CCKR. Em conjunto, as duas abordagens permitiram identificar um novo painel de genes candidatos a biomarcadores e um novo alvo terapêutico (musashi1) para tumores uroteliais de alto grau.

2. ABSTRACT

Urothelial carcinoma represents one of the most common types of urinary neoplasms, with high rates of recurrence, aggressiveness and progression to invasive muscular disease. Due to the complexity of the biological systems, little is known about the molecular mechanisms responsible for urothelial carcinomas. In recent years, the increase of bioinformatics tools has enabled the identification of new molecules and molecular mechanisms involved in carcinogenesis. In this study, two approaches were conducted aiming to identify new potential biomarkers for low and high grades urothelial tumors. Initially, data from RNA sequencing showed the levels of gene expression and splicing profile for urothelial tumors (low and high grades) and normal bladder tissues obtained from the biorepository of the University of São Paulo Medical School (FMUSP), Brazil. The gene expression profiling demonstrated modulated expression in genes related to the *TP53* pathway in both low and high grade tumors. In addition, the splicing data showed that the preferentially affected genes were those related to cell cycle, adhesion, migration and RNA processing. The high-grade tumors presented increased expression of genes related to chemotaxis (*GREM1, S100A12, NR4A1, IL6, CCL20, CXCL8, S100A9, CXCL10, CXCL11* and *CCL7*) and neuronal functions (*EPHB2, CNTNAP2, KCNQ3, TENM2, RDH12, DPF1, SHISA9, SLC30A3, MME* and *MSI1*). Furthermore, splicing modification in transcriptional factors (*GAS5, RPL10, RPL13A* and *RPL37A*) with potential impact on protein production were also identified, but, exclusively, in high-grade tumors. In the second approach, a functional study was conducted aiming to identify the role of RNA binding proteins (RBPs) in urothelial carcinogenesis. For this, the expression genes related to 1,542 RBPs were measured from 405 cases of urothelial carcinoma obtained from the TCGA (384 high and 21 low grade tumors). A total of 236 RBPs with oncogenic potential were identified in high-grade tumors, being 14 (NOCT, CELF2, ENDOU, EXO1, EZH2, IFIT2, MOV10L1, MSI, PEG10, PTRF, TERT, TRIM71, WARS and YBX2) also related to worse prognosis and, therefore, selected for the functional

study. The RBPs expression validation showed that 8/14 (EXO, EZH2, NOCT, TERT, MOV10L1, MSI1, WARS and YBX2) were also highly expressed in the tumors samples obtained from FMUSP, Brazil. Functional analysis in three cell lines (UMUC3, T24, and J82) showed that the silencing of each RBPs encoding gene (total of 14) resulted in decreased cell proliferation and viability, and increased rates of apoptosis. The *MSI1* (musashi-1 knockdown) demonstrated a strong impact on cell viability, proliferation, migration, invasion and apoptosis, and also resulted in decreased resistance to *in vitro* chemo and radiotherapies. Data from the RNA sequencing after *MSI1* knockdown showed impact on the expression of genes related to epithelial cell differentiation, Wnt-pathway and chemotaxis. The musashi-1 target analysis (by crosslinking immunoprecipitation- CLIP) showed that this RBP preferentially bound to the 3'UTR and intronic regions of mRNA related to translation, RNA processing and Wnt, TP53, PDGF,and CCKR pathways. Taken together, data from the two approaches allowed the identification of new panel of candidate biomarkers, and a new therapeutic target (musashi1) for urothelial high-grade tumors.

3. LISTA DE FIGURAS

Figura 1: Estimativa de incidência e mortalidade por câncer de bexiga para os próximos 20 anos, de acordo com levantamento realizado pelo Globocan (http://globocan.iarc.fr/Pages/burden_sel.aspx). A) Incidência e mortalidade no Brasil; B) Incidência e mortalidade no mundo.....24

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5.LISTA DE SIGLAS

Sigla	Significado
3'UTR	*3′ untranslated region*
5'UTR	*5′ untranslated regio*
A3SS	*Alternative 3'Splice Site*
A5SS	*Alternative 5'Splice Site*
APCDD1	*Protein APCDD1*
ATCC	*American Type Culture Collection*
BC	*Bladder Cancer*
BCG	*Bacillus Calmette Guérin*
BCL2	*Apoptosis regulator Bcl-2*
CCL20	*C-C motif chemokine 20*
CCTs	*Carcinoma de Células Transicionais*
CD44	*CD44 antigen*
CDI	*Coefficient of drug interaction*
CDKN2	*Cyclin-dependent kinase inhibitor 2A*
CDKN2	*Cyclin-dependent kinase 2*
CESC	*Cervical squamous cell carcinoma and endocervical carcinoma*
CIS	*Carcinoma in situ*
CLIP	*Crosslinking-Immunopreciptation*
CNTNAP2	CONTACTIN-ASSOCIATED PROTEIN-LIKE 2
CSH2	*Chorionic somatomammotropin hormone 2*
CTNNBIP1	*Beta-catenin-interacting protein 1*
CTSB	*Cathepsin B*
CXCL10	*C-X-C motif chemokine 10*
CXCL11	*C-X-C motif chemokine 11*
CXCL8	*Interleukin-8*
DMEM	*Dulbecco's modified Eagle's*
DNA	*Ácido Desoxirribonucleico*
DPF1	*Zinc finger protein neuro-d4*
dsRBD	*double-stranded RNA-binding motifs*
E2F3	*Transcription factor E2F3*
EMT	*Epitélio mesenquimal de transição*
EPHB2	*Ephrin type-B receptor 2*
FGFR3	*Fibroblast growth factor receptor 3*
FMUSP	*Faculdade de Medicina da USP*
GO	*Gene ontology*
GREM1	*Gremlin-1*
HER2	*Receptor tyrosine-protein kinase erbB-2*
HNSC	*head and neck squamous cell carcinoma*
HOXA10	*Homeobox protein Hox-A10*
ICGC	*International Cancer Genome Consortium*

IL6	*Interleukin-6*
IMP3	*Insulin-like growth factor 2 mRNA-binding protein 3*
IMP3	*U3 small nucleolar ribonucleoprotein protein IMP3*
IR	*Intron Retention*
ISUP	*Sociedade internacional de urologia e patologia*
KCNG1	*Potassium voltage-gated channel subfamily G member 1*
KCNQ3	*Potassium voltage-gated channel subfamily KQT member 3*
KH	*hnRNP K homology domains*
LGG	*low grade glioma*
LIHC	*low hepatocellular carcinoma*
LIPG	*Endothelial lipase*
LM	*Leucemia Mielóide*
lncRNA	*RNA longo não codificante*
LRP	Leucine-responsive regulatory protein
LUAD	*lung adenocarcinoma*
LUSC	*lung squamous cell carcinoma*
miRNAS	*MicroRNAs*
MME	*Macrophage metalloelastase*
MOV10L1	*RNA helicase Mov10l1*
mRNA	*RNA mensageiro*
MSI1	*RNA-binding protein Musashi homolog 1*
MVAC	*Metrotrexato, Vinblastina, Doxorrubicina e Cisplatina*
MYBL2	*Myb-related protein B*
NFE2	*Transcription factor NF-E2 45 kDa subunit*
NHUCs	*Células uroteliais humanas normais*
NOCT	*Nocturnin*
NR4A1	*Nuclear receptor subfamily 4 group A member 1*
NRAS	*GTPase NRas*
OV	*ovarian serous cystadenocarcinoma*
p53	*Proteína p53*
PBS	*Phosphate buffered saline*
PCR2	*Polycomb repressive complex2*
PDK1	*[Pyruvate dehydrogenase (acetyl-transferring)] kinase isozyme 1, mitochondrial*
PL	*Pancreatic triacylglycerol lipase*
PLD1	*Phospholipase D1*
PPR	*Pentatricopeptide repeat*
PTTG1	*Securin*
PUF	*Pumilo repeats*
RB1	*Retinoblastoma-associated protein*
RBM3	*RNA-binding protein 3*
RBPs	*Proteínas de ligação ao RNA*
RDH12	*Retinol dehydrogenase 12*

RIN	*RNA integrity number*
RNA	*Ácido Ribonucléico*
RP	*Ribossomal Protein*
RPL10	*60S ribosomal protein L10*
RPL13A	*60S ribosomal protein L13a*
RPL37A	*60S ribosomal protein L37a*
RRM	*RNA-recognition motif*
RTqPCR	*Reverse transcription quantitative polymerase chain reaction*
S100A12	*Protein S100-A12*
S100A8	*Protein S100-A8*
S100A9	*Protein S100-A9*
SA	*Splicing Alternativo*
SDS-PAGE	*sodium dodecyl sulfate–polyacrylamide gel electrophoresis)*
SE	*Skkipped exon*
SERPINE1	*Plasminogen activator inhibitor 1*
SHISA9	*Protein shisa-9*
siRNA	*RNA pequeno de interferência*
siTarget	*Small interferênce RNA Target*
SLC30A3	*Zinc transporter 3*
SMAD3	*Mothers against decapentaplegic homolog 3*
snRNA	*RNA nuclear pequeno*
SPHK1	*Sphingosine kinase 1*
TCGA	*The Cancer Genome Atlas*
TENM2	*Teneurin-2*
TERT	*Telomerase reverse transcriptase*
TNM	*Tumor, node, metastase*
TP53	*Cellular tumor antigen p53*
UCC	*Urothelial Cell Carcinoma*
UCEC	*uterine corpus endometrioid*
WARS	*Tryptophan--tRNA ligase, cytoplasmic*
WHO	*Organização Mundial de Saúde*
Wnt	*Wingles int*
YBX2	*Y-box-binding protein 2*
ZnF	*zinc-binding domains*

6. REVISÃO DA LITERATURA

6.1 Considerações gerais

Atualmente, o câncer é a segunda principal causa de morte no mundo, acometendo cerca de 8 milhões de habitantes por ano. O câncer de bexiga, ou carcinoma urotelial, representa a quinta neoplasia mais comum em homens e a décima terceira entre as mulheres, sendo 12.918 novos casos previstos para o ano de 2020 no Brasil (Figura 1A). No mundo, a neoplasia de bexiga representa a nona mais comum e a décima terceira causa de morte por câncer, sendo estimados 535.316 novos casos e aproximadamente 205.024 óbitos para o ano de 2020 (Figura 1 B) (1). Além disso, para um futuro próximo, é estimado o dobro de novos casos e mortes causadas por essa neoplasia, fenômeno explicado pelo aumento da expectativa de vida da população mundial (2). Aproximadamente metade dos casos de câncer de bexiga é causada pelo tabagismo (3); outros fatores etiológicos incluem o uso prolongado de analgésicos do tipo fenacetina, a exposição ao antineoplásico ciclofosfamida e o consumo de alimentos e água contaminada com arsênico (4–6).

Os carcinomas de células transicionais (CCTs) de bexiga correspondem a 90% dos tumores malignos nesse órgão e podem apresentar-se como pequenas papilas ou lesões extensivamente invasivas. Os demais tipos de carcinomas são representados pelos carcinomas de células escamosas (7%), pelos adenocarcinomas (2%) e pelos sarcomas (<1%) (7). Histopatologicamente, os carcinomas de células transicionais de bexiga são classificados pela extensão do tumor primário (estadiamento) e pelo grau de diferenciação histológica, podendo ser tumor papilar, carcinoma *in situ* (CIS), tumor músculo-invasivo, doença avançada e doença metastática (8).

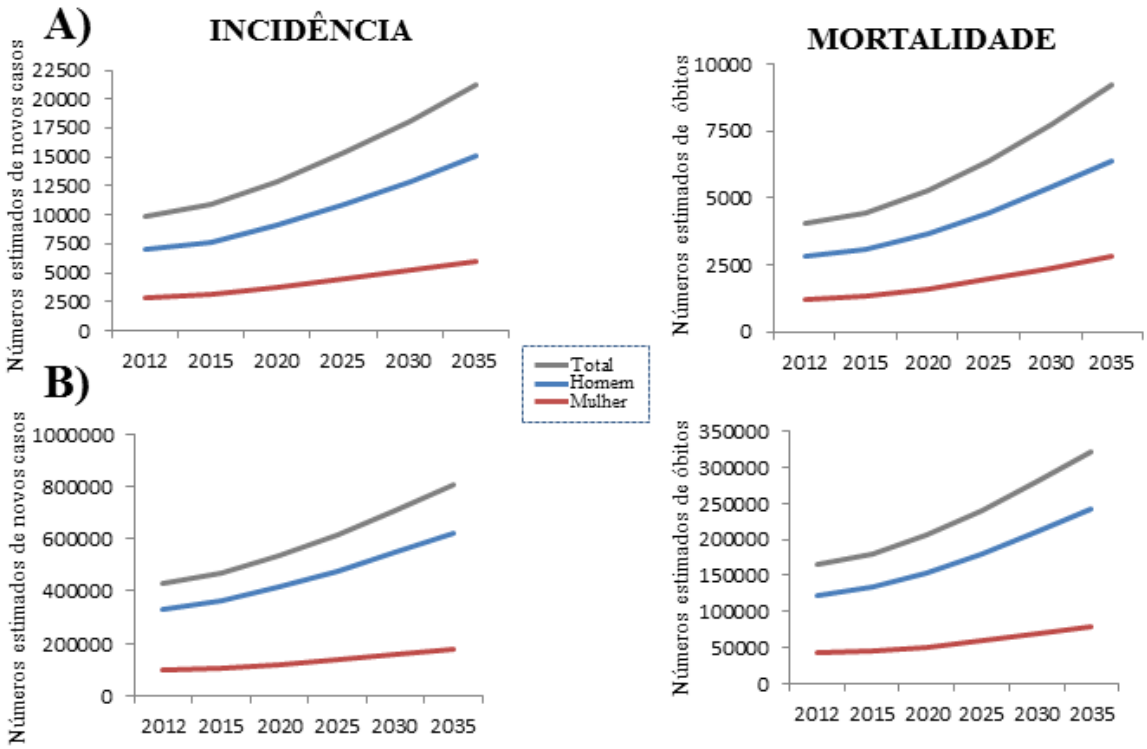

Figura 1: Estimativa de incidência e mortalidade por câncer de bexiga para os próximos 20 anos, de acordo com levantamento realizado pelo Globocan (http://globocan.iarc.fr/Pages/burden_sel.aspx). A) Incidência e mortalidade no Brasil; B) Incidência e mortalidade no mundo.

Os CCTs são superficiais quando atingem, no máximo, a lâmina própria do epitélio vesical (TA e T1), e profundos, quando invadem a camada muscular superficial e profunda (T2), tecido perivesical (T3) e tecido adiposo (T4) [Figura 2-Adaptado de (9)]. A principal característica clínica dos tumores vesicais não invasivos é o seu alto índice de recorrência, com indiferenciação progressiva e aumento da agressividade (9). Apesar da alta taxa de recorrência, esses tumores têm baixa taxa de progressão para doença músculo-invasiva (10%-20%) e uma pequena proporção progride para alto grau. Por outro lado, 20% dos pacientes têm câncer de bexiga músculo-invasivo que se desenvolve a partir de lesões displásicas de alto grau e carcinomas *in situ*, com comportamento agressivo e altas taxas de progressão e metástases. A taxa de sobrevida de cinco anos para estes pacientes é de 50% (7).

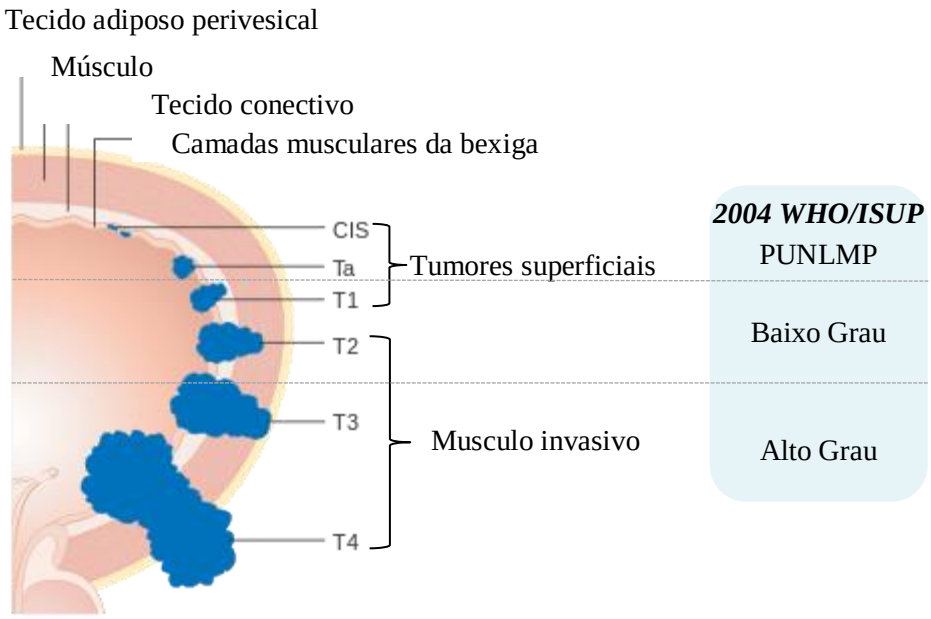

Figura 2: Estadiamento TNM (*tumour, node, metastasis*) dos tumores de bexiga (*Cancer Research UK*, adaptado). À direita, a nova classificação dos tumores de bexiga de acordo com a *WHO* (Organização Mundial de Saúde) e *ISUP* (*International Society of Urology and Pathology*) (10).

Conhecer o estágio da doença é fundamental para o estabelecimento da estratégia de tratamento para o câncer de bexiga. Diferentes tipos de tratamento estão disponíveis, incluindo cirurgia, terapia biológica, radioterapia e quimioterapia. Os procedimentos cirúrgicos periódicos para a ressecção do tumor tornam o câncer de bexiga uma doença altamente mórbida. Os pacientes submetidos a tais procedimentos são considerados grupo de risco para o desenvolvimento neoplásico *de novo* e são também mais susceptíveis a recorrências, possivelmente devido à instabilidade genética urotelial (11–13). Além da cirurgia, a radioterapia e a quimioterapia são também utilizadas para este tipo de câncer. Kotwal et al. (14) observaram que o índice de sobrevivência entre os pacientes com tumores invasivos submetidos à radioterapia é equivalente ao daqueles submetidos à cistectomia, porém com a vantagem da preservação do órgão. No caso da quimioterapia, são vários os protocolos utilizados, e envolvem combinações de drogas como metrotrexato, vinblastina, doxorrubicina e cisplatina (protocolo conhecido como MVAC) e também a combinação de

gencitabina e cisplatina. Os sinais primários da ação desses fármacos são alterações no DNA que podem resultar em bloqueio na progressão do ciclo celular e apoptose, e respostas essenciais para a manutenção da integridade genética da célula normal. A terapia com BCG (Bacillus Calmette Guérin) tem também apresentado resultados promissores como tratamento adjuvante para pacientes com riscos intermediário e alto para tumor de bexiga superficial (15), mostrando reduzir a taxa de recorrência e o risco de progressão para doença músculo-invasiva (16).

Estudos recentes mostram que os carcinomas uroteliais apresentam como características alterações genéticas heterogênicas, sem um evento patognomônico (17,18). Aproximadamente, 80% dos tumores uroteliais de baixo grau apresentam mutações de ponto no gene *FGFR3* (19), sendo, essas, um dos principais eventos genéticos subjacentes a tumorigênese urotelial (20). Mutações no gene *FGFR3* podem ativar a via RAS-MPAK e fosfolipases Cγ (PL Cγ), levando ao aumento da sobrevivência e proliferação de células uroteliais humanas normais (NHUCs) (21). Em alguns casos de carcinoma urotelial ocorre a exacerbação dessas vias, mesmo sem mutações de ponto no gene *FGFR3* (22). Entretanto, devido à complexidade do mecanismo de regulação, os níveis de expressão do *FGFR3* não o configuram como bom marcador de prognóstico para o carcinoma urotelial (23). No caso dos tumores de alto grau, estes apresentam uma série de alterações genéticas e epigenéticas como, por exemplo, a inativação dos genes *TP53, RB1* e *CDKN2*, as quais estão associadas a pior prognóstico (19-22). Recentemente, o TCGA (*The Cancer Genome Atlas*) demonstrou a presença de mutação no gene *TP53* em 49% dos tumores uroteliais de alto grau, e que aproximadamente 76% apresentavam inativação na função da p53 devido ao aumento da expressão do gene *MDM2* (antagonista do *TP5*3) (28). Modificações na via do retinoblastoma (RB1) representam outro importante evento nos tumores invasivos de alto grau. A amplificação e superexpressão do *E2F3*, o qual é normalmente reprimido pelo *RB1*, têm sido

associadas à perda da função do *RB1* em tumores de alto grau e relacionadas ao aumento da proliferação celular em linhagens uroteliais normais (29, 30). Em conjunto, o *TP53* e o *RB1* representam importantes vias em tumores de alto grau, atuando na progressão do ciclo e sobrevivência celular (31).

