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Associação entre ritmo circadiano, câncer e diabetes utilizando ferramentas da bioinformática

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RESUMO

Introdução: A tumorigênese é afetada pelos genes do relógio. Alterações da expressão dos genes do relógio podem aumentar a susceptibilidade ao câncer através dos efeitos nos mecanismos biológicos que regulam o dano e reparo ao DNA, o metabolismo energético, crescimento e morte celular em tecidos neoplásicos. **Objetivo:** analisar a associação do ritmo circadiano, diabetes tipo 2 e câncer. **Metodologia:** O estudo é uma meta-análise realizada em amostras de tecido de paciente com diabetes tipo 2 e amostras de tecido normal, genes relacionados ao ritmo circadiano e dados do transcriptoma associado ao câncer de mama, bexiga, fígado, pâncreas, cólon e reto usando a integração de perfis de expressão gênica com biomolecular em escala de genoma redes em amostras de diabetes, os bancos de dados pesquisados foram o Pubmed e TCGA. Foram utilizados os descritores ritmo circadiano, diabetes tipo 2 e câncer em inglês com operadores boleanos *and* ou *or***. Resultados:** KLF10, NTKR3, IGF1, USP2, EZH2 foram regulados negativamente em amostras de diabetes tipo 2 e câncer, enquanto ARNTL2 E AGRP foram regulados positivamente. Parece que as alterações no mRNA estão contribuindo para as alterações fenotípicas no diabetes tipo 2, resultando em alterações fenotípicas associadas à transformação maligna. Tomando esses genes para realizar uma análise de sobrevivência, encontramos apenas os genes IGF1, USP2 e ARNTL2 associados à sobrevida. Enquanto a regulação negativa de IGF1 e USP2 teve um impacto negativo, a regulação positiva de ARNTL2 foi associada a uma pior sobrevida em amostras de câncer BLCA e BRCA**. Conclusão:** Essas moléculas biológicas não apenas representam a associação de diabetes tipo 2 e biomarcadores de ritmo circadiano com câncer de mama, bexiga, fígado, cólon e reto, mas também têm potencial significativo para serem consideradas como biomarcadores em nível de sistema que podem ser usados para triagem ou terapêutica finalidades.

Palavras-chave: Genes do relógio. IGF1. USP2. Síndrome metabólica. Hipotálamo. Córtex. Câncer.

ABSTRACT

Introduction: Tumorigenesis is affected by clock genes. Changes in the expression of clock genes can increase cancer susceptibility through the effects on biological mechanisms that regulate DNA damage and repair, energy metabolism, cell growth and death in neoplastic tissues **Objective:** to analyze the association of circadian rhythm, type 2 diabetes and cancer. **Methodology**: The study is a meta-analysis performed on type 2 diabetes, genes related to circadian rhythm and transcriptome data associated with breast, bladder, liver, pancreas, colon and rectum cancer using the integration of gene expression profiles with biomolecular in genome scale networks in diabetes samples, the databases searched were Pubmed and TCGA. The descriptors circadian rhythm, type 2 diabetes and cancer in English were used with Boolean operators *and* or *or*. **Results:** several common genes deregulate in diabetes mellitus and cancer. KLF10, NTKR3, IGF1, USP2, EZH2 were both down-regulated in samples of type 2 diabetes and cancer, while ARNTL2 AND AGRP were up-regulated. It appears that changes in mRNA are contributing to phenotypic changes in type 2 diabetes, resulting in phenotypic changes associated with malignant transformation. Taking these genes to perform a survival analysis, we found only the IGF1, USP2 and ARNTL2 genes associated with patient results. While negative regulation of IGF1 and USP2 had a negative impact, positive regulation of ARNTL2 was associated with poor survival in BLCA and BRCA cancer samples. **Conclusion**: These biological molecules not only represent the association of type 2 diabetes and circadian rhythm biomarkers with breast, bladder, liver, colon and rectum cancer, but also have significant potential to be considered as system-level biomarkers that can be used for screening or therapeutic purposes.

Keywords: Clock genes. IGF1. USP2. Metabolic syndrome. Hypothalamus. Cortex. Cancer.

LISTA DE ILUSTRAÇÕES

LISTA DE SILGAS

ACS	American Cancer Society
ADA	American Diabetes Association
APC	Célula Apresentadora De Antígeno
ARNTL2	Aryl Hydrocarbon Receptor Nuclear Translocator Like 2
ATP	Adenosina Trifosfato
BLCA	Câncer Da Bexiga Urotelial
BRCA	Carcinoma Invasivo da Mama
CCG	Cuidados e Controles Gerais
CCR	Câncer Colorretal
COAD	Adenocarcinoma de Cólon
DEGs	Genes Diferencialmente Expressos
DNA	Ácido Desoxirribonucléico
EGR-1	Resposta de Crescimento Inicial 1
GLUT-4	Transportador de Glicose 4
GO	Ontologia Genética
HDL	Lipoproteína de Alta Densidade
IDF	Federação Internacional de Diabetes
IGF-1	Fator de Crescimento Semelhante a Insulina tipo 1
IL-6	Interleucina 6
IL-1β	Interleucina 1 Beta
KEGG	Enciclopédia de Genes e Genomas de Kyoto
LIHC	Carcinoma Hepatocelular do Fígado
NCBI	National Center for Biotechnology Information
NF-κβ	Fator Nucler Kappa β
PAAD	Adenocarcinoma do Pâncreas
PANTHER	Análise de Proteínas por Relações Evolutivas
PI3K	Fosfatidilinositol 3-Quinase
PTEN	Phosphatase and Tensin Homologue
READ	Adenocarcinoma do Reto
RNA	Ácido Ribonucleico
STK11	Serine/Threonine Kinase 1

TCGA	The Cancer Genome Atlas Program
TIMER	Tumor Immune Estimation Resource
TNF-α	Fator de Necrose Tumoral Alfa

SUMÁRIO

1 INTRODUÇÃO

Relógios biológicos são sistemas intrínsecos adaptados, que permitem aos organismos anteciparem as mudanças no ambiente ao seu redor, como por exemplo, a disponibilidade de comida e a predação e, com isso, permitem que eles adaptem seu comportamento e fisiologia às diferentes fases do dia, coordenando esses processos em ciclos de 24 horas (ZEE *et al*., 2013).

A nível molecular, os ritmos biológicos são mantidos por uma maquinaria composta, fundamentalmente, por um conjunto de genes, os chamados genes relógio, que apresentam uma alça de retroalimentação e regulam o padrão circadiano de transcrição e tradução deles mesmos e de muitos genes controlados por eles, os chamados genes controlados pelo relógio (KENNAWAY *et al.,* 2006; BURDELAK *et al.,* 2013).

A influência externa e interna de diversos fatores como a intensidade e a duração do tempo de luz no ambiente, o estresse e as condições de saúde também são capazes de alterar os padrões rítmicos, como de atividade e repouso. Variações fisiológicas ao longo do dia podem ser observadas durante o ciclo regular, como alterações na atividade física e mental, na função cardiovascular e regulação de temperatura corporal. Também os parâmetros do sistema imunológico, como número de leucócitos, função, proliferação e produção de citocinas apresentam uma marcada variação circadiana (ZEE *et al*., 2013; ZHU *et al.,* 2012; BERGER, 2008; KENNAWAY *et al.,* 2006).

A maquinaria molecular de transcrição circadiana não está apenas relacionada com a expressão dos genes relógio, eles estão envolvidos em diversas funções no organismo, como por exemplo, a secreção de hormônios, o envelhecimento, o ciclo celular, a resposta ao dano no DNA, dentre outros (RUTTER *et al.,* 2002; MAZZOCCOLI *et al.*, 2012; BOZEK *et al*., 2009). A desregulação do ritmo circadiano tem sido relacionada com o surgimento de diferentes patologias, como transtornos de humor, desordens metabólicas e também o câncer (MILLER *et al*., 2007).

A tumorigênese é afetada pelos genes do relógio. Alterações da expressão dos genes do relógio podem aumentar a susceptibilidade ao câncer através dos efeitos nos mecanismos biológicos que regulam o dano e reparo ao DNA, o metabolismo energético, crescimento e morte celular em tecidos neoplásicos (NIRVANI *et al.*,2018; GERY; KOEFFLER, 2010).

Além da relação entre a dessincronização e a maior incidência de alguns tipos de câncer, o ritmo circadiano também atua na regulação do metabolismo pelos genes do ciclo circadiano e afeta diferentes processos metabólicos, como o metabolismo da glicose, colesterol e função renal, pois os níveis dos hormônios metabólicos glucagon, insulina, grelina, leptina e

corticosterona oscilam de acordo com o ciclo circadiano (SINHA *et al.*, 1996; ECKEL-MAHAN; SASSONE-CORSI, 2009; SAHAR; SASSONE-CORSI, 2012). As consequências da desregulação desses genes a longo prazo podem causar síndrome metabólica e obesidade (TUREK *et al.*, 2005; MARCHEVA *et al.*, 2010; SASSONE-CORSI, 2012).

Existe uma forte associação entre obesidade, diabetes e câncer. Em 2010, a *American Diabetes Association* (ADA) *e a American Cancer Society* (ACS) publicaram um documento que afirma que o diabetes aumenta o risco de câncer de fígado, pâncreas, endométrio, cólon e reto, mama e bexiga (GIOVANNUCCI *et al*. 2010). Diabetes *mellitus* pode influenciar no surgimento de câncer de três formas principais: hiperinsulinemia, hiperglicemia ou inflamação crônica e desregulação dos hormônios sexuais (CALIMLIOGLU *et al*., 2015; GIOVANNUCCI *et al*., 2010). Cabe ressaltar que existem inúmeros fatores de riscos comuns entre diabetes mellitus e câncer como: idade, atividade física, obesidade, dieta, tabagismo e etilismo (GIOVANNUCCI *et al.,* 2010).

CALIMLIOGLU em seu estudo demonstrou a associação entre diabetes mellitus tipo 2 e câncer de pulmão, pâncreas, próstata e colorretal (CCR), demonstrando que as proteínas APC, EGFR, KPCA, MDM2, MK01, MK08, MYC, P53, TF65, TNR6, P85A 15 e SMAD3 estão associadas a mais de um tipo de câncer, as proteínas são oriundas de proto-oncogenes e genes supressores de tumor que, quando estão mutados, levam ao desenvolvimento do câncer (CALIMLIOGLU *et al.*, 2015).

Apesar da interação entre ritmos circadianos e câncer ser bastante descrita na literatura, pouco se sabe sobre genes envolvidos com o desenvolvimento de tumores que possam ser modulados pelo sistema de temporização circadiano, assim como se existe a associação entre o surgimento de câncer e diabetes relacionados a alteração do ritmo circadiano e dos genes do relógio envolvidos no processo. No presente estudo, buscou-se os elementos de ligação entre alteração do ritmo circadiano - câncer – diabetes.

2 OBJETIVOS

2.1 Objetivo geral

- Avaliar a associação do ritmo circadiano, diabetes tipo 2 e câncer utilizando ferramentas da bioinformática

2.2 Objetivos específicos

- Comparar a expressão diferencial de genes em diferentes amostras teciduais de indivíduos com e sem diabetes tipo 2;
- Diferenciar a expressão gênica em modelos animais para distúrbios do ritmo circadiano;
- Analisar a expressão gênica em tecidos de indivíduos sem câncer e em tecidos com tumores de mama, bexiga, reto, cólon, fígado e pâncreas;
- Associar a expressão diferencial de genes nas diferentes amostras de modelo animal de ritmo circadiano, diabetes tipo 2 e câncer;
- Analisar a sobrevida dos pacientes com os diferentes tipos de câncer, considerando o perfil de expressão gênica e vias metabólicas alteradas;

3 REVISÃO DE LITERATURA

Diabetes *Mellitus*

O Diabetes *mellitus* é uma doença crônica, autoimune, caracterizada por alterações na secreção e/ou atuação da insulina, com consequente hiperglicemia crônica, a qual é responsável pelas manifestações clínicas de polidipsia, poliúria e emagrecimento de forma involuntária (CALIMLIOGLU *et al.*,2015; DEEPTHI *et al.*, 2017). Outros sintomas que levantam a suspeita clínica são: fadiga,fraqueza, letargia, prurido cutâneo e vulvar, balanopostite e infecções de repetição. Algumas vezes o diagnóstico é feito a partir de complicações crônicas como neuropatia, retinopatia ou doença cardiovascular aterosclerótica (BRASIL, 2006; ADA, 2017)

De acordo com a Federação Internacional de Diabetes, (IDF), 1 a cada 11 adultos possuía diabetes em 2019, o equivalente a 423 milhões de pessoas (FEDERAÇÃO INTERNACIONAL DE DIABETES, 2019). No Brasil, há 12,5 milhões de pessoas com diagnóstico de diabetes, ocupando o 4º lugar entre os 10 países com maior número de indivíduos acometidos por ela; o 3º em número de crianças e adolescentes com diabetes tipo 1; e o 6º país do mundo em gastos com a doença (AMERICAN DIABETES ASSOCIATION, 2017). Entretanto, o Brasil não se enquadra entre os 10 países com maior investimento médio por individuo com diabetes (*Ibid.*).