6.2. Fatores epigenéticos associados à carcinogenêse

Nos últimos anos, os sequenciamentos em larga escala do genoma revelaram novos fatores genéticos e epigenéticos associados à carcinogenêse, como por exemplo, modificações pós-transcricionais resultantes de alterações em processos de *splicing* alternativo de RNAm, RNAs longos não codificantes e proteínas de ligação ao RNA.

6.2.1 Splicing alternativo de RNA mensageiro (mRNA)

O processo de *splicing* possibilita que um mesmo gene codifique diferentes proteínas com diferentes funções (32). O *splicing*, inicialmente considerado um mecanismo de controle de expressão restrito a algumas dezenas de genes de mamíferos, atualmente é visto como fundamental na interface transcrição/tradução, regulando cerca de 95% dos genes, e levando a modificação da cromatina e transdução de sinal (33). O *splicing* é executado pelo *spliceossoma* (complexo ribonucleoproteíco que reconhece cada local de junção de uma sequência consenso) (34) e pode ocorrer de duas maneiras: *splicing* constitutivo e *splicing* alternativo. O *splicing* constitutivo consiste na retirada de todos os íntrons e união de todos os éxons que compõem o pré-mRNA. Por outro lado, no *splicing* alternativo ocorre a retirada dos íntrons e a ligação de diferentes combinações de éxons, resultando em várias formas de mRNA maduro (Figura 3) (35).

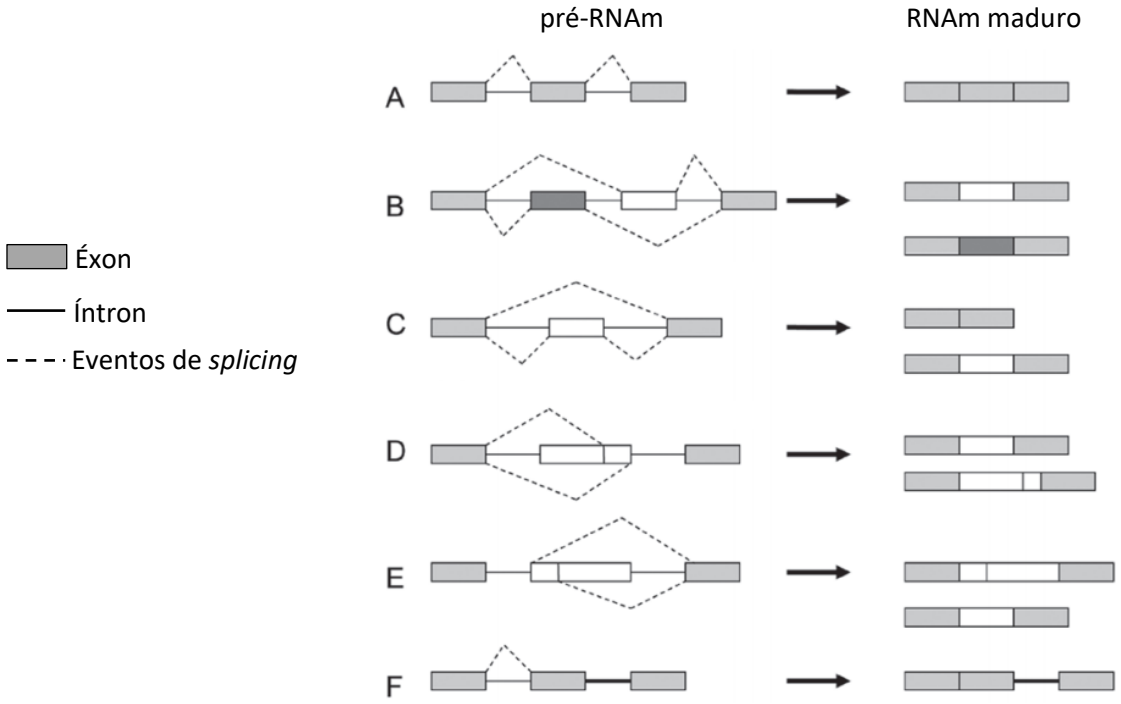

Figura 3: Principais formas de *splicing* de RNAm: A - *splicing* constitutivo; B - salto de éxons; C - éxons mutuamente exclusivos; D - sitios 5' doadores de *splicing* alternativo; E - sítios 3' receptores de *splicing* alternativo; F - retenção de íntron.

Mutações em sequências regulatórias que afetam o *splicing* alternativo são causas comuns de doença hereditária humana e câncer. Isoformas específicas de *splicing* podem também contribuir para resistência à terapia, diagnóstico e prognóstico de diferentes tipos tumorais (36). Recentes estudos sugerem que o *splicing* alternativo (SA) aberrante é um mecanismo molecular que pode levar ao desenvolvimento do câncer (36,37). A mutação no gene *U2AF1*, importante no processo de *splicing*, foi descrito em adenocarcinoma de pulmão e leucemia mielóide (LM) (38); o SA do gene *FGFR2*, que resulta na alteração das isoformas IIIb para IIIc, foi associado à transformação maligna em câncer de próstata (39); eventos de SA em *MLH* (*mutl homolog 1*) e membros da via Wnt (*Wingless int*) foram relacionados ao desenvolvimento do câncer de colón (40,41).

6.2.2 RNAs longos não codificantes (lncRNA)

Os RNAs longos não codificantes (lncRNA), possuem mais de 200 nucleotídeos e são classificados de acordo com seu tamanho, função e localização gênica (42). Inicialmente, os lncRNA eram considerados como produtos secundários da transcrição, sem nenhuma função biológica. Entretanto, nos últimos anos, centenas de estudos demonstraram que os lncRNA são capazes de interagir com moléculas de DNA, RNA e com fatores de transcrição, participando de vários processos biológicos, como metilação do DNA, modificações de histonas e remodelamento da cromatina, resultando na regulação positiva ou negativa de genes alvos (43). Alguns lncRNAs podem também atuar como "esponjas" de microRNAs (miRNA), afetando a expressão dessas moléculas (44), ou como reguladores pós-transcricionais, influenciando o processo de *splicing* alternativo de RNAm precursores, precursores de pequenos RNAs, estabilizadores de RNAm (45) e, ainda, como moléculas sinalizadoras com especificidade tecidual (46) (Fig. 4).

Há estudos que mostram a interação entre lncRNA e proteínas de ligação ao RNA, como o *polycomb repressive complex 2* (PRC2) que compete com proteínas de ligação ao RNA de genes alvos ou *miRNAs*, modificando a estrutura do RNAm (47,48). Como consequência dessas modificações ocorre a desregulação de importantes processos celulares relacionados à carcinogênese, como proliferação celular, apoptose, migração e invasão celular (49,50). O aumento da expressão do lncRNA UCA1 (*urothelial cancer associated-1*), normalmente expresso em tecidos embriogênicos, tem sido relacionado à gênese dos carcinomas uroteliais (51). Além desse, a expressão do MALAT1 (*metastasis associated lung cancer*), inicialmente indicado como biomarcador de metástases de câncer de pulmão de células pequenas, tem sido também associada ao estadiamento tumoral e metástase dos tumores uroteliais (52). Estudos recentes mostraram que o aumento de expressão do MALAT1 em culturas de células de carcinoma urotelial resultou no aumento de

sobrevivência celular, proliferação, migração e promoção do epitélio mesenquimal de transição por meio da ativação da via Wnt, sugerindo que esse lncRNA pode estar relacionado à motilidade celular, favorecendo a metástase (52,53). O LINC00092, é outro lncRNA já relacionado ao carcinoma urotelial e cuja expressão aumentada, juntamente com o aumento da ativação do regulador central de glicólise aeróbica (vias PTEN/PI3K/AKT/mTOR), pode contribuir para o metabolismo tumoral e proliferação celular (54).

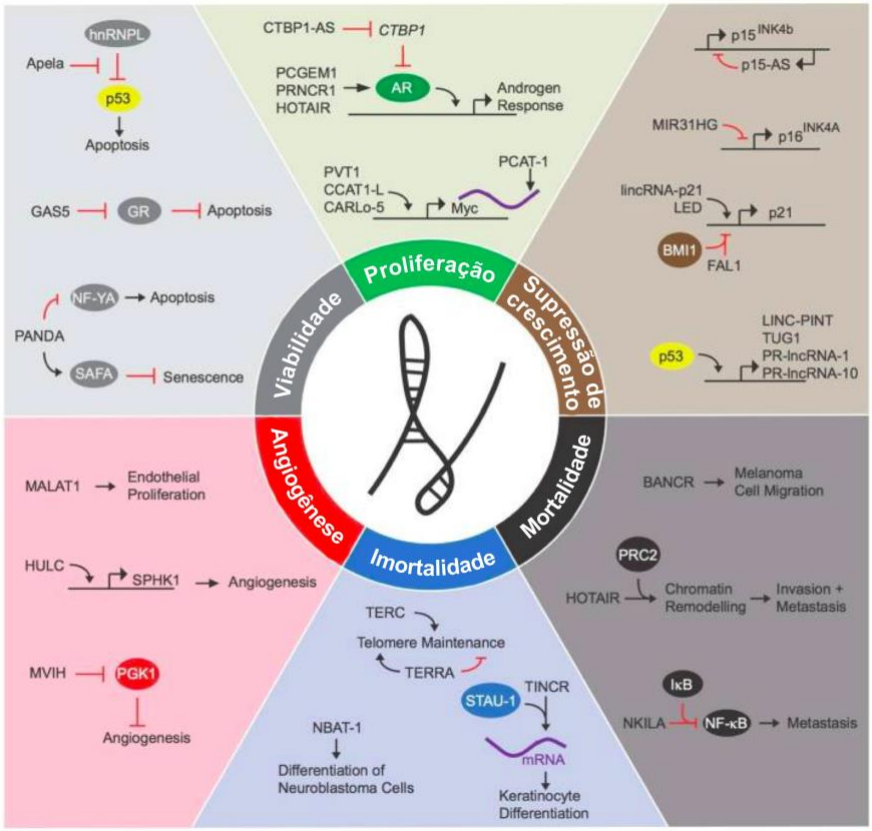

Figura 4: lncRNA contibui para alguns hallmarks do câncer (adaptado de Hanagan & Weinberg, 2011 (55)). Exemplos de lncRNA e seus respectivos alvos moleculares ou genômicos relacionados a importantes efeitos na carcinogênese: proliferação, supressão do crescimento, motilidade, imortalidade, angiogênesis e viabilidade.

Além de atuar em diversos processos carcinogênicos, alguns lncRNAs foram descritos como também desempenhando papel importante na resistência à quimioterapia. Em câncer de mama, o aumento da expressão do lncRNA-ARA foi associado à resistência ao antineoplásico adriamicina (56), enquanto o aumento do lncRNA-ATB à *trastuzumab* (57). A

expressão do lncRNA-ODRUL em linhagens celulares de osteosarcoma resultou no aumento da resistência ao tratamento com a doxorrubicina (58). Particularmente em carcinomas uroteliais, até o momento foi descrita apenas a relação entre o aumento da expressão do lncRNA-UCA1 e a resistência à cisplatina (59).

6.3 Proteínas de ligação ao RNA (RBPs) e câncer

Entre os fatores que podem estar envolvidos no desenvolvimento, recorrência ou resistência dos tumores de bexiga às terapias conhecidas, destacam-se aqueles que atuam em nível pós-transcricional, como as proteínas de ligação ao RNA (*RNA binding proteins*, RBPs). As RBPs desempenham suas funções por meio da ligação ao RNAm, abrangendo as regiões 5'UTRs, sequências de codificação e regiões 3'UTRs. Suas funções regulatórias podem ser positivas (ativadores) ou negativas (repressores), dependendo da proteína, do RNAm ou do contexto biológico (60). As RBPs atuam nos processos de *splicing*, poliadenilação, estabilidade, degradação e transporte de RNAs e tradução (Figura 5) (61,62).

Estudos recentes mostram que a maioria das RBPs tem estruturas modulares e são compostas por múltiplas repetições de apenas alguns domínios que se combinam em arranjos diferentes para criar superfícies versáteis de ligação ao RNA (63). Essa arquitetura permite que essas proteinas se liguem ao RNA com maior especificidade e afinidade do que os domínios individuais. Além disso, múltiplas cópias dos domínios de ligação ao RNA são combinadas a outros domínios enzimáticos ou efetores, permitindo que as RBPs tenham outras funções além daquela de reconhecer o alvo, o RNAm (64). Os principais domínios de ligação ao RNA são classificados em: *RNA-recognition motif* (RRM), *repeat proteins, Pumilio repeats* (PUF), *pentatricopeptide repeat* (PPR), hnRNP K homology (KH) *domains*, *zinc-binding domains* (ZnF) e *double-stranded RNA-binding motifs* (dsRBD) (64). A princípio, esses diferentes domínios, com diferentes estruturas e/ou especificidade, como os dsRBDs e RRMs, poderiam ser combinados para aumentar a afinidade e especificidade de

ligação ao RNA, contribuindo, assim, para a produção de proteinas características de cada tipo ou estado da célula. De fato, essa estratégia molecular aumenta as sequências de RNA alvos utilizando limitado repertório de domínios estruturais (64).

As RBPs vêm sendo amplamente estudadas devido suas funções na regulação da expressão gênica. O envolvimento de algumas RBPs na oncogênese e na progressão tumoral tem sido observado em diversos tipos de câncer. Em relação aos tumores de bexiga, Boman et al. (65) mostraram que a perda de expressão de RBM3 (*RNA-binding motif protein 3*) está associada a tumores clinicamente mais agressivos, sendo este gene um potencial biomarcador para estratificação do tratamento. Além disso, tem sido sugerido que altos níveis da proteína IMP3 (*insulin-like growth factor mRNA-binding protein 3*) em pacientes com câncer de bexiga podem indicar alto risco de progressão da doença e, consequentemente, a necessidade de uma terapia mais intensiva.

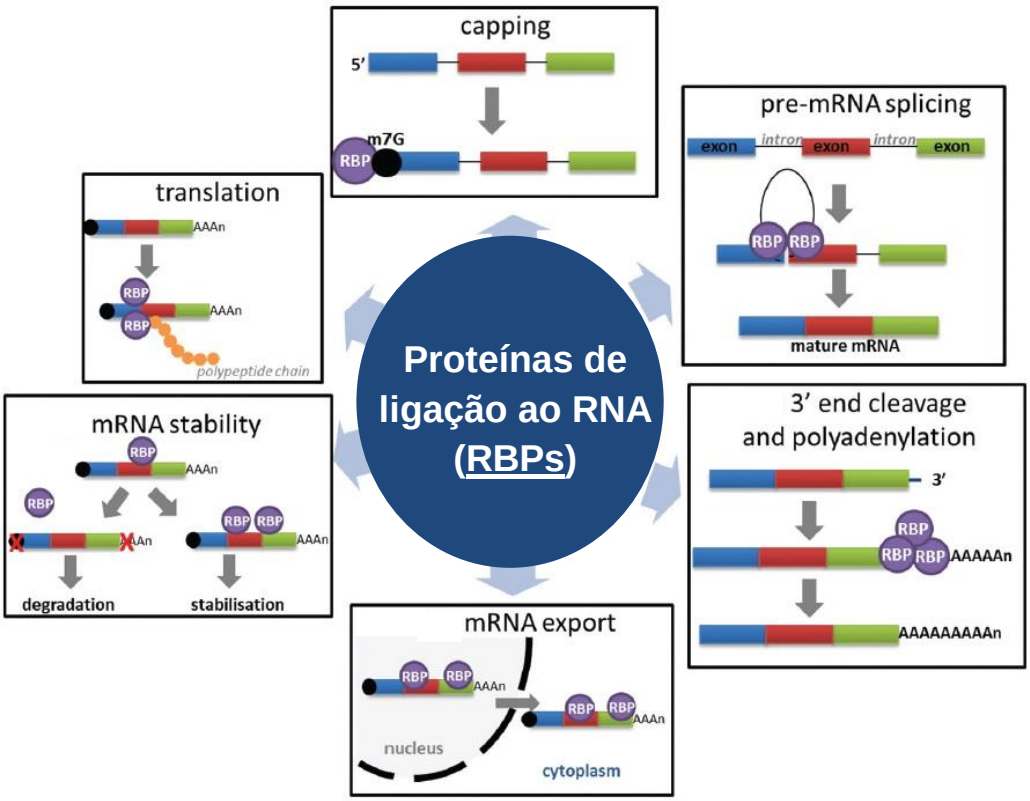

Figura 5: Mecanismos de regulação pós-transcricional por proteínas de ligação ao RNA. ***Capping*:** adição de uma 7-metilguanosina à extremidade 5' do mRNA nascente, as RBPs se ligam à capa e promovem a estabilidade do mRNA. ***Pre-mRNA splicing*:** descreve a excisão de introns não codificantes de mRNA nascentes regulados por RBPs dentro do *spliceossomo* macromolecular. **Clivagem e poliadenilação na extremidade 3':** envolve a clivagem em local definido e a extremidade 3'do pre-RNAm transcrito, seguido pela adição de 150-200 resíduos de adenosina e facilitado por um complexo de RBPs. **Transporte de mRNA:** refere-se ao transporte de mRNAs maduros através do complexo de poros nucleares para o citoplasma, mediado pela associação da RBP com o transcrito. **Estabilidade do mRNA:** pode ser modulada por associações de transcritos com RBPs específicas, alterações na cauda poli (A) e *decapping;* frequentemente precedem a rápida degradação. **Tradução**: é orquestrada por um complexo de RBPs conhecido como polissomas; as RBPs também podem modular a tradução via degradação de exonuclease ou sequestro de transcritos em compartimentos citoplasmáticos protetores (McLaughlin et al., 2015 (66))

7 - OBJETIVOS

Utilizando técnicas genômicas e ferramentas de bioinformática, o presente estudo teve como objetivo principal contribuir para o entendimento dos mecanismos moleculares da tumorigênese urotelial.

7.1 Geral

- Avaliar a expressão de genes de RBPs e variações pós-transcricionais (*splicing* alternativo e poliadenilação alternativa) nos respectivos mRNA obtidos de biópsias de carcinoma de células transcricionais (CCT) de bexiga, de baixo e alto graus, e correlacioná-los ao desenvolvimento tumoral.

7.2 Específicos

- Quantificar os níveis de mRNA em biópsias de tumor de bexiga de baixo e alto graus e de tecido normal;

- identificar as variantes de *splicing* de mRNA no transcriptoma de tecido tumoral e normal de bexiga.

- avaliar a função biológica das RBPs superexpressas em tumores de alto grau de bexiga;

- identificar as vias reguladas pela RBPs (selecionada em estudo funcional) e correlaciona-las à progressão tumoral.

NOTA DO AUTOR: o delineamento experimental, assim como os resultados do estudo e a discussão dos dados estão apresentados no formato de manuscritos para publicação.

8. MANUSCRITO 1

GENOMIC ANALYSES OF UROTELIAL CARCINOMA SHOW RNA MEDIATED MECHANISMS AS NEW CONTRIBUTORS TO MALIGNANCY

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ABSTRACT

Bladder cancer (BC) is the most common urothelial malignancy, and presents high rates of recurrence, aggressiveness and progression to invasive muscular disease. Several studies have reported that differential gene expression and aberrant alternative RNA splicing are important molecular mechanisms driving cancer development. However, little is known about such events in the transition between low- and high-grade bladder tumors. Therefore, the aim of this study was to investigate the existence of genomic markers that could characterize the malignancy of bladder cancer and its high rate recurrence. Using new generation RNA sequencing, it was analyzed the expression and splicing profiles of low- and high-grade bladder tumors and normal bladder tissues collected from a Brazilian cohort. Initially, data showed differentially expressed genes, especially related to the p53 pathway, and splicing alterations, preferentially in mRNA from genes associated to cell cycle, adhesion and migration and RNA processing, in tumor *vs.* normal bladder cells. Afterwards, data demonstrated increased expression of genes related to cell chemotaxis (*GREM1, S100A12, NR4A1, IL6, CCL20, CXCL8, S100A9, CXCL10, CXCL11* and *CCL7*), neuronal function (*EPHB2, CNTNAP2, KCNQ3, TENM2, RDH12, DPF1, SHISA9, SLC30A3, MME* and *MSI1*), and a robust set of genes related to poor prognosis (*APOE, CXCL9, CYP11A1, PTN, CCL21, MOV10L1, S100A8, NFE2, KCNG1, LIPG, MSI1, SORCS2* and *SPHK1)* in the high-grade when compared to low-grade tumor. Modifications of translation factors, with a potential impact on protein production were mostly observed in high grade tumors presented. In conclusion, besides to provide support to the classical mechanism of bladder tumorigenesis, the results indicated that a set of differentially genes (*MYBL2, LIPG, SEPT3, CTSV, MAPK8IP2, EGR4, TGFA, SLC16A10, KCNG1, LA16c-325D7.1, SOSTDC1* and *TPTEP1)* and splicing modification (GAS5, RPL10, RPL13A and RPL37A) in high-grade tumor can be considered potential genomic markers for this disease, contributing for the earlier stratification, detection of tumor recurrence and improvement of the patient survival.