As estimativas de mortalidade por diabetes e suas complicações a colocam entre as principais causas de morte por doença crônica no mundo, juntamente com doenças cardíacas isquêmicas e doenças cerebrovasculares (ZIMMET *et al.*, 2016).
Em 2019, foi estimado um total de 4,2 milhões de mortes por diabetes, o equivalente a uma morte a cada oito segundos (FEDERAÇÃO INTERNACIONAL DE DIABETES, 2019). Em Alagoas, o diabetes mellitus representa a quarta maior causa de internações por condições sensíveis à atenção primária e a terceira causa de óbitos totalizando 13.103 no período de 2007 a 2016 (SESAU, 2017).

A depender de sua patogenia, é classificado em diabetes tipo 1, tipo 2, gestacional ou de outras causas específicas, sendo o diabetes tipo 2 o mais prevalente (90-95% dos casos) (AMERICAN DIABETES ASSOCIATION, AMERICAN DIABETES ASSOCIATION, 2019), sendo comum em adultos mais velhos. No entanto, em resposta às taxas crescentes de obesidade e sedentarismo, a incidência entre crianças e adolescentes está em crescente aumento (*Ibid.*).

Indivíduos com diabetes tipo 2 apresentam produção deficiente de insulina e resistência periférica à insulina concomitantemente, que estão associados a processo inflamatório crônico e estresse oxidativo às células β pancreáticas, produtoras de insulina, e aos tecidos que

respondem à ação do hormônio (DHARMALINGAM; MARCUS, 2019). A hiperglicemia é associada ao estresse oxidativo, o qual é caracterizado por um desequilíbrio na geração de radicais livres (espécies reativas de oxigênio-ROS- e espécies reativas de nitrogênio-RNS-) e sua neutralização pelos mecanismos anti-oxidantes, o aumento de ROS E RNS acarreta destruição das ilhotas pancreáticas por apoptose e consequentemente leve à resistência à insulina (*Ibid.*). A insulina tem atividade anti-inflamatória: suprime a transcrição de fatores pro-inflamatórios como o fator nucler kappa β (NF-κβ), resposta de crescimento inicial 1 (EGR-1) e proteína ativadora 1 (AP-1) e seus genes correspondentes que medeiam a inflamação, porém, a resistência insulínica causa a ativação dessas transcrições levando à inflamação (*Ibid.*)

O desenvolvimento da resistência periférica à insulina relaciona-se à ação de mediadores pró-inflamatórios, tais como interleucina 1 beta (IL-1β), interleucina 6 (IL-6), fator de necrose tumoral alfa (TNF-α) (AKASH; REHMAN; LIAQAT, 2017; DHARMALINGAM; MARCUS, 2019). Níveis elevados de TNF-α reduzem a expressão gênica do transportador de glicose 4 (GLUT-4) responsável pelo transporte da glicose para os adipócitos e células musculares esqueléticas e cardíacas - e induzem a fosforilação do substrato-1 do receptor de insulina (IRS-1), inativando-o e reduzindo a resposta fisiológica dos tecidos à insulina (AKASH; REHMAN; LIAQAT, 2017). Devido à inibição da captação da glicose e da ação da insulina, não há estímulo para produção de adenosina trifosfato (ATP) a partir da glicose, para glicogênese e para captação de aminoácidos e produção de proteínas nos tecidos musculares esquelético e cardíaco e para a lipogênese nos adipócitos. Além disso, a insulina torna-se incapaz de inibir a lipólise nos adipócitos e produção de glicose pelo fígado. Todos esses efeitos levam à hiperglicemia e ao desenvolvimento do diabetes (PETERSEN; SHULMAN, 2018). De modo que, os níveis elevados de glicose no sangue alteram vias metabólicas teciduais e provocam estresse oxidativo. No pâncreas endócrino, o acúmulo das espécies reativas de oxigênio altera o microambiente celular, acarretando a destruição das células beta e na produção deficiente de insulina, corroborando para maior elevação dos níveis sanguíneos de glicose e para a progressão do diabetes (DHARMALINGAM; MARCUS, 2019)

Tanto o processo inflamatório quanto o estresse oxidativo parecem ser desencadeados por fatores genéticos e epigenéticos, ainda não bem compreendidos, e por fatores de risco modificáveis, sendo a obesidade central o principal (AMERICAN DIABETES ASSOCIATION, 2019; DEEPTHI, 2019), fator esse que pode estar relacionando tanto ao surgimento de neoplasias como pior sobrevida naqueles pacientes que a apresentam (GIOVANNUCCI *et al*., 2010).

Diabetes *mellitus* e Câncer

Diabetes *mellitus* pode influenciar no surgimento de câncer de três formas principais: hiperinsulinemia, hiperglicemia ou inflamação crônica e desregulação dos hormônios sexuais (CALIMLIOGLU *et al.,* 2015; GIOVANNUCCI *et al*., 2010). Muitas células neoplásicas expressam receptores de insulina e receptores de fator de crescimento semelhante à insulina tipo 1 (IGF-1), a hiperinsulinemia leva ao aumento da biodisponibilidade de IGF-1 por meio da diminuição dos níveis de suas proteínas de ligação, os receptores de IGF-1 são expressos em quase todos os tecidos do corpo e ativam vias mitogênicas na proliferação celular (GIOVANNUCCI *et al.,* 2010; ESPOSITO *et al.,* 2012). Por sua vez, os receptores de insulina, em particular, sua isoforma A podem estimular a mitogênese, mesmo em células deficientes de receptores de IGF-1, assim como é capaz de estimular a proliferação e metástase de células neoplásicas (GIOVANNUCCI *et al.,* 2010; COHEN; LEHOITH, 2012). A hiperglicemia também permite que o IGF-1 estimule a proliferação da célula do músculo liso vascular que está associado a fisiopatologia da aterosclerose assim como está presente no câncer (GIOVANNUCCI *et al.,* 2010), leva, ainda, à redução da síntese hepática e redução no sangue dos níveis de globulinas de ligação dos hormônios sexuais, acarretando aumento da biodisponibilidade do estrogênio em homens e mulheres, o aumento de estrogênio nas mulheres pós-menopausadas aumenta o risco de câncer de endométrio e de mama (GIOVANNUCCI *et al.,* 2010).