INTRODUCTION

Bladder cancer is the most common urothelial malignancy, exhibiting high rates of recurrence, aggressiveness, and progression to invasive muscular carcinoma. In 2018, were estimated 81,190 new cases and 33,170 deaths attributable to bladder cancer in the United States (1) and, according to the World Health Organization (WHO), the number of cases is anticipated to almost double in the near future (2,3). Urothelial Cell Carcinoma (UCC) represents 90% of malignancies in the bladder, being 80% superficial or low-grade bladder tumors (stages Tis/carcinoma in situ [CIS], Ta, and T1-T2a), with low progression rate (10-20%) to aggressive muscle-invasive disease. The high-grade bladder tumor (stages Ta–T4), with aggressive behavior, high progression rates and metastasis, is usually found in 20% of the patients (4,5). Due to the lack of disease-specific symptoms, diagnosis and follow-up, bladder cancer has remained a challenge to the urologic community (6).

Several studies have shown that BC is a genetically heterogeneous diseases, with no single pathognomonic molecular event (7,8). Approximately 80% of low-grade bladder cancer have activating point mutations in *FGFR3* (fibroblast growth factor receptor three)(9), which seems to be the only key genetic event contributing to tumorigenesis (10). However, due to the complexity of its regulation, *FGFR3* status is not a good final prognostic marker for BC (11). In contrast, numerous genetic and epigenetic alterations have been associated with worsening prognosis in high-grade bladder cancer (12–14). Inactivation of *TP53*, *RB1*, and *CDKN2A* are common in high-grade tumor and have a significant contribution to adverse prognosis (15). Together with amplification or overexpression of *MDM2* (a p53 antagonist), *TP53* mutation is common in 49%, and p53 function is inactivated in 76% of HGBC tumors (15,16). Modifications in the retinoblastoma (RB1) pathway represent other important event in muscle invasive bladder cancer. Taken together, *TP53* and *RB*1 play important roles in HGBC by regulating cell cycle progression and cell survival (17).

In recent years, genomic analyses of BC have been carried out by The Cancer Genome Atlas (TCGA) and the International Cancer Genome Consortium (ICGC). The published data covers mainly low-grade vs. high-grade tumor comparisons and focuses on transcriptomic differences and DNA alterations (18). Our study explores a few different aspects, including comparisons with normal tissue and changes at splicing level. Splicing alterations affect all hallmarks of cancers and there is increasing evidence connecting splicing regulators to the signals driving cell transformation (19,20). An important example of altered splicing in BC concerns the Fibroblast growth factor receptors (FGFRs). A switch between splice isoforms FGFR2-IIIb and IIIc is related to increased invasiveness in bladder cancer by promoting epithelial-mesenchymal transition (21,22). Another example is the transcription factor (RREB1); higher expression of the isoform RREB1β was associated with increased cell proliferation (23).

Aiming at identifying new contributors of bladder cancer development and malignancy, we analyzed via RNA sequencing the expression and splicing profiles of a collection of high-grade, low-grade and normal bladder tissues.

MATERIAL AND METHODS

Human subjects

Approval study protocol was obtained from the São Paulo State University - UNESP-Brazil (109640/2015).

This study includes tumor (n=20 high grade; n=11 low grade) and normal (n=4) samples obtained from the University of Sao Paulo Biorepository (São Paulo – Brazil) and Amaral Carvalho Hospital (Jau, São Paulo – Brazil). All tumor samples were collected by transurethral resection and histopathologically classified by a pathologist (K.R.M.L). The grading stage was determined according to the Tumor-Node-Metastasis (TNM) and the

World Health Organization (WHO) systems (24,25). Samples are listed in Supplementary Table IS. All samples were obtained from men.

Tissue preparation and RNA isolation

Tissue biopsies were snap-frozen and stored at -80ºC. Total RNA was extracted using the RNeasy Mini kit® (Qiagen, Hilden, Germany), according to the manufacturer's recommendation. RNA integrity was verified with the 2100 Bionalyzer (Agilent Genomics), and only samples with a RIN $\geq$ 6.0 were used.

Library preparation and sequencing

RNA sequencing libraries were generated using the Illumina TruSeq Stranded Total RNA and Ribo-Zero Gold (Illumina, Cambridge, UK), according to the manufacturer's recommendation. Purified cDNA libraries were used for cluster generation onto HiSeq Cluster Kit v4 (Illumina) and then sequenced using High-throughput RNA-sequencing (Illumina Next-Generation Sequencing) in Illumina HiSeq2500, using paired-end 2x126 cycles. An average of 7.83 million reads were obtained per sample.

Expression and splicing analyses

Data Sources

The Human genome (version GRCh37/hg19) and gene annotation (release 19) were downloaded from UCSC genome Browser (https:/genome.ucsc.edu) and GENCODE (26), respectively.

Sequencing alignment and gene expression

FASTQ read files from RNA-Seq were aligned to human genome with the Genomic Short-read Nucleotide Alignment Program (GSNAP) (Wu et al., (27); parameters: -t 24 -B 4 -N 1 -E 1 --input-buffer-size 100000 --output-buffer-size 100000 --trim-mismatch-score=0 --trim-indel-score=0). We also provide to the aligner a file with known splice sites, generated from the transcriptome data (GENCODE, release 19). In order to have only reliable

alignments, only those reads with mapping quality over 20 (Q>20, selected using samtools (28)) were kept.

For all samples, the final set of mapped reads were matched to the reference to the transcriptome (GENCODE) using the HTseq-count (29); Parameters: -f bam -r pos -a 20 -m union -s reverse). A matrix with all expressed genes and their samples was generated.

Differential Expression Analysis

We conducted three sets of comparisons: High Grade versus Normal, Low Grade versus Normal and Low Grade versus High Grade. To identify statically differentially expressed genes among those groups, we used the DEseq2 package (30), available on software R (http://www.r-project.org/). We defined genes as up-regulated those genes presenting logFC > 2 and adjusted p-value adjusted < 0.05. For down-regulated genes: logFC < -2 and p-value adjusted < 0.05.

Alternative Splicing Analysis

In order to identify events and alterations in the splicing profile of the studied groups, the alignments were analyzed using the rMATS package (31). The alternative splicing events were classified as skipped exon (SE), retained intron (RI), alternative 5 'site (A5SS), or alternative 3' site (A3SS). Those exons with a false discovery rate (FDR) smaller than 0.05 and delta-PSI >0.05 or <-0.05 were considered differentially spliced. The information about the direction of occurrence of the specific splicing events, detected by the high frequency of the event in the samples, were also kept.

Functional annotation

Functional annotation analyses (Gene Ontology and KEGG pathways) were performed using DAVID (32), having *Homo sapiens* genes as background. Terms with Benjamini-Hochberg corrected p-values < 0.05 were selected as enriched. Redundant GO

terms were summarized using REViGO (33). Pathway interaction was conduced using Cytoscape (34) and GeneMania (35).

RESULTS

Novel mRNA expression markers in urothelial carcinoma

We conducted an RNAseq analysis of normal bladder, low-grade and high-grade tumor samples aiming to identify expression and splicing alterations in cancer tissue and prevalent in high-grade tumors. Our samples come from Sao Paulo, Brazil . We decided to focus on male individuals as the incidence of bladder cancer in Brazil is moderately high in men and lowest in women when compared with world incidences (36). This is the first BC genomic study with samples just from this region.

To identify genes differentially expressed in tumor vs. normal, our strategy was to conduct two independent analyses: normal bladder tissue vs. low grade tumor (Table I) and normal bladder tissue vs. high grade tumor (Table I). We then merged the results to identify a high confident set. A total of 255 coding genes (178 upregulated and 166 downregulated in tumors) were identified in turmors compared to normal bladder (Figure 1A). The main biological categories associated with genes upregulated in tumors are cell cycle, cellular movement, chromossome segragation, DNA replication and Mitosis (Figure 1B). We performed gene ontology and pathway enrichment analyses and compiled the enriched categories using Revigo (48) (Figure 1 C). We used genes present in these categories to build a network (Figure 1S). Among the major nodes, we should highlight nuclear division and DNA replication genes. In terms pathway enrichment, the p53 pathway, cell cycle and PPAR signaling pathay were identified), in BC nearly half (49%) had *TP53* mutation, and the loss of *TP53* function was describe on 76% of BC cases (19).

Table I: Differentially mRNA expression analysis

Group	Up-Regulated	Down-Regulated	Total
High grade tumor vs. Normal bladder tissue	329	200	529
Low grade tumor vs Normal bladder tissue	222	534	756
High grade tumor vs. Low grade	108	21	129

By comparing gene expression between high-grade vs. low-grade tumor, we found 129 coding genes differentially expressed in high-grade tumor compared to low-grade (125 up and 31 downregulated). Gene ontology enrichment analysis of up-regulated genes in high grade tumors were performed and revelead a strong association with cell differentiation, cell development and anatomical structure development (Figure 2A). Network analysis showed association between up-regulated genes with neuronal genes and cell chemotaxis (Figure 2B). Recently, the new molecular classification of muscle invasive bladder cancer include and describe the neuronal subgroup like a poorest survival and aggressive phenotype (37). Beside that, nine (*CXCL9, CYP11A1, KCNG1, MSI1, NFE2, PTN, S100A8, SORCS2* and *SPHK1*) genes up-regulated in high-grade turmos were correlated with poor prognosis of bladder cancer (Figure 3). We then merged results to marged genes differentially expressed exclusive in high grade tumor (compared to normal bladder tissue or low-grade tumor). We found only 36 gene differentially expressed (25 up and 11 downregulated) exclusive in high grade, three of these genes (*KCNG1, SLC16A10* and *LIPG*) were related with poor prognosis (by Prognoscan data).

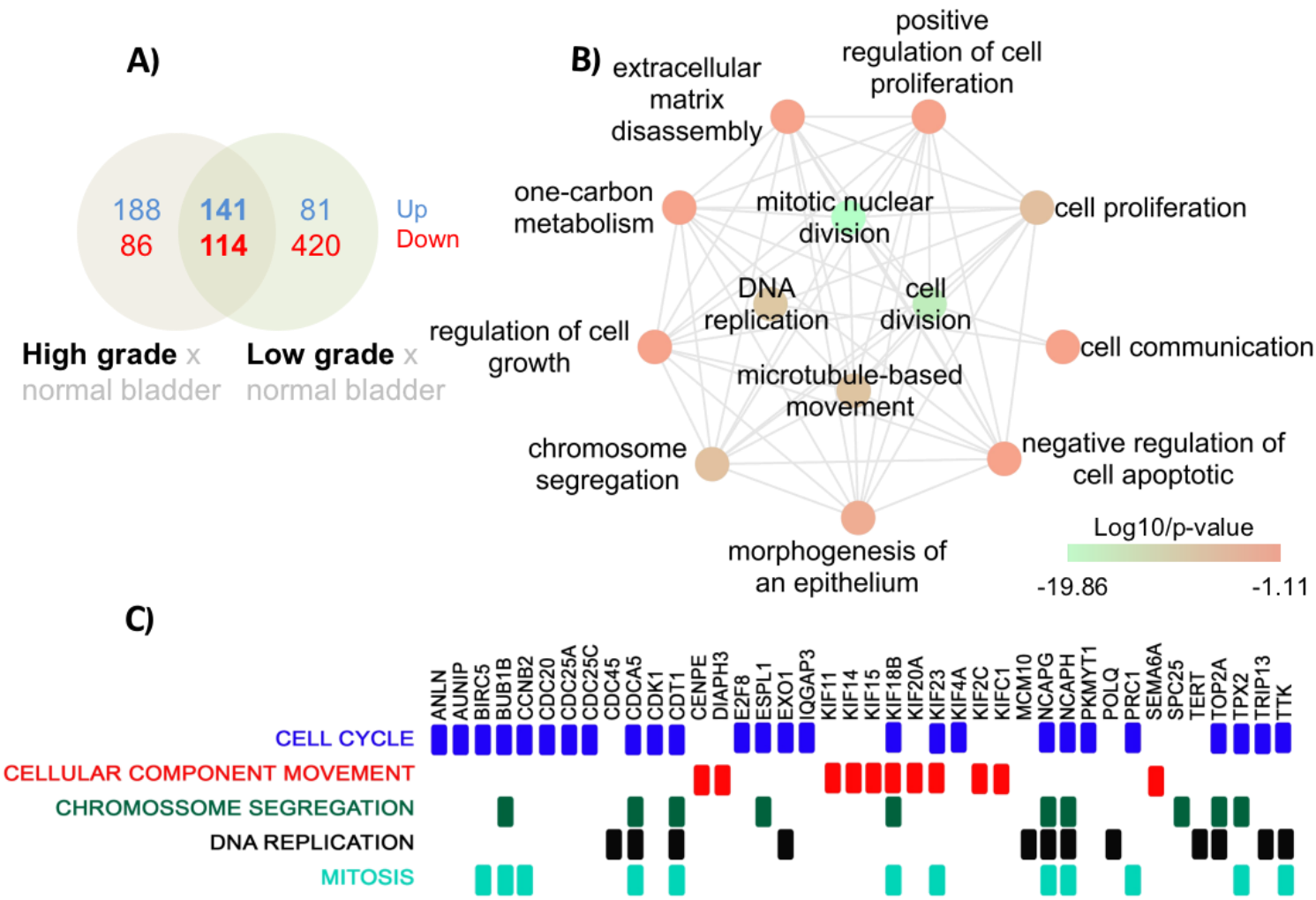

Figure 1: Venn diagram of differentially mRNA expression and enriched GO terms (Revigo) analysis . **A)** Comparison between tumor (Low and High grade) vs. normal bladder. Overlaps were considered for events in the same direction. **B)** Up-regulated genes in tumor are related to cell cycle, cell division and mitotic division. **C)** Up-regulated genes and the respective GO terms.

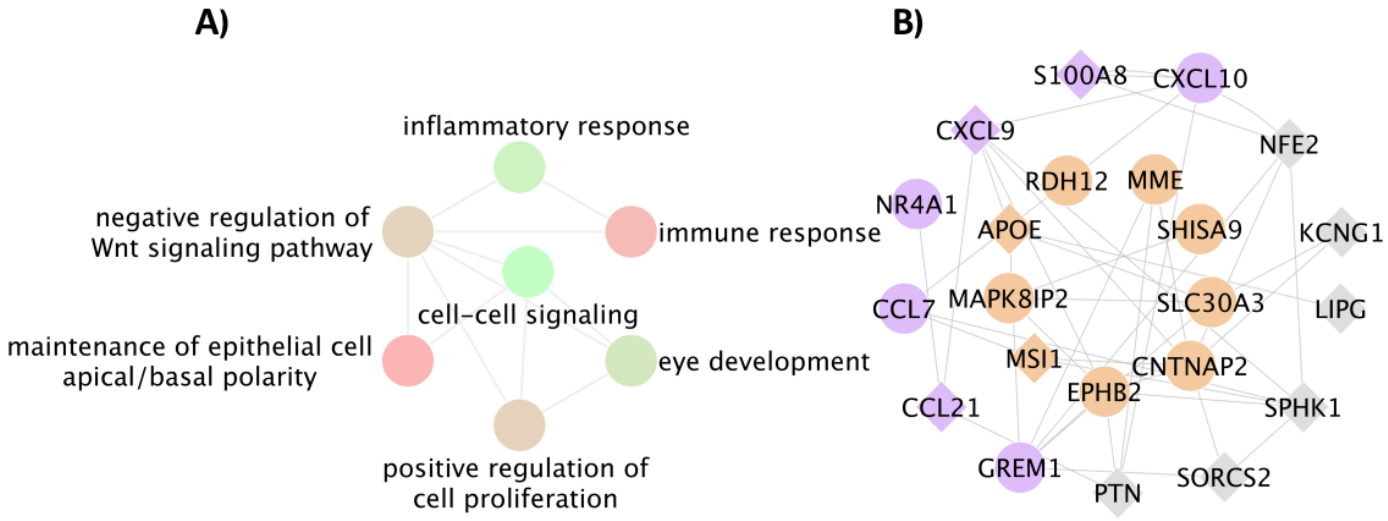

Figure 2: Up-regulated genes in high grade tumor compared with low grade bladder tumor. Purple symbols represent genes related to cell chemotaxis; orange symbols represent neuronal genes; diamond symbols represent genes related with poor prognosis for bladder cancer (PrognoScan data)

RNA processing revealed new candidate targets in urothelial carcinoma

The second part of this study we performed a map of splicing isoforms related to bladder tumors and tumor grade. First, we conducted an individual group analysis to determine the differentially regulated splicing events (Table II). Next, we merged the results to found the high confidence events between tumors (low and high-grade tumors) vs. normal bladder. We identify a total of 247 events (ES: 215; IR:16; A5SS:11; A3SS:5) in 191 genes (Figure 3A) differentially splicing between tumors vs. normal bladder tissue, related to cell cycle, actin binding and RNA processing (Figure 3 B). Network analysis of these select genes, showed strong association with cell division (by *TP53* pathway targets) and RNA processing (Figure 3C).

Table II: Number of splicing events in bladder cancer

Group	Splicing events				
	SE	**IR**	**A5SS**	**A3SS**	**TOTAL**
High grade tumor vs. Normal bladder tissue	604	53	41	23	721
Low grade tumor vs. Normal bladder tissue	481	33	27	17	558
High grade tumor vs. Low grade tumor	226	26	24	20	296

Skipping Exon (SE); Intron Retention (IR); alternative 5'splice site (A5SS); alternative 3'splice site (A3SS)

The analysis between high-grade vs. low grade, resulted in a total of 296 events (226 ES, 26 IR, 22 A5SS, 20 A3SS), in 242 genes (Table II), enriched GO terms showed these genes were related to RNA processing (Figure 4A). Specifically, in high grade, the lncRNA *GAS5* showed retention of introns 8, 9 and 11. Regarding of the ribosomal genes, we identified intron retention that coding stop codon in the genes *RPL10* (Intron 3), *RPL13A* (Intron 2) and *RPL37A* (Intron 1), being the last related with poor prognosis (*Prognoscan data*) for bladder cancer when down-regulated ($p<0.0075$) (see event example in the supplementary Figure 3S). Protein-protein interaction analysis revealed a network of ribosomal proteins with intron retention in the high-grade tumor (Figure 4B).

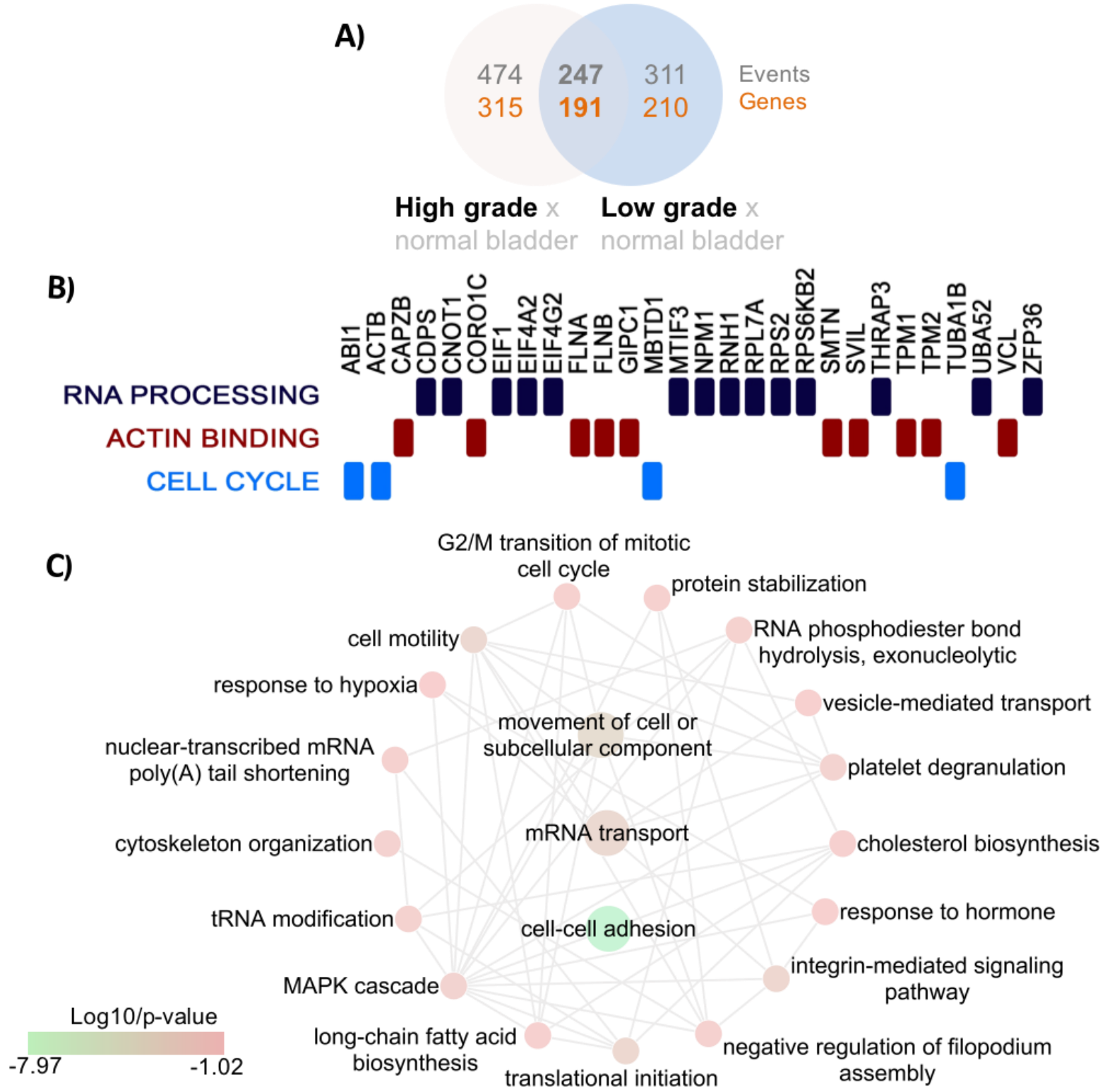

Figure 3: Venn diagram of splicing and enriched GO term (Revigo) analysis. **A)** Comparison between tumors (low and high grades) vs. normal bladder; green is the number of genes with splicing events; red represents number of splicing events. Overlaps were considered for events in the same direction. **B)** Genes with splicing modification in tumors (low and high grades) compared with normal bladder. **C)** Pathways of genes with splicing modification.

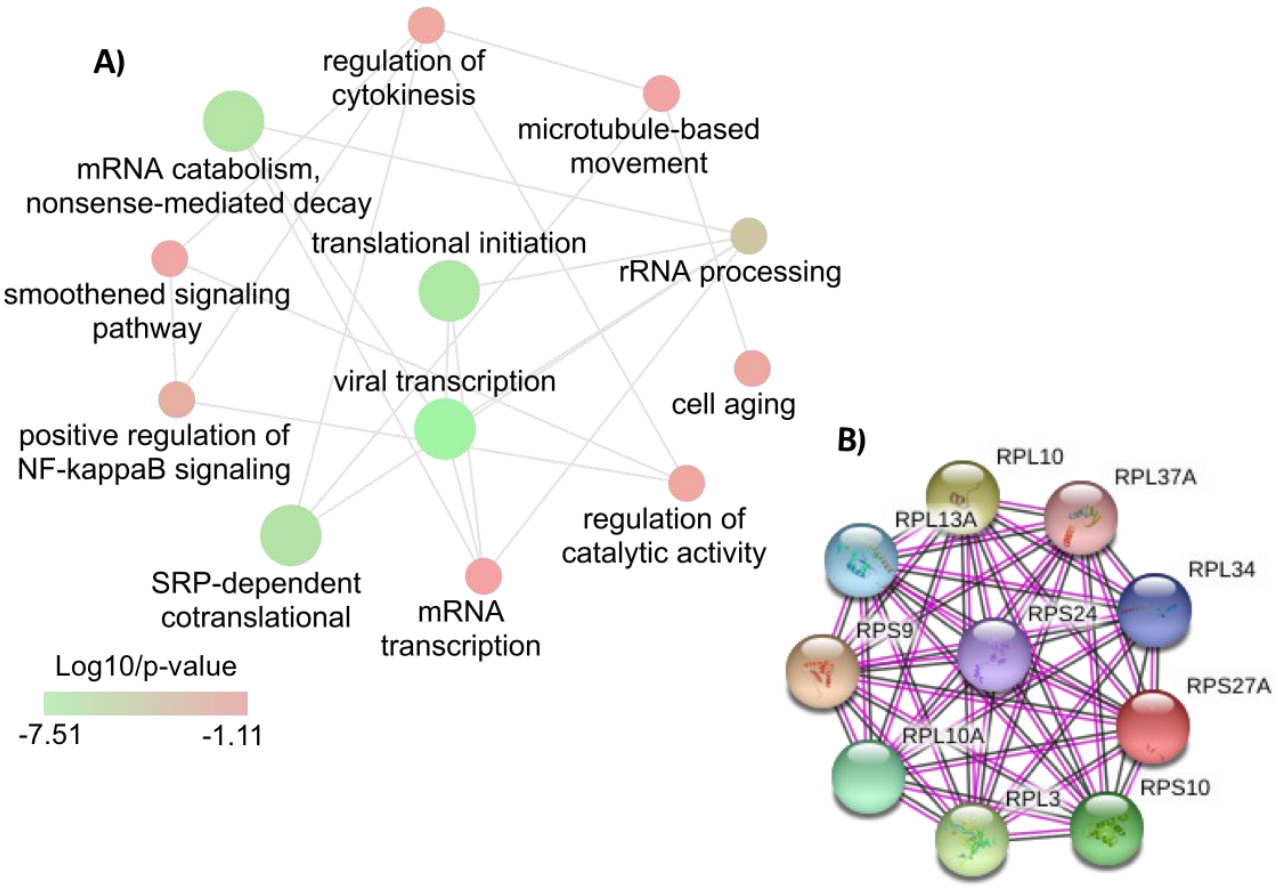

Figure 4: Splicing modification in high grade compared with low grade bladder tumors. **A)** Gene ontology pathway showing the strong association between genes with splicing modification in high grade tumor and RNA processing and translation. **B)** Ribossomal protein network with splicing events in high grade tumors.

DISCUSSION

In the last decades, a number of genomic studies has identified new taxonomy of several cancers (38). However, compared to other neoplasias, bladder cancer had a relative latecomer. Herein, using mRNA expression and splicing profiles of low- and high-grade bladder tumors, we demonstrated an increased expression of genes related to cell chemotaxis, neuronal function and a robust set of genes related to poor prognosis in the high grade tumors. In addition, we supported the classical mechanism of bladder tumorigenesis previously described in literature. In agreement with Weinstein et al. (16) and Hussain et al. (39) our data showed differential expression of genes related to p53 pathway and defense response in both low-and high-grade tumors compared to normal bladder tissue.

Interesting findings were obtained when low-and high-grade tumors were compared. The upregulation of genes related to cell chemotaxis in the high-grade tumors can indicate important step for tumor progression and aggressiveness. Chemotaxis is mediated by

chemokines, chemokines receptors, growth factors and growth factor receptor (33) and, in cancer, the pathway can be reprogrammed in favor of tumor cell dissemination (34). In fact some of these chemokines (*CCL21, CCL7, CCL3, CXCL8* and *CXCL10)* were also previously related to breast, cervical, colorectal, prostate cancer and melanoma (42–46).

Another set of upregulated genes in high-grade tumors was related to neuronal function (*MSI1, EPHB2, CNTNAP2, KCNQ3, TENM2, RDH12, DPF1, SHISA9, SLC30A3,* and *MME*). Some of these genes have been associated to epithelial-mesenchymal transition phenotype (EMT) (47,48). EMT is a reversible process that involves changes in cell morphology, differentiation, and motility, facilitating invasion and metastasis (49,50). EMT alterations have been associated with high-grade bladder tumor, since they can lead to resistance to therapeutic agents and poor outcome (12). Taken together, chemotaxis and EMT phenotype could have important role to the tissue invasion and increase tumor grade-staging.

Among the other genes exclusively modulated in the high-grade bladder tumors, three of them were related with poor prognosis (*KCNG1, SLC16A10* and *LIPG*). Interestingly, the pathway in which the protein (endothelial lipase G) encoded by *LIPG* is evolved, was recently associated to increased cell proliferation, tumor initiation and metastasis in breast cancer (51,52). According to Lo et al. (51) *LIPG* possesses a lipase-dependent function that supports cancer cell proliferation and a lipase-independent function that promotes invasiveness, stemness and basal/ epithelial-mesenchymal transition features of breast cancer. Therefore, the up-regulation of *LIPG* might have contribute to increase expression of genes that induce the EMT phenotype, making the tumor more aggressive.

In cancer, the splicing process is commonly disrupted, resulting in both functional and non-functional end-products (53). Among the mechanism of gene regulation, alternative RNA splicing has been investigated as potential markers for cancer (54). However, despite previous studies have described splicing modification in important genes related to adhesion,

cellular matrix and actin (16), a comprehensive characterization of these events in bladder cancer is still not clear. The higher number of events characterizing bladder tumor (compared to health tissue) denotes that cancer-relevant genes are thought to have functional diversity as a result of alternative splicing. Surely, cell communication, cell cycle, adhesion and cellular biogenesis are mechanisms implicated in all steps of carcinogenesis. When we looked for specific splicing events in low and high-grade tumors, we detected that low-grade tumors presents splicing modification in genes related to cell-cell adhesion.

Four ribosomal genes with increased intron retention were identified specificaly in high-grade bladder turmors, but without alteration in their respective mRNA. Herein, the intron retentions occurred as stop codon in three ribosomal genes (*RPL10, RPL13A*, and *RPL37A*), and might result in a truncated protein. The other gene, *RPL3*, presented intron retention inside an open ready frame with a new domain that could result in loss or novel function. Indeed, previous study has demonstrated that intron retention can be related to tumor suppressor inactivation through protein truncation (55). In the last decade, various studies have described the presence of ribosomal protein (RP) gene mutations in some diseases, including cancer (56), with increased. Nevertheless, such increase of RP expression was not detected in bladder and breast carcinomas.

Other interesting finding of our study was the decreased *GAS5* transcripts expression in high-grade tumors. This finding could be associated with the increased intron retention and, consequently, to the reduction of intron processing and snoRNA, and increased cell proliferation and resistance to apoptosis. GAS5 is a non-coding RNA which acts a putative tumor suppressor and apoptosis-promoting lncRNA that host multiple small nucleolar RNAs (57). The functions of GAS5 lncRNA are not very clear, but it is known that from its intronic sequences it expresses multiple small noncoding nucleolar RNAs (snoRNAs) that are involved in the biosynthesis of ribosomal RNA (58). We identified the retention of the

introns 8, 9 and 11, which encode SNORD79 (U79), SNORD80 (U80) and SNORD81 (U81) snoRNAs, respectively. Mourtada-Maarabouni et al. (59) have previously reported that overexpression of U81, U80/U47/U81 and U74 *GAS5* transcripts can induce growth arrest and apoptosis in several mammalian cell lines, and that the *GAS5* transcripts were substantially reduced in human breast cancer, suggesting this gene may be acting as a tumor suppressor (59). Other studies have showed that increased expression of *GAS5* results in cell-cycle arrest or cell apoptosis in several human cancer (lung, bladder, kidney, gastric and renal cancers) (60–63).

In summary, our study revealed two set of biomarkers candidates for bladder cancer diagnosis and specific biomarkers for high grade tumors. The first set consisting of three mRNA (*KCNG1, SLC16A10* and *LIPG*) and the second set, splicing events in *GAS5* lncRNA, and in the mRNA of *RPL3, RPL13A, RPL10* and *RPL37A* ribosomal genes, are new biomarkers candidates for bladder high-grade tumors. Taken toghether data generated by this study might offer new directions for bladder cancer diagnosis, earlier detection of tumor recurrence and treatment, resulting in improved patient survival.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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Supplementary Figures

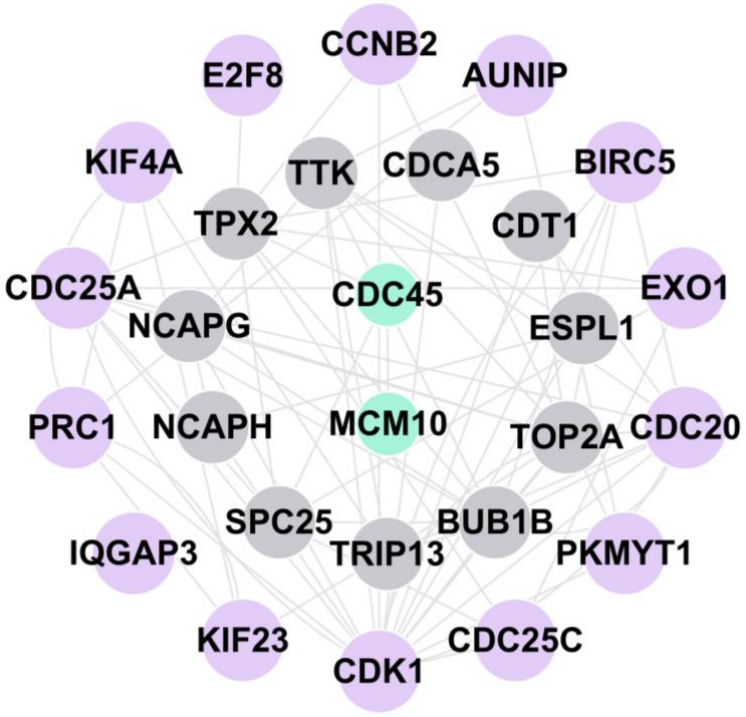

Figure 1S: Up-regulated genes in tumors compared with normal bladder. Green symbols represents genes related to DNA replication; Purple represent genes related to cell cycle; Grey represents related to cell cycle, DNA replication, chromosome segregation and cell division.

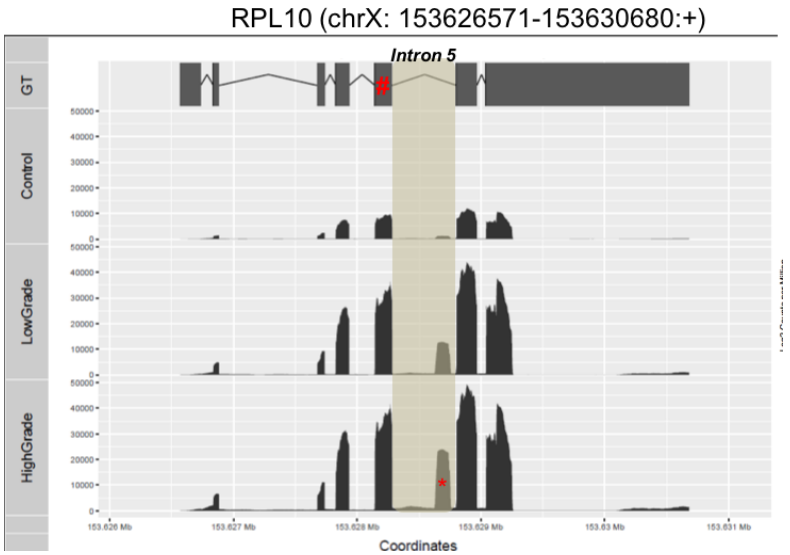

Figure 2S: RNA-seq read coverage of RPL10 for a control sample from bladder, low grade and high grade tumors. Shaded boxes mark introns that are most frequently retained in high grade tumors. #Exon that encodes a START codon. *Intron retained inside of open ready frames encodes new STOP codon.

Table IS. Demographic and histopathological characterization of the study population

Parameters	Bladder tumors		Normal bladder tissue
	Low grade	High grade	Control
Number of cases	11	20	4
Age	75 ±9	66 ±9	66 ±2
Tumor stage			
Ta	9	1	-
T1	-	5	-
T2	1	9	-
T3	-	2	-

The grading stage was determined according to the Tumor-node-metastasis (TNM) and the World Health Organization (WHO) systems (29,30)

9. MANUSCRITO 2

Musashi-1 can promote bladder tumorigenesis by Wnt signaling pathway

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ABSTRACT

RNA- binding proteins (RBPs) are emerging as fundamental players in tumor development. Among the RBPs, musashi1 (*MSI1*), together with their mRNA targets, form a complex network that affects multiple molecular pathways, including tumorigenesis. This study aimed to identify RBPs and investigate their impact on urinary bladder tumor development. In addition, the effect of luteolin as a possible adjuvant therapeutic compound for bladder cancer was also examined. Initially, we used the TCGA data bank to investigate the expression of 1,542 RBPs in 405 bladder cancer cases (384 high-grade and 21 low-grade tumor). Then, the findings were validated in 30 tumor samples (20 high-grade and 11 low-grade) obtained from a Brazilian tumor repository (BTR - USP). A total of 236 RBPs genes with oncogenic potential (up-regulated in high-grade compared to low-grade bladder tumors) were identified from the TCGA bank, being 14 (*NOCT, CELF2, ENDOU, EXO1, EZH2, IFIT2, MOV10L1, MSI, PEG10, PTRF, TERT, TRIM71, WARS* and *YBX2*) associated with a poor prognosis, and further selected for functional analyses. The RT-qPCR analysis showed that 8/14 RBPs (*EXO, EZH2, NOCT, TERT, MOV10L1, MSI1, WARS* and *YBX2*) were also up regulated in the BTR-USP high-grade tumor samples. The functional analyses using three high-grade tumor cell lines (UMUC3, T24 and J82) showed that the knockdown of each of RBPs genes (14) resulted in decreased cell proliferation and viability, and in increased apoptosis rates. The musashi RNA binding protein 1 (*MSI1*) gene knockdown (KD) showed the strongest impact on cell viability, proliferation, migration, invasion and apoptosis rates, and also in the cell chemo-radiotherapy resistance. *MSI1*-KD had also a large impact on gene expression profiling. Gene regulation, skeletal muscle and epithelial cell differentiation, Wnt-pathway and chemotaxis were among the biological process with the highest number of genes affected by the *MSI1*-KD. Data also showed the musashi1 RBP preferentially bond to 3'UTR and intron regions in mRNA related to translation, RNA processing, DNA replication and DNA repair. The pathways with greater number of musashi1 mRNA targets were Wnt, TP53, PDGF and CCKR. Regarding to luteolin, a strong effect was detected on cell proliferation, confirming its ability to increase cell sentivity to cisplatin and radiation. In conclusion, this study showed *MSI1* as an important key gene in bladder carcinogenesis, and highlighted musashi1 as a potential therapeutic target.

1. INTRODUCTION

Bladder tumors are highly prevalent in industrialized countries, with more than 50,000 new cases diagnosed per year in Europe and North America (1). The urothelial cell carcinomas (UCCs) represent about 90% of malignancies in the bladder, and 80% are superficial, low-grade and papillary non-invasive tumors. Despite the high rate of recurrence, UCC has low progression rate (10%-20%) for muscle-invasive diseases, and small probability to progress to high grade tumor. Moreover, 20% of the patients present muscle-invasive bladder cancer, which develop from high-grade dysplastic lesions and carcinomas *in situ*, with aggressive behavior and high progression rate and metastasis. The five-year survival rate for such patients is about 50% (2). The muscular wall impairment in bladder implies in worse prognosis, requiring aggressive medical intervention, such as radical cystectomy (3). Several studies have showed numerous genetic and molecular changes in bladder UCCs, although the mechanisms that lead to a malignant phenotype are not fully understood (4). However, it is known that not a single event, but the accumulation of genetic changes will be determine the clinical behavior of UCC (5).

Among the factors involved in the development, recurrence or resistance of bladder tumors are those that act at post-transcriptional level, such as the RNA binding proteins (RBPs). The RBPs act by binding to mRNA, covering 5'UTRs regions, coding sequences, and 3'UTRs regions and their regulatory functions can be positive (activators) or negative (repressive), depending on the protein, mRNA and the biological context (6). These binding proteins operate on splicing, polyadenylation, stability, degradation and transport of RNAs and translation (7,8). The major domains RNA binding are classified into: repeat proteins, pentatricopeptide repeat (PPR), RNA-recognition motif (RRM), hnRNP K homology (KH) domains, zinc-binding domains (ZnF), pumilio repeats (PUF) and double-stranded RNA-binding motifs (dsRBD) (9). These different domains, with different structures and/or

specificity, such as dsRBDs and RRMs, can be combined to increase the affinity and specificity of RNA, contributing to the protein production according to the type or state of cell. Actually, this molecular strategy increases the sequences of RNA targets using a limited repertoire of structural domains (9).

Mutations and alterations in RBPs have been related to tumor initiation and growth (10–12), and some studies have demonstrated that abnormally expressed RBPs are correlated with patient prognosis (13,14). The overexpression of the RBP Musashi family found in leukemia, brain, breast and colon cancers, has been linked to evading apoptosis, epithelial-mesenchymal transition phenotype and sustained cell proliferation (15–19). Similarly, the pumilio protein family has been related to genomic imbalance, sustained cell proliferation and tumor suppression in bladder and breast cancers (20–23), and the HuR RPBs with bladder cancer progression (24).