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Fatores relacionados ao diabetes como a doença hepática não gordurosa, a qual se manifesta com esteatose e cirrose podem aumentar a suscetibilidade ao câncer hepático (GIOVANNUCCI *et al.*, 2010; BHATT; SIMTH, 2015). Em relação ao câncer de pâncreas, o mesmo pode ser a causa do metabolismo anormal da glicose, porém uma associação positiva foi encontrada quando o diagnóstico de diabetes mellitus antecede o câncer em 5 anos, diminuindo as chances da causa do diabetes mellitus ser o próprio câncer, provavelmente, pela associação entre a presença do diabetes e o surgimento de câncer pelos mecanismo de hiperinsulinemia, hiperglicemia ou inflamação crônica e desregulação dos hormônios sexuais (CALIMLIOGLU *et al.,* 2015; GIOVANNUCCI *et al.*, 2010).

Obesidade é um fator de confusão com relação a associação entre diabetes mellitus e o câncer de mama, já que ambas estão associadas à resistência periférica à insulina, o aumento dos níveis de insulina e de IGF-1 levam ao aumento dos níveis de estrogênio circulante pela redução das proteínas de ligação dos hormônios sexuais (estrógeno e andrógenos) ambos associados ao câncer de mama (AMERICAN DIABETES ASSOCIATION, 2017; DANTAS *et al.,* 2009; BUONO *et al.*, 2017).

HARDEFELDT *et al.* (2012,) em sua metanálise demonstraram um aumento de 20% no risco de câncer de mama em mulheres com diabetes mellitus e de 29% em homens (*Ibid.*). Por sua vez, BUONO demonstrou que pacientes que tinham a associação de obesidade, diabetes mellitus e câncer de mama apresentavam menores taxas de sobrevida livre de doença, independentemente do tamanho e subtipo do tumor, idade e/ou tratamento realizado, destacando-se, assim, o diabetes como um fator de mau prognóstico de forma isolada (BUONO *et al.,* 2017).

Mutações nos genes BRCA 1 e 2 estão relacionados com o câncer de mama hereditário, mulheres com mutações no BRCA 1 apresentam 87% de chance de desenvolver câncer e 85% quando a mutação é em BRCA 2 (HARDEFELDT *et al* ,2012). Outros genes como p53, PTEN, STK11/LKB1, MLH1 e MLH2 também podem ter associação com síndromes hereditárias e seu risco aumentado para câncer de mama (LOFRANO *et al*., 2009).

O gene PTEN foi descrito com papéis tanto no crescimento celular quanto na sinalização metabólica. Ele atua como gene supressor de tumor, principalmente devido à sua atividade de fosfatase lipídica que regula negativamente a via de sinalização da fosfatidilinositol 3-quinase (PI3K) -AKT que funciona como um mediador da maioria dos receptores de tirosina quinases (WANG *et* al, 2014), ele também foi implicado no diabetes tipo 2, pois a via PI3K-AKT desempenha papel na sinalização de insulina (PAL *et al.,* 2012). Mutações germinativas do

PTEN predispõem ao processo de desenvolvimento neoplásico, assim como ao desenvolvimento de obesidade (PAL *et al.,* 2012).

O mecanismo biológico entre diabetes mellitus e CCR, não está claramente definido, havendo a possibilidade de estar relacionado aos fatores de risco comuns associados à obesidade e inflamação, assim como hiperinsulinemia e hiperglicemia (ZHU *et al*., 2017). Del Puerto-Nevado *et al.* (2017) demonstraram a expressão de alguns genes no diabetes mellitus que têm relação com câncer colorretal: APC; KRAS; p53; MSH2; MSH6 estão aumentados, assim como o aumento de STK11 que está associada à síndrome de Peutz-Jegher (*Ibid.*).

Ritmo circadiano

Relógios biológicos são sistemas intrínsecos adaptados, que permitem aos organismos anteciparem as mudanças no ambiente ao seu redor, como por exemplo, a disponibilidade de comida e a predação e, com isso, permitem que eles adaptem seu comportamento e fisiologia às diferentes fases do dia, coordenando esses processos em ciclos de 24 horas (ZEE *et al*., 2013). Essa sincronização aos ciclos ambientais provoca a sincronização interna dos eventos fisiológicos circadianos. Como consequência, os organismos conseguem antecipar os eventos fisiológicos, economizando energia e otimizando as reações. Essas observações evidenciam a importância de manter a ordem temporal interna, com as relações de fase bem estabelecidas entre as variáveis fisiológicas (*Ibid.*).

O termo "ritmo circadiano" (do Latim *circa diem* que significa "cerca de um dia") foi criado para descrever oscilações endógenas em organismos, que foram assim classificadas por conter um período aproximado ao da rotação diária do planeta Terra (ZEE *et al.,* 2013; ZHU *et al.,* 2012). Uma das características dos relógios circadianos é a capacidade de serem sincronizados por estímulos externos, mantendo, entretanto, as oscilações mesmo na ausência desses estímulos, ou seja, são autossustentáveis (ZEE *et al*., 2013; ZHU *et al.,* 2012; KENNAWAY *et al.,* 2006).

Um grande número de processos biológicos é regulado pelo relógio circadiano, como por exemplo: comportamento alimentar, ciclos de sono-vigília, variação hormonal, temperatura corporal e metabolismo energético (ZEE *et al*., 2013; KENNAWAY *et al.,* 2006). Contudo, a influência externa e interna de diversos fatores como a intensidade e a duração do tempo de luz no ambiente, o estresse e as condições de saúde também são capazes de alterar os padrões rítmicos, como de atividade e repouso. Variações fisiológicas ao longo do dia podem ser observadas durante o ciclo regular, como alterações na atividade física e mental, na função

cardiovascular e regulação de temperatura corporal. Também os parâmetros do sistema imunológico, como número de leucócitos, função, proliferação e produção de citocinas apresentam uma marcada variação circadiana (ZEE *et al*., 2013; ZHU *et al.,* 2012; BERGER, 2008; KENNAWAY *et al.,* 2006).

A nível molecular, os ritmos biológicos são mantidos por uma maquinaria composta, fundamentalmente, por um conjunto de genes, os chamados genes relógio, que apresentam uma alça de retroalimentação e regulam o padrão circadiano de transcrição e tradução deles mesmos e de muitos genes controlados por eles, os chamados genes controlados pelo relógio (CCGs, do inglês *clock controlled genes*) (KENNAWAY *et al.,* 2006; BURDELAK et *al,* 2013).

Ritmo Circadiano, Câncer e Síndrome metabólica

A maquinaria molecular de transcrição circadiana não está apenas relacionada com a expressão dos genes relógio, eles estão envolvidos em diversas funções no organismo, como por exemplo, a secreção de hormônios, o envelhecimento, o ciclo celular, a resposta ao dano no DNA, dentre outros (RUTTER *et al.,* 2002; MAZZOCCOLI *et al.*, 2012; BOZEK *et al*., 2009). A desregulação do ritmo circadiano tem sido relacionada com o surgimento de diferentes patologias, como transtornos de humor, desordens metabólicas e também o câncer (MILLER *et al*., 2007).