RNA binding proteins have been described as potential therapeutic targets for cancer prevention and treatment (25). Thus, some natural compounds, such as gossypol (26), azaphilone-9 (27) and luteolin (28), have been tested, due to their interaction with RBPs binding sites. Luteolin is one of the most common flavonoid in various plants, including peppermint, rosemary, thyme, pinophytes and pteridophytas (31). Recently, some studies described luteolin as a chemo- and radiosensitizer compound used for improving the therapeutic activity on drug-resistant cells (28–30). An antitumorigenesis activity of this flavonoid was observed in colon (31), gastric (32) and prostate (33) cancers.

In order to identify novel RBPs potentially implicated in bladder cancer development and their mechanisms of action, we conducted transcriptomic analyses followed by functional screenings. In addition, the effect of luteolin was investigated in a bladder cancer cell line treated X-ray and cisplatin, a widely used antineoplastic drug.

2. MATERIAL AND METHODS

2.1 TCGA data analysis

RNA-Seq raw reads from 412 samples of bladder cancer were downloaded from The Cancer Genome Atlas - TCGA (34). Reads were mapped against the human genome (version hg19/GRCh37 – UCSC Genome Browser (35). Mapped reads with quality $(Q) \geq 20$ (Phred scale) were selected using the SAMtools (36). Read counts per gene were defined using the HTSeq (37) and GENCODE - version 19 (38) as the reference transcriptomes. Differential expression analysis was performed using DESeq2 (39), comparing high- to low-grade tumors. All differentially expressed genes between high- and low-grade tumors (Benjamini-Hochberg corrected p-value < 0.05 and log2 fold change $\geq |1|$) were selected. The catalog containing 1542 human RBPs from Gerstberger et al. (40) was used as a reference to identify all differentially expressed RBPs.

2.2 RBPs validation in human bladder cancer tissues

2.2.1 Tumor samples

Approval study protocol was obtained from the São Paulo State University – UNESP, Botucatu – SP, Brazil (109640/2015). The tumor samples (n=20 high grade; n=11 low grade) were obtained from the University of Sao Paulo Biorepository (São Paulo – SP, Brazil) and from the Amaral Carvalho Hospital (Jau – SP, – Brazil). All tumor samples were collected by transurethral resection and histopathologically classified by a medical pathologist (K.R.M.L). The grading stage was determined according to the Tumor-Node-Metastasis (TNM) and the World Health Organization (WHO) systems (41,42).

2.2.2 Tissue preparation, RNA isolation and RTqPCR

Tissue biopsies were snap-frozen and stored at -80ºC. Total RNA was extracted using TRIzol reagent (Invitrogen), according to the manufacturer's instructions. Reverse transcription of mRNAs was performed using a high-capacity cDNA reverse transcription kit

(Applied Biosystems) with random priming. For mRNA analysis, quantitative PCR was carried out using specific Taqman for each RBPs (Applied Biosystems). Real-time PCR was using the ViiA™ 7 Real-Time PCR System (Applied Biosystems). Data were acquired by the ViiA 7 RUO software (Applied Biosystems), and analyzed using the $2^{-\Delta\Delta CT}$ method with GAPDH as an endogenous control. Same methodology was used for the *in vitro* knockdown quantification.

2.3 *In vitro* functional assays

2.3.1 Cell growth, RBPs expression and transfection

UMUC3, T24 and J82 human urothelial carcinoma cell lines from high-grade tumors were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's (DMEM; Gibco, Grand Island, NY, USA) or McCoy's 5A medium (Gibco, Grand Island, NY, USA), supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin (Life Technologies, Carlsbad, CA). RBPs expression was investigated by RT-qPCR, as described before. Cells were transfected with control siRNA or siTarget (Dharmacon) small interfering RNA (siRNA) using Lipofectamine RNAiMax reagent (Invitrogen). All experiments were perfomed in triplicate.

2.3.2 Cell proliferation

UMUC3, T24 and J82 transfected cells were grown in 96-well tissue culture plates (8×10^2 cells/well). The percentage of confluence was monitored for 96 hours using a high-definition automated imaging system (IncuCyte; Essen BioSicence). Data were analyzed using the ANOVA statistical method and were presented as mean ± standard error.

2.3.3 Cell viability and caspases 3/7 activity assay

Transfected cells were seeded in a 96-wells tissue culture plate (1.5 x 10^3 cells/well for UMUC3 and T24, and 3 x 10^3 cells/well for J82 cell lines). Seventh-two hours upon transfection, cell viability and caspases 3/7 activities were measured using the CellTiter-Glo (Promega, Madison, CA) and Caspases-Glo 3/7 assay (Promega), respectively, according to the manufacturer's instructions. The absorbance and luminescence were measured by the Molecular Devices SpectraMax M5 microplate reader. Data were analyzed using the t-test and presented as mean ± standard error.

2.3.4 Colony formation assay

UMUC3 transfected cells were plated onto 12-well plates (1,000 cells/well). After 10–14 days incubation, colonies were fixed with 4% formaldehyde and stained with 0.5% crystal violet. The stained cells were extracted using 200µL of 33% acetic acid solution and the optical density was measured at 560nm.

2.3.5 *In vitro* scratch assay

UMUC3 transfected cells were grown in 96-wells tissue culture plates until 100% confluence. Afterwards, a 96-pin WoundMaker (Essen BioScience) was used to create reproducible and precise wounds in all 96-wells. Migration was monitored using a high-definition automated image-lock system (IncuCyte; Essen BioScience). The software was set to scan every 2 hours, for two days. Data were measured as % wound confluence, analyzed using the ANOVA and presented as mean ± standard error.

2.3.6 Migration and invasion – Boyden chamber

Cell migration and invasion were measured by the Boyden chamber assay using the BiocatMatrigel Invasion chamber and Control Inserts (Corning Life Sciences). UMUC3

transfected cells were harvested (trypsin) and counted. For the invasion assay, the chamber was rehydratated with fresh medium for two yours. Then, 1×10^4 cells were resuspended in serum-free medium. A total of 500 µL of medium with 10% fetal bovine serum was added to the lower chamber, and 300 µL of suspended cells were placed into the upper chamber. The assay mixture was incubated at 37°C and 5% CO_2 atmosphere, for 16 h. The plate was removed, and the medium inside the insert was aspirated. The chamber was stained using a cell stain solution, and washed with deionized water. Afterward, stained cells were extracted using 200 µL of extraction solution. Finally, 100 µL of the extracted solution were added to a 96-well microtiter plate and the optical density at 560 nm was measured by the BioTek Synergy HT microplate reader (BioTek, Winooski, VT).

2.3.7 Treatment of MSI1 knockdown cells (KD) with cisplatin or ionizing radiation

UMUC3 cells were transfected with siRNA control or siMSI1 and, 48 hours later, treated with cisplatin (Sigma- Aldrich; Cat# C2210000) at doses ranging from 1 to 1.5 µM, or irradiated (X-ray) at doses ranging from 1 to 5 Gy (CP-160 Cabinet X-radiatior; Faxitron X-ray Corporation, Tucson, AZ). After chemical or radiation exposure, cells were incubated at 37ºC for 2 hours to stabilize. Then, cells were suspended in fresh medium and plated into 96-well plate for the proliferation assay, and into 12-well plate for colony formation assay, as described before. Data were analyzed using the ANOVA, and presented as mean ± standard error. The coefficient of drug interaction (CDI) was used for analyzing possible synergistic effect of the *MSI1* knockdown and radiation. According to CDI values, the interactions were categorized as synergism, additive or antagonism. CDI was calculated as follows: CDI = AB/(A × B), where: AB = value for the combination/value for the control; A and B = value for the single agent/value for the control. CDI<1, CDI=1 and CDI>1 means synergistic, additive and antagonistic, respectively (43–45).

2.3.8 RNA sequencing and analysis

The libraries used for RNA sequencing were prepared using the TruSeq RNA Library Preparation kit (Illumina, San Diego, CA), following manufacturer's instructions, and sequenced in a HiSeq-3000 machine in the UT Health San Antonio Genomic Facility. All experiments were performed in triplicate.

For data analysis, the Human genome (version GRCh37/hg19) and gene annotation (release 19) were downloaded from the UCSC genome Browser (https:/genome.ucsc.edu) and GENCODE (38), respectively. FASTQ read files from the RNA sequencing were aligned to human genome with the Genomic Short-read Nucleotide Alignment Program (GSNAP) (46); parameters: -t 24 -B 4 -N 1 -E 1 --input-buffer-size 100000 --output-buffer-size 100000 --trim-mismatch-score=0 --trim-indel-score=0). We also provided to the aligner a file with known splice sites, generated from the transcriptome data (GENCODE, release 19). In order to have only reliable alignments, only those reads with mapping quality over 20 (Q>20, selected using the SAMtools) (47) were kept. For all samples, the final set of mapped reads were matched to the reference to the transcriptome (GENCODE) using the HTseq-count (37); parameters: -f bam -r pos -a 20 -m union -s reverse). A matrix with all expressed genes and their samples was generated.

The DEseq2 package was used to determine differentially expressed gene between *MSI1* KD-UMUC3 cells vs. control UMUC3-cells (39). Up-regulated genes were defined as those presenting logFC > 1 and p-value adjusted < 0.05; down-regulated genes as logFC < -1 and p-value adjusted < 0.05.

2.3.9 Crooslinking immunopreciptation (iCLIP)

For the iCLIP experiments, UMUC3 cells were grown in 10 cm Petri dishes, washed with cold PBS and UV irradiated in 5 mL PBS (on ice) at 150 mJ/cm^2 and 254 nm. Cells

were scraped, transferred to microtubes and spun at top speed for 10 seconds at 4°C. The irradiated cells were treated with low (1:1000) and high concentrations (1:50) of RNase I (48), and the cross-linked endogenous MSI1-RNA complexes were immunoprecipitated using anti-MSI1 antibodies and radiolabeled with T4 polynucleotide kinase. Normal rabbit IgG was used as negative control. Immunoprecipitated MSI1-RNA complexes were separated using SDS–PAGE and transferred onto nitrocellulose membranes. The autoradiogram of the equivalent gel showed the radiolabeled complexes. Samples were sequenced in the Illumina Hiseq3000.

2.3.10 Functional annotation

Functional annotation analyses (Gene Ontology and KEGG pathways) were performed using the DAVID (49), and the Homo sapiens genes as background. Terms with Benjamini-Hochberg corrected p-values < 0.05 were determined as enriched. Redundant GO terms were summarized using REViGO (50). Networks of GO terms were built using the Cytoscape (51).

2.4 Cell treatment with luteolin and cisplatin or ionizing radiation

UMUC3 cells were plated into 35mm dishes and, 24 hours later, treated with luteolin (Sigma-Aldrich; Cat# L9283). After 48 hours, cells were exposed to cisplatin (1 and 1.5 μM) for 24 hours or irradiated at doses of 1, 2.5 and 5 Gy (CP-160 Cabinet X-radiator; Faxitron X-ray Corporation). Afterwards, cells were suspended in fresh medium and plated into 96-well plate for proliferation assay, as described before.

3. RESULTS

Several RBPs were aberrantly expressed in high grade bladder tumors

To identify RBPs potentially involved in high grade tumor development, the expression profiles of all 1,542 human catalogued RBP coding genes were examined (40).

Raw RNA sequencing (RNA-seq) data for 412 bladder cancer samples from TCGA database were obtained. This approach allowed the identification of 236 upregulated and 193 downregulated RBPs genes in high grade compared to low grade tumors (Supplementary file 1). Then, we focused on the up-regulated RBPs, because they tend to be more attractive in the therapeutic contexts (52). A survival analysis by PrognoScan data base was conducted (53), and 14/236 upregulated RBPs showed association with reduced patient survival ($p < 0.05$; log-rank test) (Fig. 1) (Table I). Next, the expression of these 14 RBP genes was investigated in tumor samples, and the RT-qPCR analysis showed 7/14 RBPs also overexpressed in high-grade compared to low-grade tumor (Fig. 2).

Table I: RNA binding proteins (RBPs) up-regulated in High grade tumors and related to poor prognosis

Gene Symbol	Gene ID	Full name	Function	Reference
NOCT	25819	*Nocturin*	This gene are related to complex controls as mRNA metabolism through of mRNA elongation (deadenylation), degradation of mRNA, activation and repression of mRNA initiation, the gene can be yet, effects anti-proliferative by cell cycle arrest via p21-dependend and p53-independent pathway and an important relation in carcinogenesis	(54–57)
CELF2	10659	*Elav-like family member 2*	The gene have been demonstrated play a role in regulating alternative splicing, mRNA editing	(58–60)
*ENDOU**	8909	*Endonuclease, poly(U) specific*	P11 were demonstrated be detected in ovarian malignant cells but not in normal ovaries, suggesting this protein can be a marker for early diagnosis, and also participates in the biosynthesis of small nucleolar RNAs, involved in ribosome biogenesis	(61–63)
*EXO1**	9156	*Exonuclease 1*	Encodes a protein with 5' to 3' exonuclease activity as well as an RNase H activity, also interact with components of mismatch repair	(64–66)
*EZH2**	2146	*Enhancer of zeste 2 polycomb repressive complex 2 subunit*	This gene is involved for mechanism of methylation of Lys 9 and, more highly, Lys 27 of histone H3 and Lys 26 on histone H1. Although the gene is overexpression in various type of cancer, the oncogenic mechanism of this gene is unknown.	(67–71)
IFIT2	3433	*interferon induced protein with tetratricopeptide repeats 2*	Play a significant role in the mediation of cellular apoptosis in response to viral infection or IFN signaling.	(72)
*MOV10L1**	54456	*Mov10 RISC complex RNA helicase like 1*	Is a predicted RNA helicase that is essential for primary piRNA biogenesis. However, there is no information in the literature about the mechanism and expression this gene in other tissues.	(73)

*MSI1**	4440	*Musashi RNA binding protein 1*	MSI seems to be strongly associated with tumor progression in human somatic tumors as endometrial cancer, esophageal adenocarcinoma and gastric cancer. MSI also can module gene involved in cell proliferation, cell differentiation, cell cycle and apoptosis.	(74, 75, 76, 77)
PEG10	23089	*Paternally expressed 10*	PEG10 was demonstrated promoted proliferation, migration and invasion in human lung cancer cell line and can be regarded as a potential prognostic marker for this cancer. Related to the carcinogenesis, progression and prognosis of gallbladder adenocarcinoma.	(78, 79)
PTRF	284119	*polymerase I and transcript release factor*	Involved in a variety process including cell repair and senescence, beside glucose metabolism and regulation of lipid. Also are related with transcription termination by RNA polymerase I. i) Extension of the transcription complex is paused; ii) Then release of pre-rRNA and RNA polymerase I from the paused transcription complex occurs.	(80,81,82)
*TERT**	7015	*Telomerase reverse transcriptase*	Over-expression of TERT have been associated with metastasis, invasion and cell proliferation.	(83)
TRIM71	131405	*Tripartite motif containing 71, E3 ubiquitin protein ligase*	TRIM71, A regulator of stem and progenitor cell proliferation and differentiation, can too silence mRNA and drive protein ubiquitylation.	(84–87)
*WARS**	7453	*Tryptophanyl-tRNA synthetase*	WARS, are involved in regulation of RNA transcription and translation, angiogenic signaling pathways and protein synthesis.	(88)
*YBX2**	51087	*Y-box binding protein 2*	YBX2 have been related with DNA repair , DNA replication and RNA splicing.	(89,90,91)

*also differentially expressed in high–grade tumors in samples from Brazil.

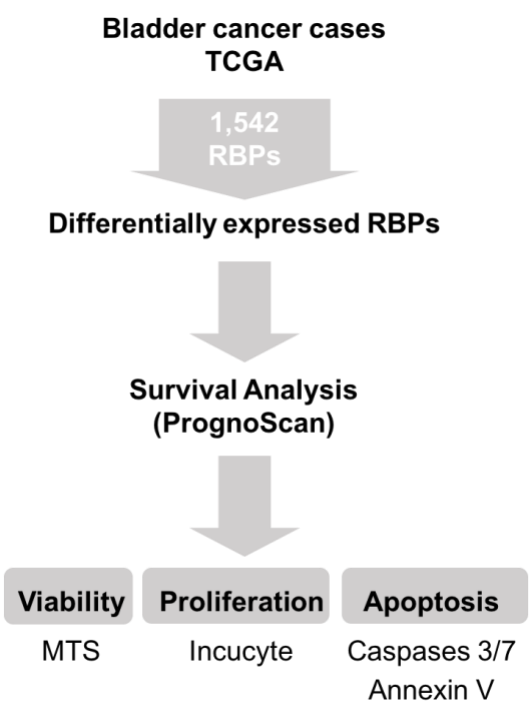

Figure 1. Study design. Differentially expressed RNA binding protein (RBP) genes in high-grade vs. low-grade tumors. The RBPs were evaluated regarding their association with poor prognosis. Their functional impact on bladder tumor was assayed by a set of functional assays.

RBPs impacted on cancer relevant process

The 14 selected RBPs were also investigated in a functional screening. Transient knockdowns of each RBPs were obtained in J82, UMUC3 and T24 cell lines using specific siRNAs (median knockdown efficiency ~75%), and their impact on cell proliferation (Incucyte), viability (MTS assay), and apoptosis (caspase-3/7 and annexin V assays, Incucyte) were evaluated. Results of these four assays are summarized in Table II.

Table II: Summary of *in vitro* assays results

Gene	Proliferation			Viability (MTS)			Annexin V			Caspases-3/7		
	T24	UMUC3	J82	T24	UMUC3	J82	T24	UMUC3	J82	T24	UMUC3	J82
CELF2	*	-	*	-	-	-	-	***	-	-	-	-
ENDOU	***	-	*	-	-	-	-	***	-	-	**	-
EZH2	***	-	-	-	***	-	***	***	-	*	***	-
EXO1	-	-	***	-	-	-	**	-	**	-	-	-
IFIT2	**	-	-	-	-	-	-	-	-	-	***	-
MOV10L1	***	-	**	-	-	-	-	***	**	-	***	-
MSI1	***	***	***	**	***	***	***	***	***	***	*	***
NOCT	***	-	***	-	-	-	-	-	-	-	*	-
PEG10	***	-	**	-	***	-	**	-	-	-	*	-
PTRF	-	-	**	-	-	-	***	-	-	***	-	-
TERT	***	-	***	***	***	***	-	**	-	-	-	-
TRIM71	***	-	**	-	***	**	-	***	*	-	***	-
YBX2	-	-	**	-	**	*	*	-	-	-	*	-
WARS	***	-	***	-	***	-	**	-	-	-	*	**

*$p<0.05$; **$p<0.01$; ***$p<0.001$

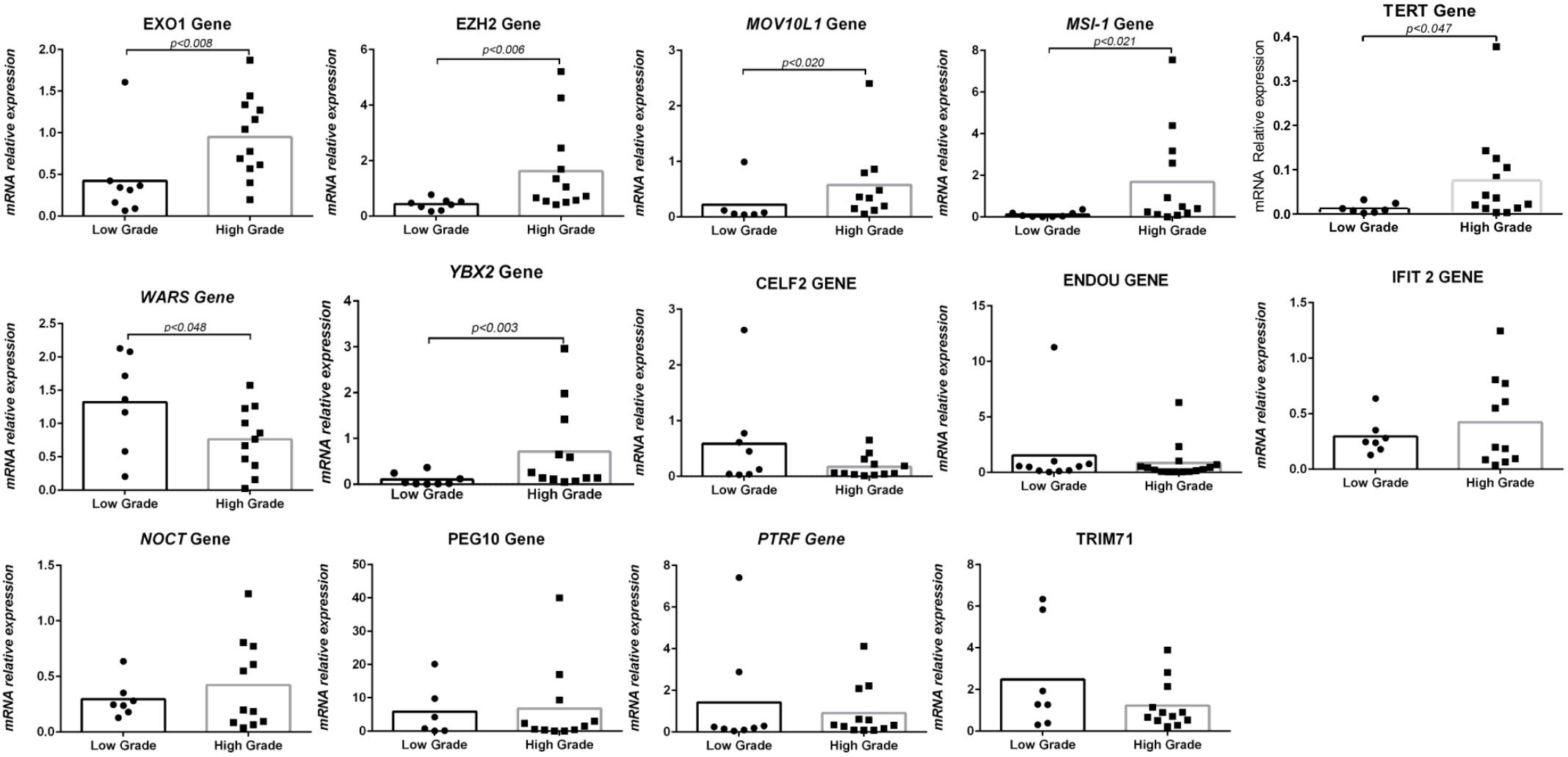

Figure 2. mRNA levels of 14 RNA binding proteins in bladder tumor samples from Brazil

MSI1 oncogenic properties in high-grade bladder tumors

The *MSI*-knockdown (KD) exhibited exciting results in the functional screening: it caused a decrease in cell proliferation (Fig. 3A), and increases of caspases 3/7 (Fig. 3B) and annexin V (Fig. 3C) activities in UMUC3 and T24 cells. In addition, decreased colony growth (Fig. 3D), migration (Fig. 3E, 3G) and invasion (Fig. 3F) were also observed in UMUC3 cells.