O relógio circadiano e o ciclo celular possuem semelhanças conceituais e moleculares, pois ambos são constituídos por processos que apresentam alças de regulação interligadas e apresentam sequências de transcrição, tradução, modificação e degradação de proteínas (MAZZOCCOLI *et al*., 2012; LANDGRAF; SHOSTAK; OSTER, 2012; SOTÁK *et al.,* 2013). Existem interações entre esses dois ciclos e, quando há uma desregulação no ritmo circadiano, há uma alteração no ciclo celular, fazendo com que as células se proliferem desordenadamente.

Alterações da expressão dos genes do relógio podem aumentar a susceptibilidade ao câncer através dos efeitos nos mecanismos biológicos que regulam o dano e reparo ao ácido desoxirribonucléico (DNA), o metabolismo energético, crescimento e morte celular em tecidos neoplásicos (NIRVANI *et al.*, 2018; GERY; KOEFFLER, 2010). Genes que fazem parte do mecanismo de regulação do ciclo celular, como Myc, p53, Cyclin D1 e Wee1, já foram descritos por apresentarem alteração em sua expressão em pacientes com leucemias associadas à desregulação de genes do relógio (ZEE *et al.,* 2013; MAZZOCCOLI *et al*., 2012; LANDGRAF; SHOSTAK; OSTER, 2012)

Além da relação entre a dessincronização e a maior incidência de alguns tipos de câncer, o ritmo circadiano também atua na regulação do metabolismo pelos genes do ciclo circadiano e afeta diferentes processos metabólicos, como o metabolismo da glicose, colesterol e função renal, pois os níveis dos hormônios metabólicos glucagon, insulina, grelina, leptina e corticosterona oscilam de acordo com o ciclo circadiano (SINHA *et al*., 1996; ECKEL-MAHAN; SASSONE-CORSI, 2009; SAHAR; SASSONE-CORSI, 2012). As consequências da desregulação desses genes a longo prazo podem causar síndrome metabólica e obesidade (TUREK *et al*., 2005; MARCHEVA *et al.,* 2010; SASSONE-CORSI, 2012).

A incidência de síndrome metabólica está aumentando no mundo ocidental e sua correlação com o câncer tem se tornado mais aparente (BRAUN *et al.*, 2011). A síndrome metabólica é uma combinação de condições patológicas que coexistem num mesmo indivíduo, incluindo hiperglicemia, hipertensão, hipertrigliceridemia, baixo nível da lipoproteína de alta densidade (HDL) e aumento da circunferência abdominal (ALBERTI *et al*., 2009; BATTELLI *et al*., 2019). Essa combinação de fatores predispõe à obesidade, ao aumento de doenças cardiovasculares, diabetes tipo 2 e vários estudos têm associado à prevalência de certos tipos de câncer (BITZUR *et al*., 2016; BELLASTELLA, G. *et al.,* 2018). No estudo de Esposito (2012), nos homens, a síndrome metabólica foi associada com os cânceres de fígado, cólon e bexiga. Enquanto, que, nas mulheres, esse estudo revelou uma associação com cânceres endometrial, pancreático, mama pós-menopausa, retal e colorretal (ESPOSITO *et al*., 2012).

A fisiopatologia da síndrome metabólica está relacionada principalmente com a capacidade do tecido adiposo secretar uma ampla variedade de mediadores bioativos que modulam várias cascatas de sinalização, os quais são chamados de adipocinas (OUCHI *et al*., 2011; MENDONÇA *et al*., 2015). Nos pacientes com síndrome metabólica, ocorre uma desregulação na produção de adipocitocinas, as quais podem desencadear um estado inflamatório crônico (BRAUN *et al*., 2011; MENDONÇA *et al*., 2015). A inflamação e seus mediadores aumentam o risco de desenvolver câncer de esôfago, mama, vesícula biliar, renal, pancreático, colorretal e gástrico (GRAI *et al*., 2015; HRISTOVA, 2018).

Na obesidade, há um aumento dos ácidos graxos livres, citocinas inflamatórias no plasma, infiltração e ativação de macrófagos e decréscimo dos níveis de adiponectina, os quais podem contribuir para a resistência à insulina (BRAUN *et al*., 2011). O aumento da biodisponibilidade da insulina causa o diabetes tipo 2, podendo ativar as vias de efeito mitogênico (Ras /Raf/ MAPK ou PI3K /AKT/ mTOR), diminuir a apoptose e aumentar a síntese de proteínas (WONG

et al., 2010; BELLASTELLA *et al*., 2018). A ativação dessas vias carcinogênicas predispõe ao aumento do risco de câncer (WONG *et al*., 2010; BELLASTELLA *et al*., 2018).

Pacientes obesos têm níveis elevados de leptina, que tem atividade mitogênica/anti-apoptótica, estimula a migração e invasão das células neoplásicas, pode aumentar a produção de citocinas pelos macrófagos e pode promover a neoagiogênese através da indução e ativação de fatores pro-angiogênicos (COHEN; LEROITH, 2012; LIGIBEL; STRICKLER, 2013) e possuem baixos níveis de adiponectina que tem atividade anti-mitótica/pro-apoptótica (LIGIBEL; STRICKLER, 2013).

Apesar da interação entre ritmos circadianos e câncer ser bastante descrita na literatura, pouco se sabe sobre genes envolvidos com o desenvolvimento de tumores que possam ser modulados pelo sistema de temporização circadiano, assim como se existe a associação entre o surgimento de câncer e diabetes relacionados a alteração do ritmo circadiano e dos genes do relógio envolvidos no processo.

4 METODOLOGIA

O estudo é uma metanálise, onde os bancos de dados pesquisados foram o Pubmed e *The Cancer Genome Atlas Program* (TCGA). Foram utilizados os descritores ritmo circadiano, diabetes tipo 2 e câncer em inglês com operadores boleanos *and* ou *or.*

4.1 Critérios de coleta e inclusão de estudos

Foi pesquisado o banco de dados GEO (https://www.ncbi.nlm.nih.gov/geo/) para estudos disponíveis publicados. Após uma revisão sistemática, 6 estudos de GSE foram encontrados. Os critérios de inclusão para os estudos foram os seguintes:

(1) amostras diagnosticadas com diabetes mellitus tipo 2 e amostras normais

(2) experimentos que envolviam ritmo circadiano e / ou análise do sono

(3) perfil de expressão gênica de mRNA (ácido ribonucléico).