To determine the relationship between *MSI1* expression and chemo/radiotherapy resistance, we combined *MSI1*-KD and treatment with cisplatin or x-ray. *MSI1*-KD resulted in a decreased cell resistance to both types of treatments. Knockdown cells showed a strong decrease of cell proliferation and colony formation after treatment with cisplatin (Fig. 4 A, B) and x-ray (Fig. 4 C, D).

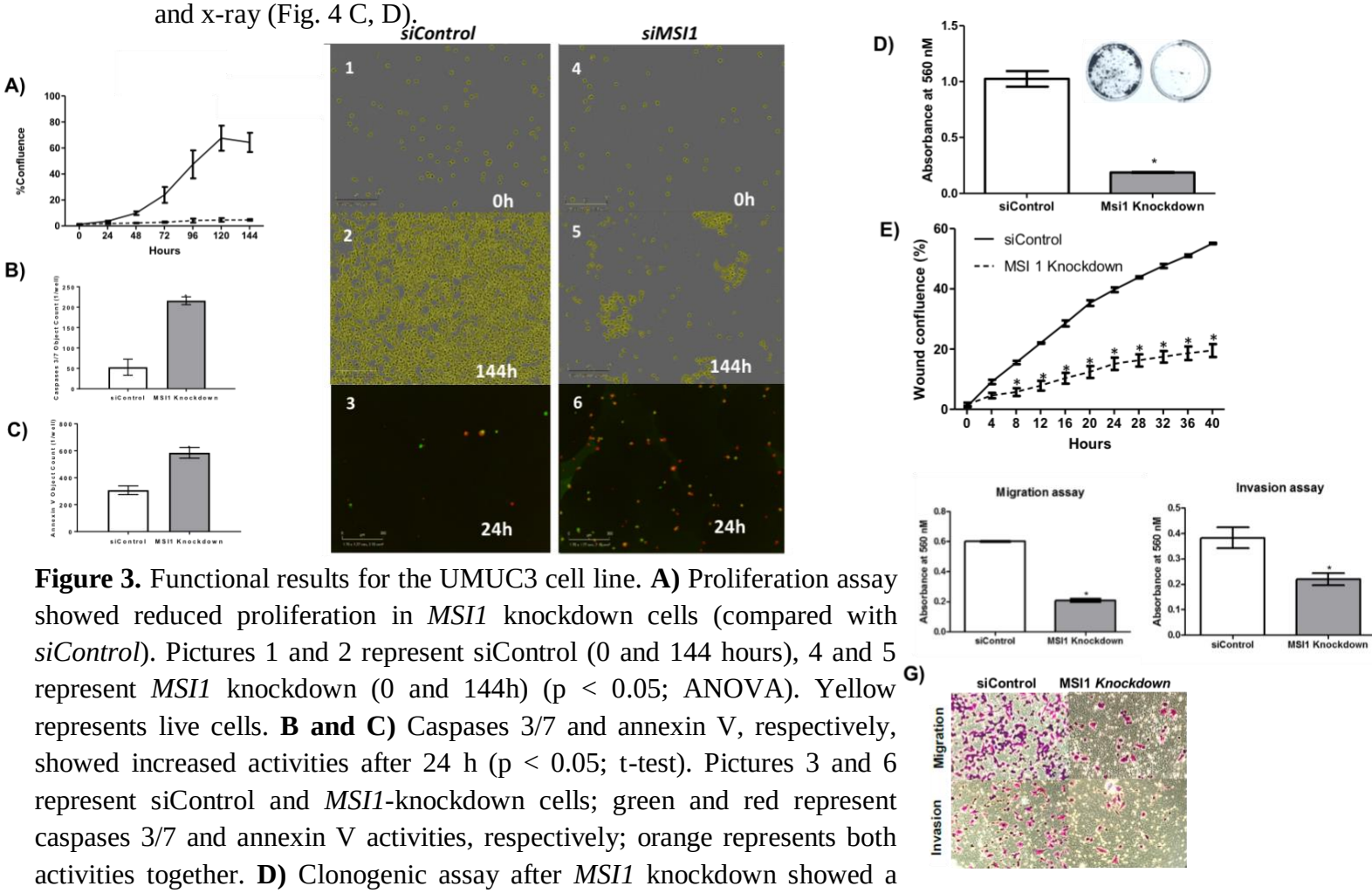

Figure 3. Functional results for the UMUC3 cell line. **A)** Proliferation assay showed reduced proliferation in *MSI1* knockdown cells (compared with *siControl*). Pictures 1 and 2 represent siControl (0 and 144 hours), 4 and 5 represent *MSI1* knockdown (0 and 144h) ($p < 0.05$; ANOVA). Yellow represents live cells. **B and C)** Caspases 3/7 and annexin V, respectively, showed increased activities after 24 h ($p < 0.05$; t-test). Pictures 3 and 6 represent siControl and *MSI1*-knockdown cells; green and red represent caspases 3/7 and annexin V activities, respectively; orange represents both activities together. **D)** Clonogenic assay after *MSI1* knockdown showed a decrease of colony formation (compared with siControl; $p < 0.05$; t-test). **E)** *In vitro* scratch assay showed a decrease of cell migration (compared to control after 8 hours; $p < 0.05$; ANOVA). **F)** Transwell migration and invasion assays: *MSI1* knockdown resulted in a reduced cell migration and invasion compared to control ($p < 0.05$; t-test). The dye absorbed by cells was dissolved in 33% acetic acid solution and quantified by absorbance at 560 nm. **G)** Photomicrograph of a transwell chamber migration and invasion cells. All results are representative of three independent experiments.

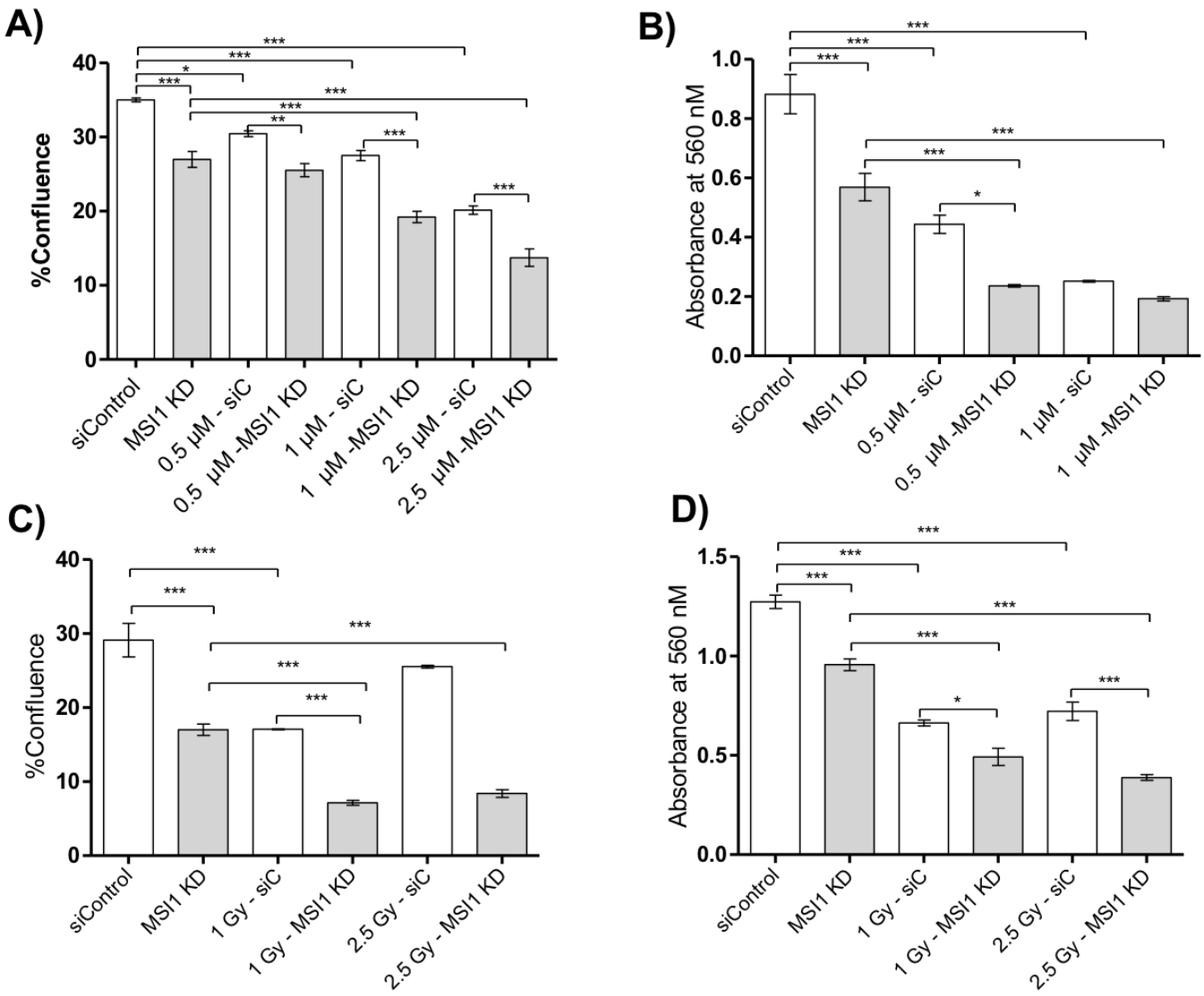

Figure 4. Graphs showing the synergistic effect between MSI1 knockdown and chemical and radiation treatments (Coefficient of drug interaction < 0.9). UMUC3 MSI1- knockdown cells showed decreased resistance to cisplatin and radiation. **A)** Proliferation assay showed decreased proliferation of UMUC3 MSI1 knockdown cells with the lower dose of cisplatin (compared to siControl). **B)** Clonogenic assay showed decreased colony formation in the UMUC3 MSI1-knockdown cells treated with 0.5 µM of cisplatin. **C)** Proliferation assay after exposure to X-rays. UMUC3 MSI1- knockdown cells showed decreased cell proliferation after low doses of X-rays (1 and 2.5 Gy). **D)** Clonogenic assay after radiation showed decreased colony formation in UMUC3 MSI1- knockdown cells (dose response effect). *p < 0.05; **p < 0.01; ***p < 0.001; ANOVA, Tukey.

MSI1 knockdown affected gene expression

To determine the contribution of *MSI1* to bladder cancer development, UMUC3 cells were knockdown and RNA-seq analysis was carried out. Then, we mapped changes in the transcriptomic profiling and compared to that of the control UMUC3 cells. A total of 1,090 differentially expressed (193 upregulated and 897 downregulated) genes (log2 fold change > |1| *p*-value < 0.05) was found (Supplementary file 2). Among the upregulated genes,

we observed strong enrichment for biological process related to negative regulation of transcription from RNA polymerase II, G1/S transition of mitotic cell cycle, skeletal muscle cell differentiation (Fig. 5). The downregulated genes showed strong association with Wnt-pathway, cell differentiation, embryo development and locomotion (Fig. 6). Moreover, we found nine downregulated lncRNAs (SBF2-AS1, CTD-213N18.2, JHDM1D-AS1, LINC01152, CTD-2339F6.1, RP4-785G19.5, AC074286.1, RP11-428J1.5 and RP11-977B10.2) after *MSI1* knockdown, all of them usually related to poor prognosis when upregulated in bladder cancer (Table III).

Table III: Differentially expressed lncRNAs in MSI1 knockdown (KD) cells related to poor prognosis for bladder cancer.

ENSBL	GENE ID	P-value	Bladder Tumors	MSI 1 KD Cells	Others tumors
ENSG00000246273	SBF2-AS1	0.05	Up	Down	CESC*, LUAD**
ENSG00000267221	CTD-2132N18.2	0.04	Up	Down	OV*
ENSG00000260231	JHDM1D-AS1	0.04	Up	Down	
ENSG00000256124	LINC01152	0.04	Up	Down	
ENSG00000254139	CTD-2339F6.1	0.04	Up	Down	
ENSG00000250917	RP4-785G19.5	0.01	Up	Down	HNSC*, LUAD*,
ENSG00000213963	AC019080.1	0.01	Up	Down	LUSC**
ENSG00000272142	RP11-428J1.5	0.009	Up	Down	LGG*, LIHC*, UCEC**
ENSG00000258101	RP11-977B10.2	0.006	Up	Down	LGG*

CESC: cervical squamous cell carcinoma and endocervical adenocarcinoma; LUAD: lung adenocarcinoma; OV: ovarian serous cystadenocarcinoma; HNSC: head and neck squamous cell carcinoma; LUSC: lung squamous cell carcinoma; LGG: low grade glioma; LIHC: low hepatocellular carcinoma; UCEC: uterine corpus endometrioid carcinoma; *up-regulated; ** down-regulated

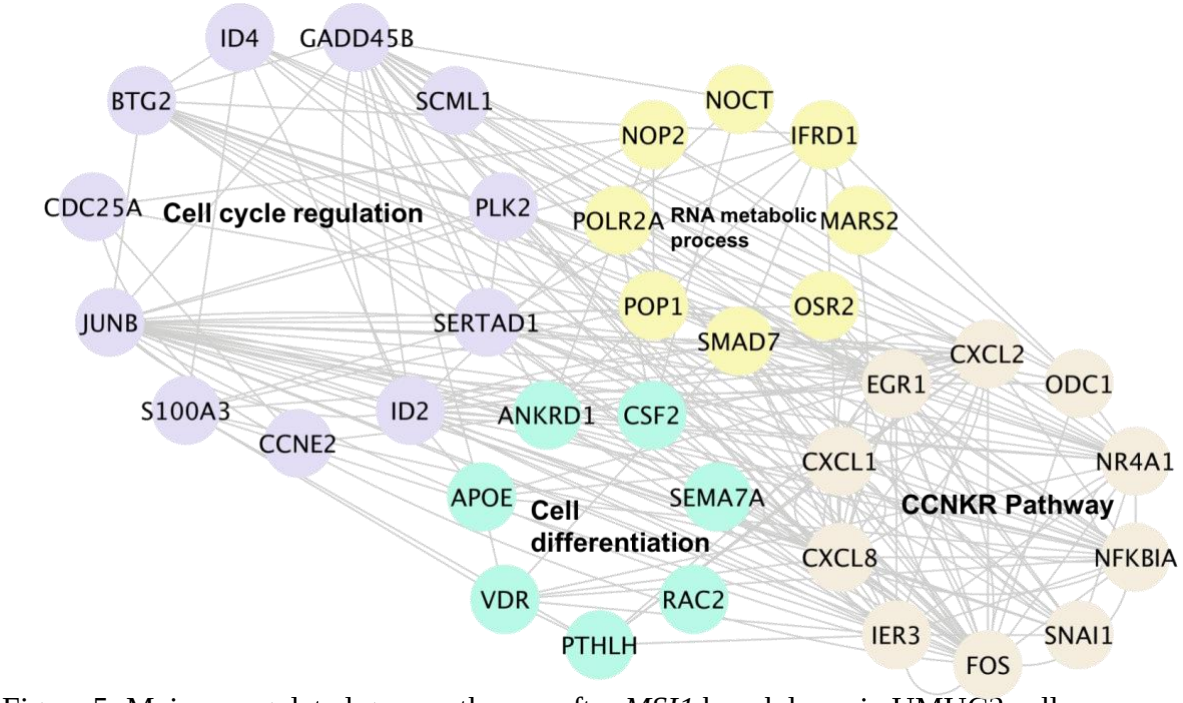

Figure 5: Main upregulated gene pathways after *MSI1* knockdown in UMUC3 cells.

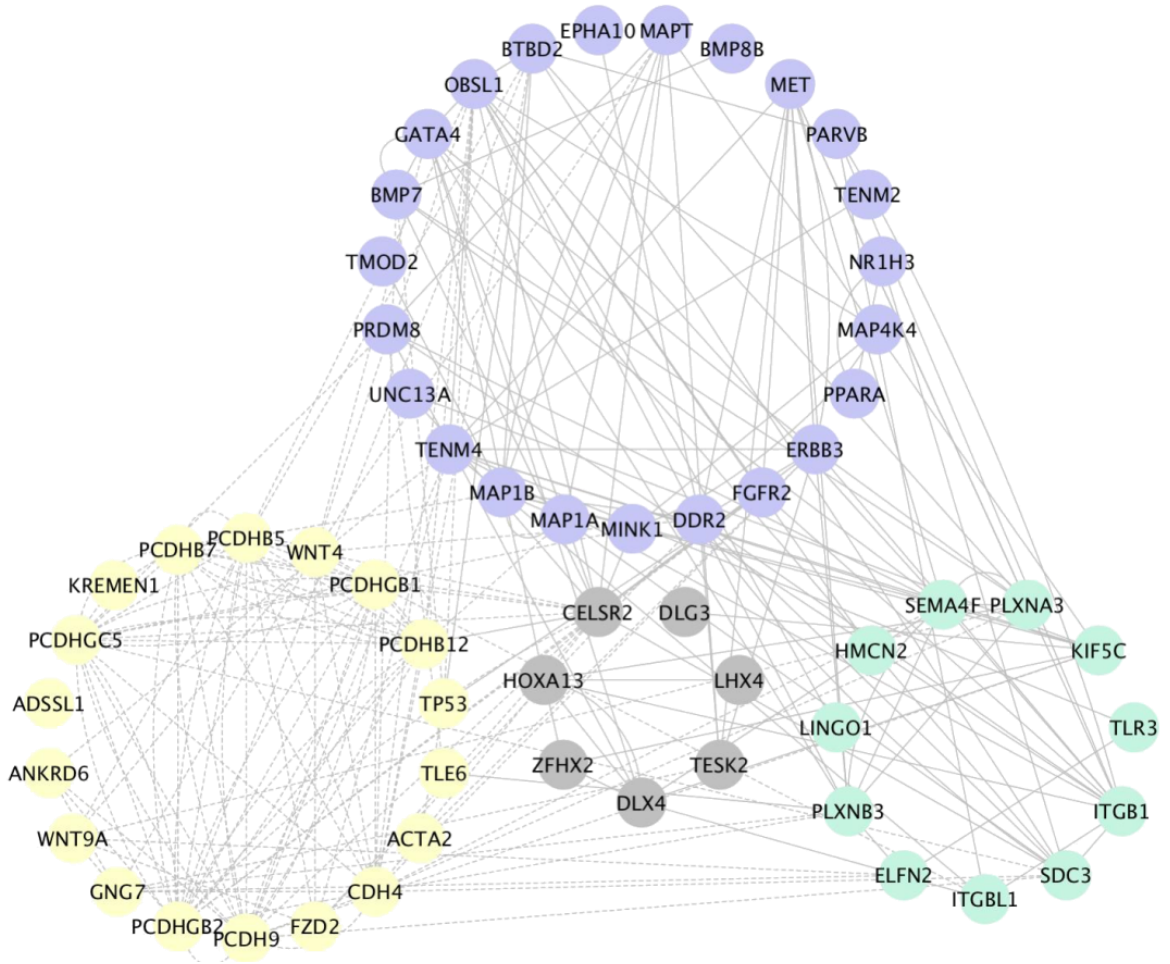

Figure 6: Main downregulated gene pathways after *MSI1* knockdown in UMUC3 cells.

Musashi-1 targets were strongly associated with translation genes

To determine the musashi-1 targets in UMUC3 cells, we carried out the iCLIP analysis. A total of 363 targets were identified (Supplementary file 3). Among them, 44% had the mRNA intronic region as the binding site; 33% the 3'UTR, 17 % the coding sequencing (CDS), 2% the 5'UTR and 4% miRNA and lncRNA regions (Figure 7A). Data enrichment showed a strong relationship between these targets and post-embryonic development, positive regulation of transcription and protein phosphorylation (Fig. 7B). Furthermore, several of these targets were related to RNA processing, translation, DNA replication and repair (Fig. 7C). The pathway enrichment showed that musashi-1 binding in targets related to *Wnt*, *TP53*, *PDGF* and *CCRKR* pathways and angiogenesis (Fig. 7D). Twenty-two of the musashi-1 target genes were also differentially expressed after the *MSI1* knockdown.

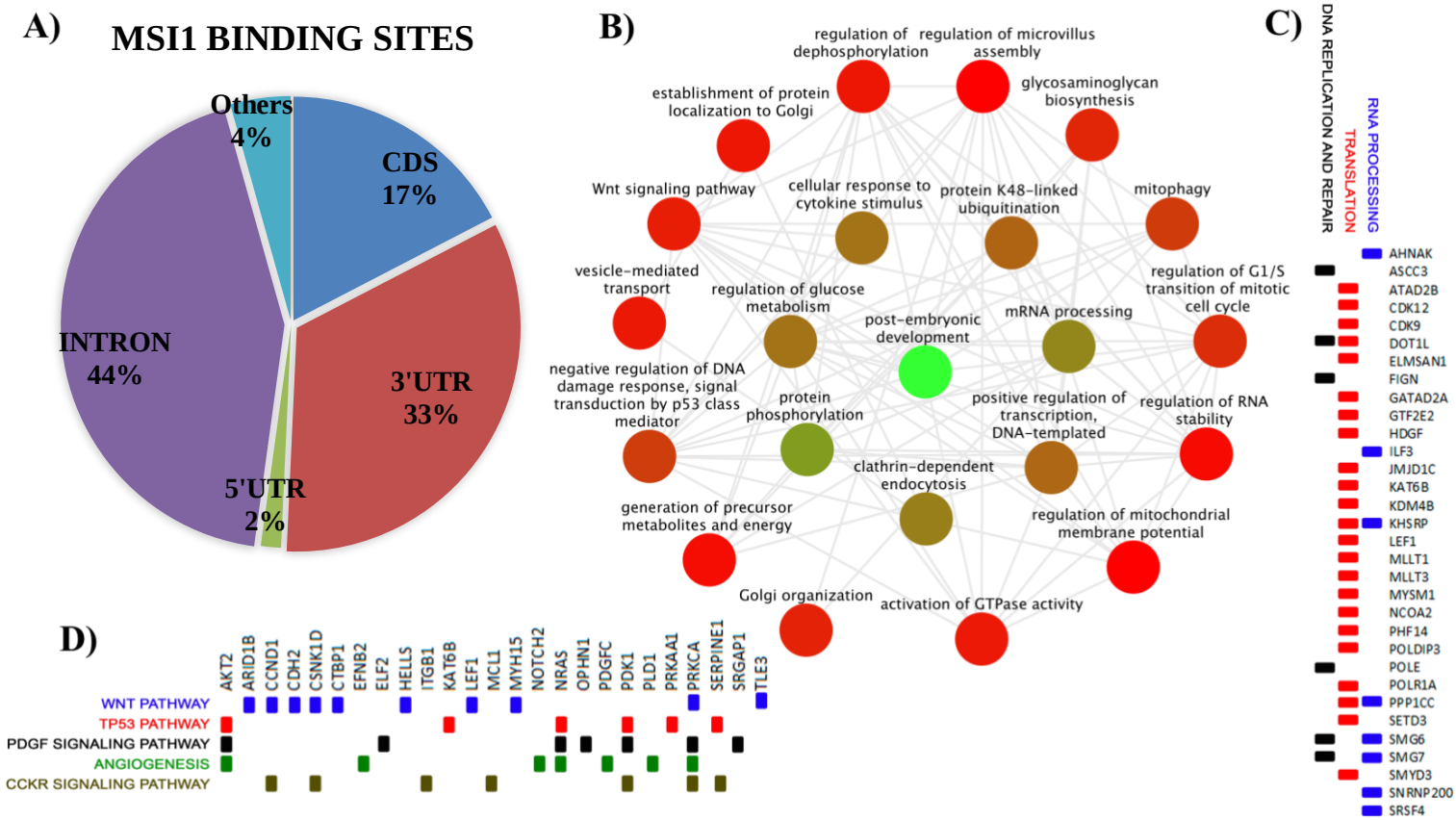

Figure 7. *MSI1* targets enrichment functional analysis. **A)** Musashi1 binding sites. **B)** Biological enrichment showed strong association with post-embryonic development, mRNA processing and protein phosphorylation. **C)** *MSI1* targets were associated with RNA processing, translation, DNA replication and repair. **D)** *MSI1* targets showed association with important pathways in carcinogenesis, such as *Wnt, TP53, PDGF* and *CCKR* signaling pathways and angiogenesis.

Luteolin inhibited cell proliferation and increased sensitivity to cisplatin and radiation

Herein, we also investigated whether the treatment with luteolin could produce similar impact than the *MSI1*-KD in UMUC3 cells. Firstly, we evaluated the isolated effect of lutein in UMUC3 cells. Data showed that luteolin was able to inhibit cell proliferation at the four concentrations tested (range of 15-30 µM) (Fig. 8A). Then, we checked the impact of lutein in cells treated with cisplatin (1 and 1.5 µM) or radiation (range of 1-5 Gy). Data demonstrated that luteolin increased cell sensitivity to cisplatin and radiation, similarly to the effect previously observed with the *MSI1*-KD (Fig. 8B and C; Supplementary Fig. 3B). In addition, a synergistic (CI<1) effect of luteolin and x-ray was detected in UMUC3 cells (Fig. 8D).

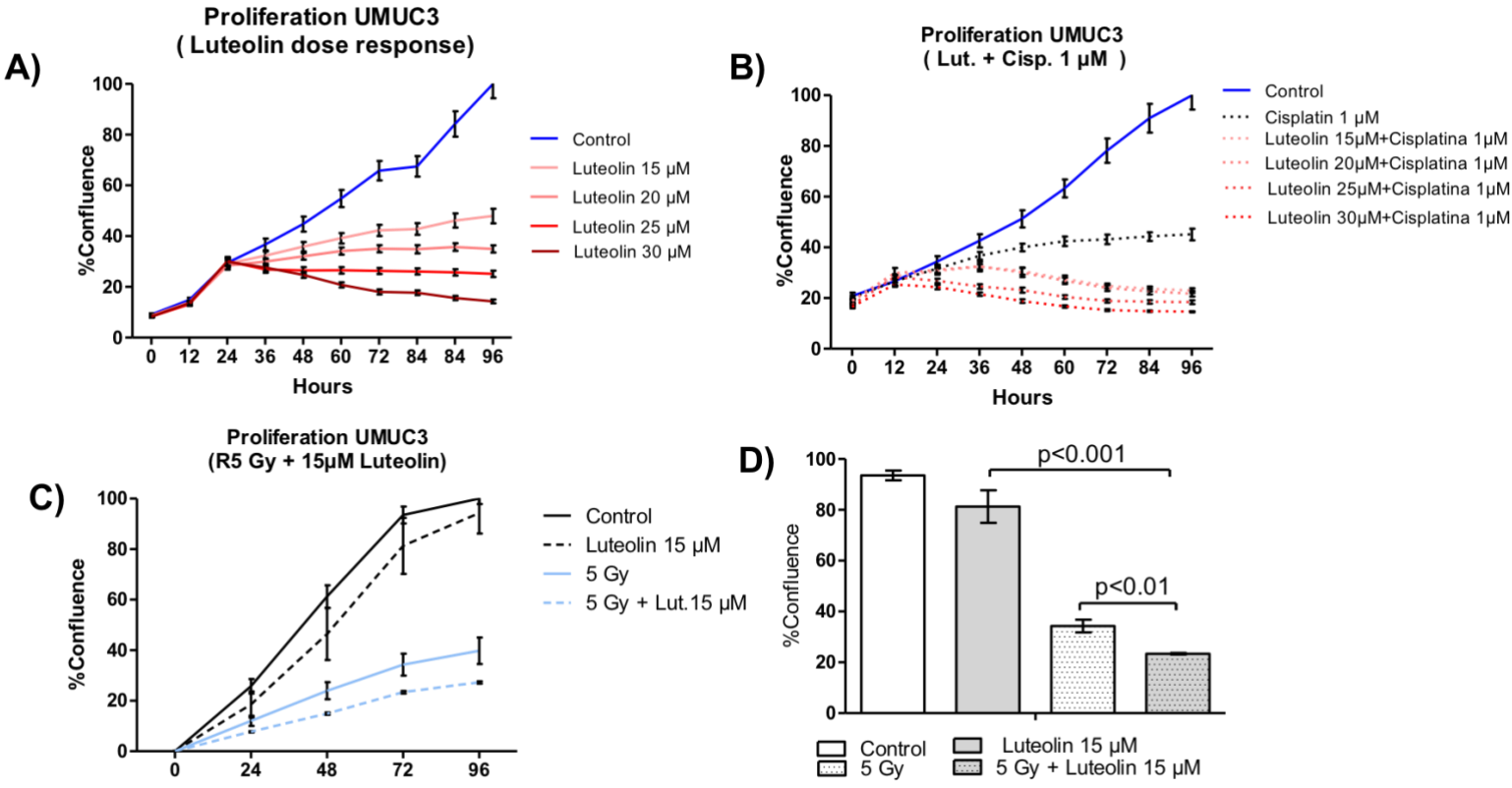

Figure 8. Luteolin sensitizes bladder cancer cell UMUC3 to cisplatin and radiation. UMUC3 cells were treated with luteolin for 48 hours, then the cells were exposed to cisplatin from 1-1.5 µM or radiation (x-ray) from 0-5 Gy. Treated cells were harvest and seed for proliferation assay. **A)** UMUC3 cell proliferation after treatment with luteolin were monitored over a period of 96-hours. **B)** UMUC3 cell proliferation assays after Luteolin and Cisplatin combined treatment. **C)** UMUC3 cell proliferation after Luteolin and X-ray combined treatment. **D)** The graph shows side-by-side differences in proliferation between single and combined treatment at 96 hours. All experiments

were done in triplicate. The combination treatment of luteolin and 5 Gy radiation showed synergistic effect in proliferation, which was judged by the Combination Index (CI) (CI< 1). Statistical significance was calculated by one-way ANOVA and multiple t test.

4. DISCUSSION

RBPs are feasible molecular markers for high grade bladder tumors

Several studies have demonstrated that RBPs are abnormally expressed in various types of cancer, and they are correlated with the patient prognosis (15,16, 45–47). Herein, we confirmed such findings and expanded the knowledge in concerning RBPs role in bladder carcinogenesis. By comparing data from the TCGA, we identified 14 up-regulated RBPs in high-grade *vs.* low-grade tumors, which were also related to poor-prognosis. Seven of these RBPs (*EXO1, EZH2, MOV10L1, MSI1, TERT, WARS, YBX2*) were also up-regulated in high-grade tumor samples obtained from a Brazilian biorepository (University of São Paulo). In addition, the *in vitro* functional analyses confirmed the strong link between such RBPs and important steps of carcinogenesis (cell proliferation, viability and apoptosis). Based on such findings, we believe these RBPs could be considered as feasible biomarker candidates for high-grade bladder tumors.

Does *MSI1* behave as an oncogene in human urinary bladder carcinogenesis?

Data obtained after *in vitro* UMUC3 *MSI1*-KD showed that this gene was related to reduced cell migration and invasion, and resistance to chemo and radiotherapy. Actually, some studies have demonstrated that the *MSI1* knockdown is associated to decreased tumor growth (95–98). However, the mechanisms by which *MSI1* exerts such effect are not completely known. Liu et al. (95) have showed that *MSI1* promotes growth by targeting the cell cycle checkpoint protein p21, p27 and p53. Recently, Master et al (99) reported that *MSI1* also promotes epithelial mesenchymal transition (EMT) through activation of the canonical Wnt signaling pathway in cervical cancer. EMT is a reversible process that

involves changes in cell morphology, differentiation and motility, facilitating invasion and metastasis (100,101). Alterations in the EMT have been associated with high-grade bladder tumor, resistance to therapeutic agents and poor outcome (102). Similarly, some studies have showed that the upregulation of the Wnt/β-catenin pathway stimulates resistance to the combined therapy interferon-alpha and 5-fluouracil for hepatocellular carcinoma (103), and radioresistance in head and neck cancers (104). Additionally, increased cell proliferation and activation of Wnt-β-catenin signaling were described in colon cancer cells with overexpression of *MSI1* (66). In agreement with these previous results, our data demonstrated that the canonical Wnt/β-catenin was one of the main pathway affected by the *MSI1*-KD. Namely, *CTNNBIP1*downregulation (consequently reduced level of β-catenin), decreased level of the lncRNA ZEB1-ASI l and, consequently, a downreguation of *ZEB1* (a gene involved in the EMT) (53), increased expression of *DKK* and *APCDD1* genes (Wnt-pathway inhibitors) (105) were detected in UMUC3-KD cells. Furthermore, the *MSI1-KD* was associated to decreased expression of *LRP*, a transmembrane receptor which supports wnt pathway activation (106,107). Taken into account that the Wnt/β-catenin pathway activation seems to play a role in the maintenance of bladder epithelium stem cell and tumor initiating cells (108,109), we speculate that the *MSI1* expression might have important role in bladder cancer aggressiveness through the Wnt/β-catenin pathway. Besides this relationship between *MSI1* and the Wnt/β-catenin pathway, other interesting finding in our study was the association between *MSI1*-KD and the downregulation of nine lncRNAs related to bladder cancer poor prognosis, six of them (SBF2-AS1, JHDM1D-AS1, RP4-785G19.5, AC074286.1, RP11-428J1.5, and RP11-977B10.2) related to high-grade bladder tumor. Therefore, taken together our data indicated *MSI1* as having important role in bladder cancer carcinogenesis.

Musashi1 binding preferentially to 3'UTR and intronic region

Recently, the new molecular classification of muscle invasive bladder cancer included a sub-group called neuronal-like, with *MSI1* overexpression, poorest survival and aggressive phenotype (110). Musashi1 is highly expressed protein in the neuronal progenitor and stem cells for maitenance of self-renewal and differentiation (111). Biochemical and structural studies have demonstrated mustachi1 has two N-termiSERPInal RNA recognition motifs (RRM) mediate Musashi1 binding to a group of target mRNAs. RRM1 contributes the majority of the binding energy and specificity, while RRM2 has a more supportive role. In musashi-targeted genes, RRM1 and RRM2 recognition sequences are found in a close proximity at the 3′-end of the mRNAs (112). Various studies have reported that *MSI1* functions as a translational repressor on a variety of mRNA species by binding to a specific consensus sequence in the 3'UTR (113,114). The high resolution iCLIP assay provided us the opportunity to examine the musashi1 binding site in UMUC3 bladder cancer cell line. The 3′ UTR binding sites (nearly to 33%) identified in our study, were consistent with the long-held hypothesis that musashi1 targets the 3′ UTR to execute its primary function as a regulator of mRNA stability and/or translation (115–117). Our data showed that musashi1 bound to downstream sites of important mRNA, such as SERPINE1, NRAS, CD44, PDK1, CTSB, PTTG1, PLD1, SMAD3 and TIMP2, involved in multiple carcinogenesis processes (83-91). However, the majority (nearly 44%) of the musashi1 binding sites were in intronic regions, suggesting an additional and unexpected role of this protein in pre-mRNA processing. Interestingly, some of the genes with binding sites in intronic region are associated with important cancer process, such as cell cycle control, MAPK cascade and Wnt pathway.

Luteolin also impacts on cell proliferation and cancer therapeutic resistance

Some studies have suggested the use of luteolin as adjuvant therapy for cancer (31,32,120,121). The *in vitro* synergistic effect betweeen luteolin and bacillus Calmette-Guerin (BCG) induced a greater inhibition of cell proliferation and apoptosis rate in a bladder cancer cell line (122). Huang et.al (30) described a chemo-sensitize effect of luteolin on cisplatin-resistant ovarian cancer cell line, by enhancing cell death. According to these authors the flavonoid caused a down-regulation of Bcl-2 gene and protein. Luteolin ability to increase the sensitivity of glioblastoma cell to PARP inhibitor and x-ray treatment was also reported. Recently, Yi et.al (28) demonstrated that luteolin interacts with the Msi1 RNA binding domain and blocks musashi1 effect on the expression of some target genes. Herein, we confirmed the strong effect of luteolin on cell proliferation and its ability to increase cell sentivity to cisplatin and radiation also in urinary bladder cancer cells. Therefore, these finding confirmed the relevance of luteolin as a potential adjuvant therapeutic compound and highlighted musahi1 as a potential therapeutic target in bladder cancer.

CONCLUSION

In summary, the results provided evidences of the strong link between some RBPs and important steps of carcinogenesis, and indicated such RBPs as feasible biomarker candidates for high-grade bladder tumors. In addition, the study showed *MSI1* as an important key gene in bladder carcinogenesis, and the musashi1 as evolving in bladder tumor chemo/radio resistance, highlighting these RBP as a potential therapeutic target. Furthermore, the data confirmed luteolin as a possible adjuvant therapeutic compound also for bladder cancer.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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Supplementary figures

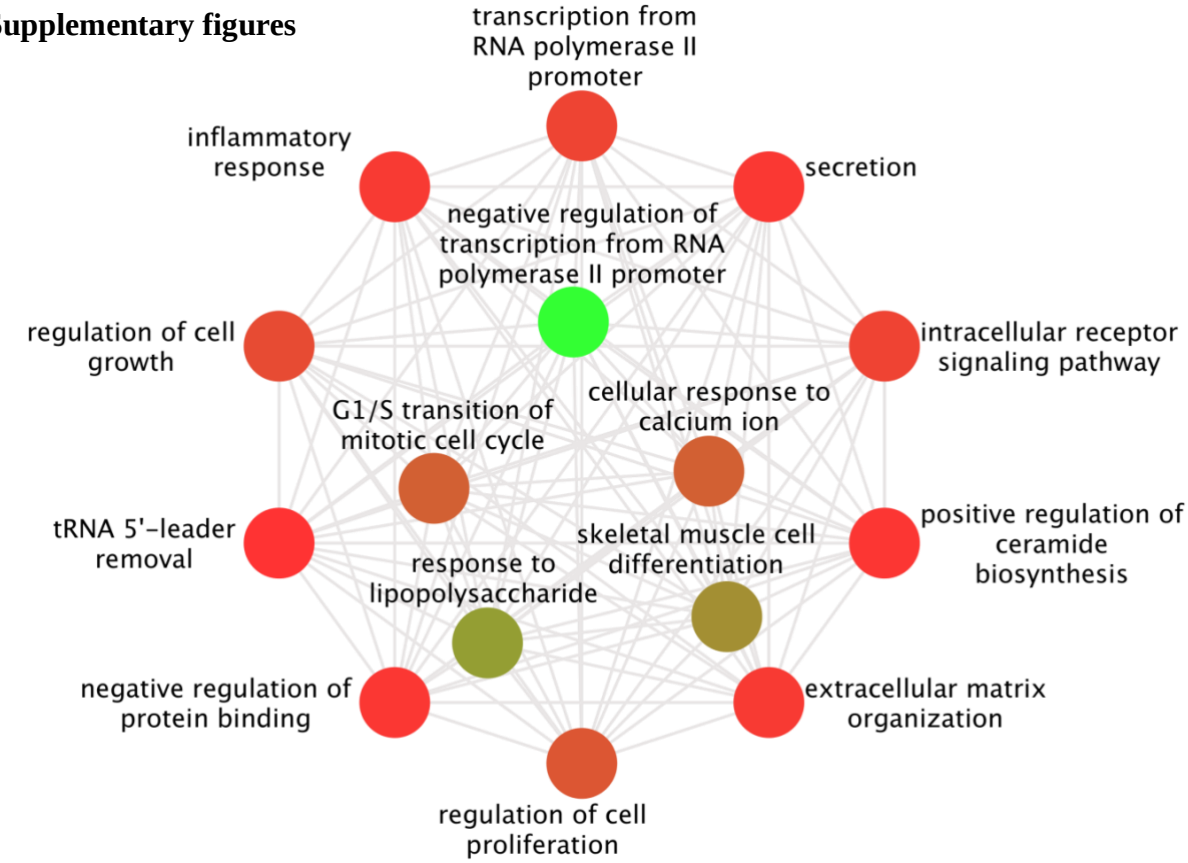


Figure 1S: Gene ontology pathway of upregulated genes in UMUC3 MSI1 KD cells.