Seis perfis de diabetes de expressão gênica tipo 2 (GSE56931, GSE20966, GSE25724, GSE26168, GSE23343 e GSE29221) foram baixados do banco de dados GEO para análise, já para avaliação de privação de sono, apenas o GSE6514 foi baixado

Os perfis de expressão de genes humanos de pacientes diabéticos tipo 2 e não diabéticos de tecido enriquecido com células beta foram obtidos por microdissecção de captura a laser foram:

- GSE20966 (20 amostras / 7 mulheres e 13 homens)
- GSE25724 (ilhotas pancreáticas - 13 amostras / 6 mulheres e 7 homens)
- GSE26168 (sangue - 24 amostras masculinas)
- GSE23343 (fígado - 17 amostras / 7 mulheres e 10 homens)
- GSE29221 (músculo esquelético - 24 amostras masculinas)

As coletas de sangue a cada 4 horas durante um estudo de 3 dias: linha de base normal de 24 horas, 38 horas de vigília contínua e sono de recuperação subsequente, para um total de 19

pontos de tempo por sujeito, com cada avaliação de teste de vigilância psicomotora de 2 horas quando acordado (GSE56931).

Experimentos de privação de sono GSE6514 foram realizados em camundongos machos amostras C57 / BL6 com, aproximadamente, 10 semanas de idade com ± 1 semana de diferença. Os animais foram alojados em um ciclo claro/escuro de 12:12 h com água disponível *ad libitum*, foram submetidos a 14 dias de aclimatação, durante os quais foi estabelecido um padrão alimentar noturno. Os camundongos foram sacrificados após 3, 6, 9 e 12 horas de privação total de sono. A privação foi iniciada com a luz acesa e realizada por meio de um manuseio delicado. As amostras de GSE82113 foram analisadas em condições normais de privação de sono e condições de tempo de sono anormais para avaliar a robustez do preditor. Amostras de sangue cobrindo pelo menos um ciclo circadiano para medir a abundância de mRNA (ácido ribonucleico) e medir o ritmo da melatonina. Também foram realizadas análises em perfis de tecidos periféricos. GSE4239 foi usado para determinar genes regulados pelo relógio na glândula adrenal. Animais Per2Brdm1 / Cry1− / - foram usados para análise de expressão. Os animais foram conduzidos a um ciclo de 12 horas de luz: 12 horas de escuridão por duas semanas e depois liberados em escuridão constante. A alimentação restrita impacta o relógio circadiano hepático de Cry1, camundongos duplo *Knockout* Cry2 sem um relógio circadiano foi realizada usando o perfil GSE13062. Da mesma forma, o tecido do fígado de tipo selvagem, mutante de Clock e C57BL / 6 deficiente em Cry camundongos machos de 8 a 10 semanas de idade foi examinado no perfil GSE454. Os camundongos foram arrastados sob 12 h de luz branca e 12 h de escuridão por 14 dias. O perfil GSE6904 mostrou que os dados de camundongos foram expostos a partir de 1 h após as luzes apagadas a um pulso de luz de 30 minutos. No final do pulso de luz, os ratos foram sacrificados e os cérebros extraídos. As células do núcleo supraquiasmático foram extraídas usando microscopia de captura a laser e a expressão gênica foi quantificada usando *Affymetrix microarrays.*

4.2 Dados de microarray e processamento de dados

GEO2R foi aplicado para rastrear mRNAs diferencialmente expressos entre diabetes mellitus tipo 2 e amostras de tecido normal.

GEO2R (http://www.ncbi.nlm.nih.gov/geo/geo2r/) é uma ferramenta da web interativa para comparar dois grupos de dados que podem analisar qualquer série GEO. Os valores de p

ajustados usando o método de de Benjamini e Hochberg padrão foram aplicados para corrigir a ocorrência de resultados falso-positivos.

4.3 Lista de genes

Entrez Gene da NCBI (www.ncbi.nlm.nih.gov/gene/) e GeneCards (https://www.genecards.org/) foram usados como identificadores dos genes relacionados ao ritmo circadiano. A lista de genes e DEGs downregulated / upregulated dos perfis de expressão gênica foram combinados e identificados com um Diagrama de Venn 2.1.0 (http://bioinfogp.cnb.csic.es/tools/venny/index.html).

4.4 Análise funcional e de enriquecimento de vias

A análise da ontologia genética (GO) dos processos biológicos relevantes, componentes celulares e funções moleculares foi realizada usando o programa Análise de Proteínas por Relações Evolutivas (PANTHER, www.pantherdb.org), um banco de dados que reúne famílias de proteínas, funções e vias. Os termos GO atribuídos às moléculas identificadas foram classificados de acordo com a sua função (BASTOS *et al*., 2011). A Enciclopédia de Genes e Genomas de Kyoto (KEGG) é um banco de dados integrado para interpretação biológica de sequências de genoma e outros dados de alto rendimento (KANEHISA *et al.,* 2016). As análises do KEGG estavam disponíveis no banco de dados DAVID (https://david.ncifcrf.gov/), um recurso de dados composto por uma base de conhecimento de biologia integrada e ferramentas de análise para extrair informações biológicas significativas de grandes quantidades de genes e coleções de proteínas. As análises KEGG foram realizadas usando o banco de dados DAVID para identificar DEGs (genes expressos diferencialmente). Um valor de $p < 0,05$ foi estabelecido como o critério de corte (HUANG *et al.,* 2009).

4.5 Dados de RNA-seq e dados clínicos do TCGA

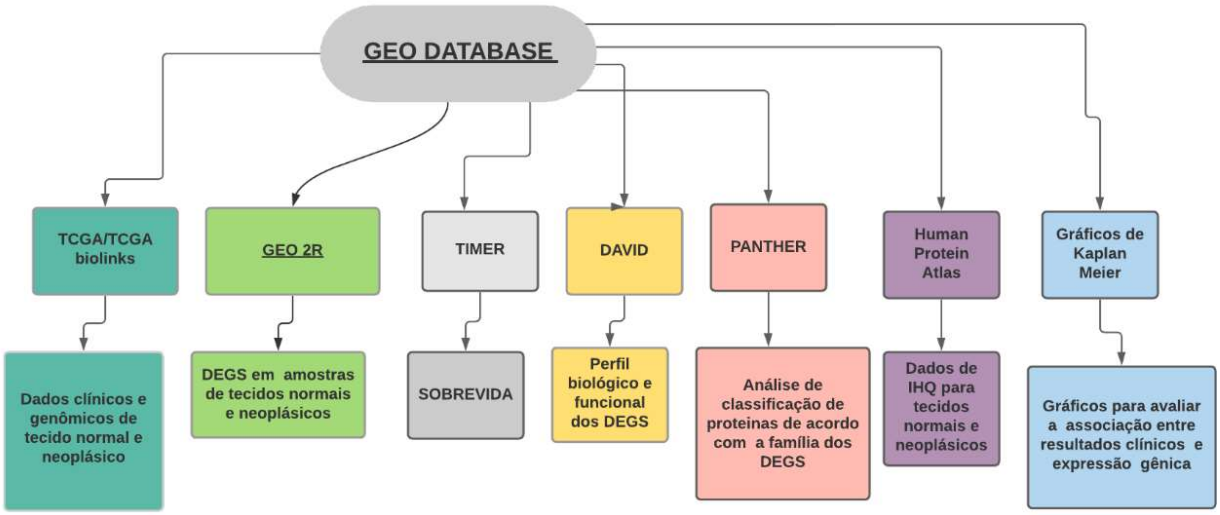
Foi utilizado o TCGAbiolinks em R/Bioconductor (http://bioconductor.org/packages/release/bioc/html/TCGAbiolinks.html) (COLAPRICO *et al.,* 2016) e o TCGAbiolinksGUI (SILVA *et al.,* 2018) para baixar dados genômicos e clínicos de tecidos normais e de tumores sólidos para seis tipos de câncer do TCGA. Os tipos de câncer foram: câncer da bexiga urotelial (BLCA), carcinoma invasivo da mama (BRCA), adenocarcinoma do

cólon (COAD), carcinoma hepatocelular do fígado (LIHC), adenocarcinoma do pâncreas (PAAD) e adenocarcinoma do reto (READ). Foram recuperados dados de nível para a expressão de mRNA e miRNA (ILLUMINA HISEQ, 2000).

4.6 Análises de expressão de câncer

Utilizamos o servidor da w*eb Tumor Immune Estimation Resource* (TIMER) (https://cistrome.shinyapps.io/timer/), uma ferramenta analítica abrangente que reanalisou dados do TCGA, para detectar a expressão gênica em vários tipos de câncer (LI *et al.,* 2017). Os perfis de expressão de BLCA, BRCA, COAD, LIHC, PAAD e READ para amostras normais comparadas com as neoplásicas foram obtidos usando a opção TIMER diff.exp (https://cistrome.shinyapps.io/timer/). A análise de sobrevida dos dados do TCGA foi realizada utilizando o módulo *Survival* do *Tumor Immune Estimation Resources* (TIMER). Gráficos de Kaplan-Meier foram desenhados para explorar a associação entre o resultado clínico e a expressão gênica e para visualizar as diferenças de sobrevida. Dados de proteína baseados em imagens de imunohistoquímica para amostras normais e de câncer estão disponíveis no Atlas de Proteínas humanas (https://www.proteinatlas.org/).

Figura 1 - Resumo dos bancos de dados utilizados e as finalidades para qual foram utilizados



Fonte: elaborado pelos autores

5 PRODUTOS

1. Correlation between circadian rhythm related genes, type 2 diabetes, and cancer: insights from metanalysis of transcriptomics data, segundo as normas da Molecular and Cellular Endocrinology

2. Patente: Privilégio de Inovação. Número do registro: BR10202002668, título: "Painel genético para diagnóstico e prognóstico do câncer de mama", Instituição de registro: INPI - Instituto Nacional da Propriedade Industrial. Depósito: 24/12/2020

5.1 PRODUTO 1

Correlation between circadian rhythm related genes, type 2diabetes, and cancer: insights from metanalysis of transcriptomics data. Publicado no periódico Molecular and Cellular Endocrinology

Correlation between circadian rhythm related genes, type 2 diabetes, and cancer: insights from metanalysis of transcriptomics data

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ABSTRACT

Clock genes work as an auto-regulated transcription-translational loop of circadian genes that drives the circadian rhythms in each cell and they are essential to physiological requests. Since metabolism is a dynamic process, it involves several physiological variables that circadian cycling. The clock genes alterations can affect multiple systems concomitantly, because they constitute the promoter factors for relevant metabolic pathways. Considering the intertwined structure of signaling, regulatory, and metabolic processes within a cell, we employed a genome-scale biomolecular network. Accordingly, a meta-analysis of diabetic-associated transcriptomic datasets was performed, and the core information on differentially expressed genes (DEGs) was obtained by statistical analyses. In the current study, meta-analysis was performed on type 2 diabetes, circadian rhythm-related genes, and breast, bladder, liver, pancreas, colon and rectum cancer-associated transcriptome data using the integration of gene expression profiles with genome-scale biomolecular networks in diabetes samples. First, we detected downregulated and upregulated DEGs in mouse cortex and hypothalamus samples of mice with sleep deprivation. In summary, upregulated genes active genes associated with oxidative phosphorylation, cancer and diabetes, mainly in hypothalamus specimens. In cortex, we observed mainly downregulation of immune system. DEGs were combined with 214 circadian rhythm related genes to type 2 DM and cancer samples. We observed that several common genes deregulated in both diseases. *Klf10*, *Ntkr3*, *Igf1*, *Usp2*, *Ezh2* were both downregulated in type 2 DM and cancer samples, while *Arntl2* and *Agrp* were upregulated. It seems that the changes in mRNA are contributing to the phenotypic changes in type 2 DM, resulting in phenotypic changes associated with the malignant transformation. Taking those genes to perform a survival analysis, we found only *Igf1, Usp2* and *Arntl2* genes associated with patient outcomes. While *Igf1* and *Usp2* downregulation had a negative impact, *Arntl2* upregulation was associated with poor survival both in BLCA and BRCA cancer samples. Our data stimulate efforts in news studies to achieve the experimental and clinical validation about these biomolecules.

Key-words: clock genes; IGF1; USP2; metabolic syndrome; hypothalamus; cortex; cancer

INTRODUCTION

Tumorigenesis is affected by clock genes. Clock genes control gene expression and cell cycle being directly involved in the regulation of cell division (Gery et al., 2006; Reddy et al., 2005), cell proliferation or apoptosis (Hua et al., 2006; Rana et al., 2014), control of cell-cycle checkpoints (Ben-Shlomo, 2014; Borgs et al., 2009), and response to DNA damage (Fu et al., 2002; Gery et al., 2006). When these functions are altered, raise cancer development propensity (Gery and Koeffler, 2010).