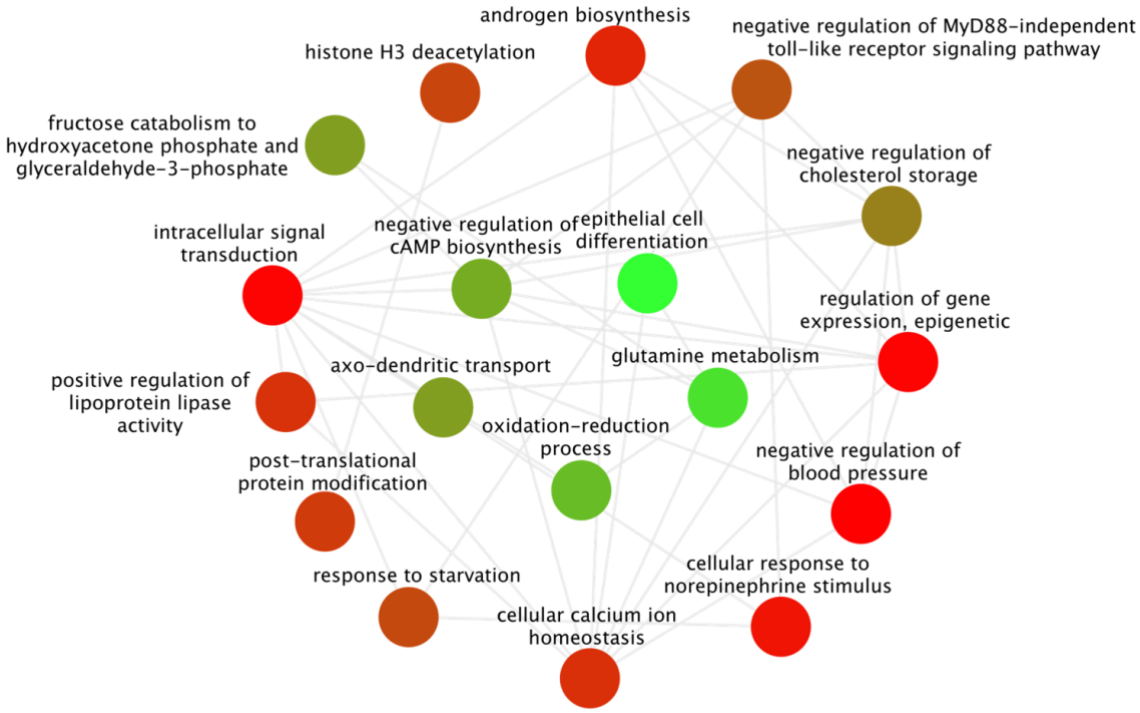


Figure 2S: Gene ontology pathway of downregulated genes in UMUC3 MSI1 KD cells.

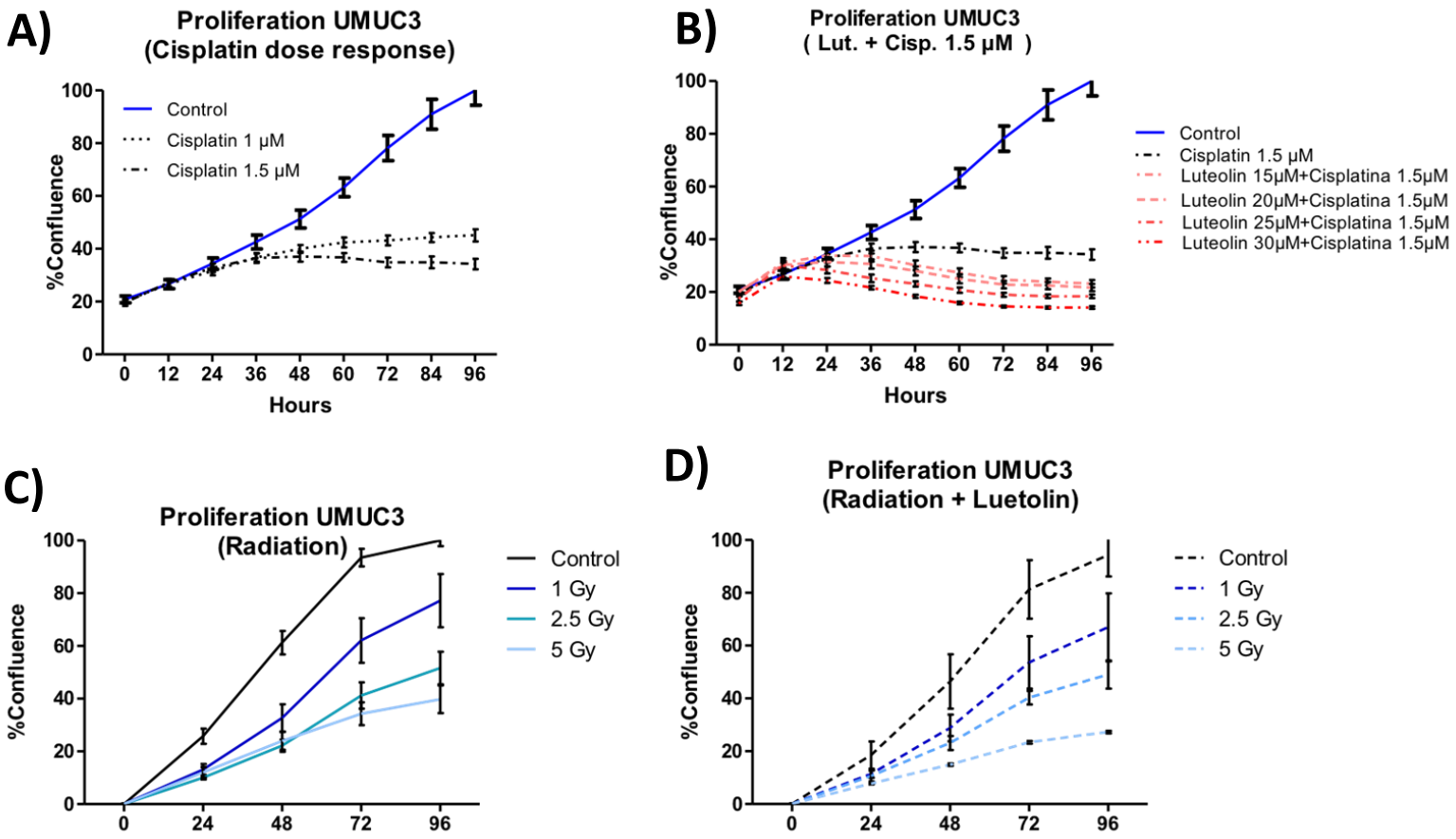


Figure 3S. UMC3 cell line treatments. **A)** Dose response of cisplatin treatment in UMUC3 cell line. **B)** Dose response of combination luteolin and cisplatin treatment in UMUC3 cell line. **C)** Dose responte of x-ray treatment. **D)** Dose response of combination luteolin (15 µM) and x-ray.

Manuscrito 1

(n=35)

Amostras de tecido uroletial

Controle$_{(4)}$
Baixo Grau$_{(11)}$
Alto Grau$_{(20)}$

Sequenciamento de RNA

	Tumor vs Normal	Alto vs Baixo Grau
Expressão gênica	↑ genes relacionados com o ciclo celular, segregação cromossômica, replicação do DNA e mitose ↓ genes relacionados com sistema imune	↑ genes relacionados com o quimiotáxia e função neuronal (*EPHB2, CNTNAP2, KCNQ3, TENM2, RDH12, DPF1, SHISA9, SLC30A3, MME* e ***MSI1***) ↓ genes com expressão diferencial exclusivo em alto grau (KCNG1, SLC16A10 e LIPG).
Splicing	Eventos de splicing em genes relacionados com ciclo celular, ligação de actina e processamento de RNA Enriquecimento funcional revelou associação desses genes com a via do *TP53*.	Retenção dos íntrons 8, 9 e 10 no lncRNA GAS5. Eventos de *splicing* em mRNAs codificadores de proteínas ribossomais Exclusivamente em tumores de alto grau foram dectados retenção de íntrons nos mRNA (*RPL10, RPL13A, RPL37A*).

Manuscrito 2

NIH
THE CANCER GENOME ATLAS
Baixo grau (21)
Alto grau (384)
(n=405)

1.542
RBPs no total

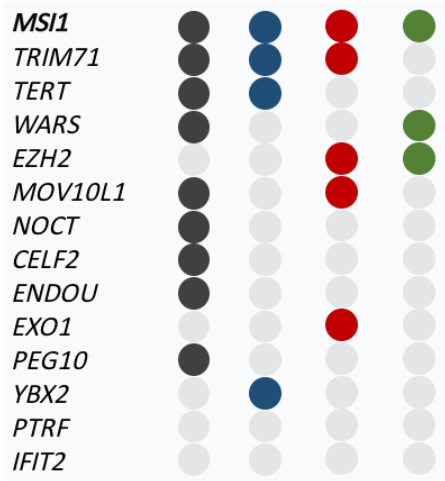
236
up-regulated
(alto vs baixo grau)

14
pior prognóstico

Ensaios Funcionais

Ensaios Funcionais
com 14 RBPs

Efeito do *knockout* de genes



Nota: p<0,05 em duas ou mais linhagens celulares UMUC3, T24 e J82.

Alvos do MSI1

Expressão Gênica (RNAseq)

↑ genes relacionados com ciclo celular e diferenciação de músculo esquelético;

↓ genes relacionados com a via Wnt, diferenciação celular e desenvolvimento embrionário;

↓ lncRNAs relacionados com pior prognóstico para tumores de bexiga.

CLIP *assay*

MSI1 se liga preferencialmente nas regiões 3'UTR e íntrônica do mRNA alvo;

MSI1 alvos foram relacionados a via Wnt, TP53, PDGF e CCRKR.

mRNAs que são alvos do MSI1 atuam no processamento do RNA, tradução, replicação e reparo no DNA.

11. CONSIDERAÇÕES FINAIS

Os carcinomas uroteliais possuem como característica o fato de serem geneticamente heterogênicos. A partir de estudos conduzidos pelo *"The Cancer Genome Atlas"* (TCGA), houve importante avanço para o entendimento da gênese dos tumores de bexiga, e a caracterização molecular permitiu identificar marcadores de desenvolvimento tumoral e a detecção de novos alvos terapêuticos (2, 28). A maioria dos tumores de baixo grau apresenta mutação de ponto no gene *FGFR3,* favorecendo a proliferação celular (21). A perda do último éxon do mRNA do gene *FGFR3*, a qual resulta de alterações no processamento da molécula, tem sido associada à atividade oncogênica do gene (67). Em contraste, os tumores de alto grau apresentam aumento da atividade de oncogenes clássicos, como *E2F3, MDM2* e *HER2* (68,69). Aproximadamente 49% dos tumores de alto grau analisados pelo TCGA possuíam mutação no gene *TP53* e, como resultado do aumento da expressão do gene *MDM2* (antagonista do *TP53*), cerca de 76% dos tumores apresentavam inativação da via do gene *TP53* (28).

As novas tecnologias de sequenciamento de larga escala e ferramentas de bioinformática estão sendo fundamentais para a identificação dos eventos moleculares envolvidos no desenvolvimento tumoral, dentre os quais as alterações no processamento de mRNAs e proteínas de ligação ao RNA (RBPs). Nesse contexto, o presente estudo foi delineado com o objetivo de identificar o papel das RBPs na carcinogênese urotelial, com ênfase na caracterização dos tumores de baixo e alto graus.

Quimiotaxia e epitélio mesenquimal de transição associados à progressão tumoral

Nossos resultados do sequenciamento do RNA mostraram aumento na expressão de genes relacionados à quimiotaxia, funções neuronais e a pior prognóstico para os tumores de bexiga. Confirmando dados da literatura, foi observada expressão diferencial de genes

relacionados à via da p53 e de resposta imune, tanto em tumores de baixo como de alto graus. Diferentemente, a hiperexpressão dos genes associados à quimiotaxia foi característica dos tumores de alto grau (com capacidade de invadir tecidos adjacentes). O processo de quimiotaxia permite que as células se locomovam em resposta a estímulos químicos mediados pela ação de citocinas, receptores de citocinas e fatores de crescimento. Sabe-se que na carcinogênese esse mecanismo pode ser reprogramado favorecendo a disseminação tumoral (70,71).

Roberson et al. (72) descreveram um novo sub-grupo de tumores invasivos, o *"neuronal-like",* considerado mais agressivo e com pior prognóstico. Realmente, nossos achados, a partir das amostras do TCGA, mostraram que um conjunto de genes neuronais (*MSI1, EPHB2, CNTNAP2, KCNQE, TENM2, RDH12, DPF1, SHISA9, SLC30A e MME*) apresentava-se hiperexpresso nos tumores de alto grau e estava também relacionado a pior prognóstico do tumor. Dentre os eles, o *MSI1* e o *EPHB2* vêm sendo relacionado à promoção e progressão tumoral por meio da indução do epitélio mesenquimal de transição (EMT) (73,74). De fato, o EMT tem sido associado a tumores de bexiga de alto grau e à resistência a quimioterapia (24). Em conjunto, os genes diferencialmente expressos nos tumores de alto grau, relacionados à quimiotaxia e ao EMT (neuronal), podem contribuir para invasão tecidual e aumento do estadiamento tumoral. A hiperexpressão do *MSI1* também foi detectada nas análises de genes diferencialmente expressos a partir dos dados do TCGA, reforçando a relevância desse gene como biomarcador candidado em tumores de alto grau. A a caracterização funcional e molecular do *MSI1* será discutida posteriormente, no item **"Musashi-1 como novo biomarcador e alvo terapêutico em tumores uroteliais".**

Processamento do mRNA de proteínas ribossomais e do RNA longo não codificador (lncRNA) GAS5 em tumores uroteliais de alto grau

Alterações no processamento do RNA têm sido descritas em todos os tipos de câncer e associadas a tumores com fenótipos invasivos e agressivos (36). No presente estudo, identificamos em tumores de alto grau importantes eventos de retenção de íntron em mRNA de genes codificadores de proteínas ribossomais (RPL10, RPL13A, RPL37A) e que estavam relacionados com o processamento do RNA. Interessante, é que os íntrons retidos alteram a matriz de leitura dos respectivos mRNA e resultam na inserção de um códon de parada (*UAA*, *UAG* ou *UGA*), afetando, consequentemente, a tradução proteica e podendo resultar em proteínas truncadas e com sua função basal comprometida. Estudos recentes mostram que a diminuição da expressão do gene *RPL10* está relacionada ao desenvolvimento inicial dos tumores de próstata (75) e que este gene está também associado a alteração na biossíntese ribossomal durante a tumorigênese (76). Por outro lado, o mRNA RPL3 apresentou retenção de íntron dentro da região codificadora, fato que pode resultar em uma proteína com novo domínio e, como consequência, levar à perda ou ganho de função Também têm sido descritas a atividade extra-ribossomal do *RPL3*, além da sua ação como indutor da parada do ciclo celular e apoptose, por meio da p21, na ausência funcional da p53 (77,78). A perda de função de ambas proteínas ribossomais nos tumores de alto grau pode estar associada ao fenótipo agressivo do tumor.

Eventos de retenção de íntron (8, 9 e 11) também foram identificados no mRNA do lncRNA GAS5. O lncRNA GAS5 hospeda em seus íntrons importantes pequenos RNAs nucleares (snRNAs) que atuam como supressores tumorais (79). Em nosso estudo foram identificados a retenção dos íntrons 8, 9 e 11, os quais codificam respectivamente os snRNAS SNORD79 (U49), SNORD80 (U80) e SNORD81 (U81), sugerindo desta forma uma diminuição no processamento desses snoRNAs em tumores de alto grau. De fato, nos últimos anos, vários estudos demonstraram a ação do lncRNA GAS5 como um novo agente supressor

em tumores uroteliais. Recentemente, foi identificado que a inibição de expressão do lncRNA GAS5 em linhagens celulares de tumores de bexiga resultava no aumento da proliferação celular (80). Diferentemente, contudo, Wang et al (81) mostraram que a indução da expressão do lncRNA GAS5 resultou no aumento das taxas de apoptose *in vitro* e na inibição do crescimento tumoral *in vivo.*

Proteínas de ligação ao RNA envolvidas na carcinogênese urotelial

A expressão diferencial de proteínas de ligação ao RNA (RBPs) tem sido relatada em diversos tipos de cânceres e associada a pior prognóstico (15,16, 45–47). No presente estudo foram identificadas 14 RBPs com aumento de expressão em tumores de bexiga de alto grau e associadas a pior prognóstico em amostras do TCGA. A análise da expressão diferencial dessas RBPs revelou aumento de expressão de 8/14 RBPs também nas amostras tumorais obtidas do biorrepositório da Universidade de São Paulo e do Hospital Amaral Carvalho. O estudo funcional *in vitro* mostrou a relação entre os níveis de expressão das RBPs e importantes processos celulares envolvidos na carcinogênese, como proliferação, apoptose e viabilidade celular. Em conjunto, esses dados sugerem que as RBPs poderiam ser consideradas candidatas a biomarcadores para tumores de bexiga de alto grau.

Musashi-1 como um novo candidato a biomarcador e alvo terapêutico em tumores uroteliais

Conforme descrito, as amostras de tumores uroteliais de alto grau, obtidas do biorrepositório de São Paulo, apresentaram aumento da expressão do gene *MSI1* (que codifica a RBP musashi-1). As análises a partir de dados do TCGA corroboraram tais achados e também revelaram forte associação entre o aumento de expressão do *MSI1* e o pior prognóstico para carcinoma urotelial. A musashi-1 é uma importante proteína associada à manutenção e diferenciação de células tronco (87), e sua expressão aberrante tem sido observada em diferentes tipos de câncer, como o glioblastoma (88), câncer de cólon (89) e

câncer cervical (73). A análise funcional *in vitro* mostrou que o silenciamento do *MSI1* por pequenos RNAs de interferência (siRNA) resultou na diminuição da proliferação, viabilidade, taxas de migração e invasão, aumento de apoptose e, também, na diminuição da resistência celular aos tratamentos com cisplatina e raio X.

De maneira similar aos efeitos do silenciamento do *MSI1,* outro resultado importante deste estudo foi o efeito da luteolina inibindo a proliferação celular e aumentando a sensibilidade a quimio e radioterapia. Esses achados indicam a musashi-1 como potencial alvo terapêutico em tumores de bexiga e reforçam a relevância da luteolina como potencial composto adjuvante para a terapia tumoral e destacam.

Musashi-1 promove a carcinogênese urotelial por meio da via Wnt/β-catenina

O sequenciamento do RNA após o silenciamento do *MSI1* e a realização da técnica de CLIP (*crooslinking imunopreciptation*) na linhagem UMUC3, sugerem que o *MSI1* promove a proliferação celular por meio da via canônica Wnt/β-catenina. Esta hipóstese tem por base quatro evidências: i) redução na expressão do *CTNNBIP1*, que resultou na diminuição dos níveis de β-catenina; ii) diminuição na expressão do lncRNA ZEB1-AS1 e consequente redução da expressão do *ZEB1* (relacionado ao fenótipo de EMT); iii) aumento na expressão do *DKK* e *APCDD1* (Wnt inibidores); iv) diminuição na expressão de *LRP*, receptor transmembrana relacionado à ativação da via Wnt.

12. CONCLUSÃO

Os resultados do presente estudo permitiram as seguintes conclusões:

1 – a modulação na expressão de genes relacionados a quimiotaxia e epitélio mesenquimal de transição (EMT) está associada a tumores de bexiga de alto grau;

2 - alterações no processamento de genes ribossomais e do lncRNA GAS5 podem ser potenciais novos marcadores para tumores de bexiga de alto grau;

3 - proteínas de ligação ao RNA estão associadas à carcinogenese urotelial e podem ser consideradas potenciais biomarcadores para tumores de alto grau;

4 – a musashi-1 atua como um produto de oncogene, promovendo a carcinogênese urotelial por meio da via Wnt- β-catenina;

5 – a musashi-1 promove resistência a quimio e radioterapia *in vitro*;

6 – a luteolina apresentou evidência que suportam seu potencial como agente adjuvante para o tratamento dos carcinomas uroteliais.

Finalizando, nossos resultados forneceram informações que poderão contribuir para o melhor entendimento do processo de carcinogênese urotelial, para caracterização molecular de baixo e alto graus e para o estabelecimento de novas estratégias terapêuticas para tumores resistentes aos tratamentos padrão.

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