Clock genes work as an auto-regulated transcription-translational loop of circadian genes that drives the circadian rhythms in each cell and they are essential to physiological requests (Dunlap, 1999; Lowrey and Takahashi, 2011). The circadian proteins are altered in tumor cells when compared to the adjacent normal cells in several tissues, such as breast tumors (Chen et al., 2005); endometrial carcinoma (Yeh et al., 2005); and lung cancer (Gery et al., 2006). Several clock genes may function as oncogenes, such as *Arntl2*, *Nr1d1*, and *Npas2*, while other clock genes may function as a tumor suppressors, such as *Pers*, *Crys* and *Rors* (Ye et al., 2016). Once they control de cell cycle, the metabolism is also controlled by the internal temporal system. In this system, the central oscillator (the suprachiasmatic nucleus of hypothalamus) orchestrates the peripheral oscillators, which include systems, organs, tissues and cells.

Since metabolism is a dynamic process, it involves several physiological variables that circadian cycling. Clinical and experimental studies have demonstrated that clock genes expression are closely related to metabolic syndrome (Gómez-Abellán et al., 2008; Scott et al., 2008) and to an increased risk for cardiometabolic disorders (de Oliveira et al., 2019; Golombek et al., 2013; Karatsoreos et al., 2011). This increased risk includes alterations on the hepatic insulin pathway, indicative of hepatic insulin resistance (de Oliveira et al., 2019). The gene expression of pancreatic islet cells (Perelis et al., 2015; Saini et al., 2016) and rennin-angiotensin system (Herichova et al., 2014; Ohashi et al., 2017) are regulated by the core clock genes. The clock genes alterations can affect multiple systems concomitantly, since they constitute promoter factors for type 2 diabetes (Cao and Wang, 2017). We recently published biological molecules not only represent association of type 2 diabetes and breast, bladder, liver, pancreas, colon and rectum cancer but also have significant potential to be considered as systems-level biomarkers that may be used for screening or therapeutic purposes (Pereira et al., 2019); however, the link between them and clock genes altered expressions are still unknown.

The cellular metabolism relies on circadian control, both within normal or tumor cells, in this way, the circadian desynchronization can occur at molecular level and/or systemically. Individuals with shift work are prone to develop cancer and metabolic syndrome. The International Agency for Research on Cancer of the World Health Organization determined that shift work at night is the most disruptive for the circadian clock and, for this reason, increase the tendency to cancer development (Blair et al., 2010). It was classified as “probably carcinogenic to humans” (International Agency for Research on Cancer, 2019). All these data converge to the same point: the circadian disruption can lead to the metabolic disorders and cancer development. We, in the present study, are searching the cross-talk elements to close the circuit: chrono disruption – cancer – diabetes.

METHODS

Collection and inclusion criteria of studies

We searched the GEO database (https://www.ncbi.nlm.nih.gov/geo/) for publicly available studies. The inclusion criteria for studies were as follows: (1) samples diagnosed with type 2 DM and normal samples, (2) experiments that involve circadian rhythm and/or sleeping analysis and, (3) gene expression profiling of mRNA. After a systematic review, seven gene expression profiles (GSE56931, GSE20966, GSE25724, GSE26168, GSE23343, GSE29221 and GSE6514) were collected. GSE20966 (20 samples/ 7 female and 13 male) is a human gene expression profiles of beta-cell enriched tissue obtained by Laser Capture Microdissection from subjects with type 2 diabetes. GSE25724 (pancreatic islets – 13 samples/ 6 female and 7 male), GSE26168 (blood – 24 male samples), GSE23343 (liver – 17 samples/ 7 female and 10 male), GSE29221 (skeletal muscle – 24 male samples) are human expression data from type 2 diabetic and non-diabetic male and female patients.

Blood draws every 4 hours during a 3-day study: 24-hour normal baseline, 38 hours of continuous wakefulness and subsequent recovery sleep, for a total of 19 time-points per subject, with every 2-hr psychomotor vigilance test assessment when awake (GSE56931). GSE6514 sleep deprivation experiments were performed on male mice (C57/BL6), 10 weeks of age ±1 week. Animals were housed in a light/dark cycle of 12:12 h with water available *ad libitum*. Animals were subjected to 14 days of acclimatization during which a nighttime feeding pattern was established. Mice were euthanized following 3, 6, 9 and 12 h of total sleep deprivation. Deprivation was initiated at lights on and performed through gentle handling.

GSE82113 samples was analyzed under normal, sleep-deprivation and abnormal sleep-timing conditions to assess robustness of the predictor. Blood samples covering at least one circadian cycle to measure mRNA abundance and measuring melatonin rhythms. We also performed analysis in peripheral tissue profiles. GSE4239 was used to determine clock regulated genes in the adrenal gland. *Per2Brdm1/Cry1*$^{-/-}$ animals was used for expression analysis. Animals were entrained to a 12 hr light:12 hr dark cycle for two weeks and after released into constant darkness. Restricted feeding impacts the hepatic circadian clock of *Cry1*, *Cry2* double KO mice lack a circadian clock was performed by using GSE13062 profile. Similarly, liver tissue of wildtype, Clock mutant and Cry deficient C57BL/6 8- to 10-week-old male mice was examined in GSE454 profile. Mice were entrained under 12 h of white light and 12 h of darkness for 14 days. GSE6904 profile showed data from mice were exposed starting at 1 hour after lights off to a 30-minute light pulse. At the end of the light pulse, mice were

euthanized and the brains extracted. Cells from the suprachiasmatic nucleus were extracted using laser capture microscopy and gene expression was quantified using Affymetrix microarrays.

Microarray data and Data processing

GEO2R was applied to screen differentially expressed mRNAs between type control and experimental tissue samples. GEO2R (http://www.ncbi.nlm.nih.gov/geo/geo2r/) is an interactive web tool for comparing two groups of data that can analyze any GEO series. The p-values using Benjamin and Hochberg false discovery rate method by default were applied to correct the occurrence of false positive results.

Cancer expression analyses

Gene list collection, Functional and pathway enrichment analysis, RNA-seq and clinical information data from TCGA were performed as previously described (Pereira et al., 2019). We performed survival analysis using Tumor Immune Estimation Resource (TIMER) a web server (https://cistrome.shinyapps.io/timer/) (Li et al., 2017). Kaplan–Meier for BLCA, BRCA, COAD, LIHC, PAAD and READ expression profiles plots were drawn to explore the association between clinical outcome and gene expression. Immunohistochemistry protein data for normal and cancer samples were performed with the Human Protein Atlas (https://www.proteinatlas.org/).

RESULTS

Identification of differentially expressed genes, gene ontology enrichment and functional classification

In an attempt to characterize the molecular signaling events in temporal changes in gene expression during spontaneous sleep and extended wakefulness, we adopted a dataset of expression profiling of mRNA from GEO database. This dataset contains the total mRNA of animals euthanized at different times during the lights on period. The experiments address temporal changes in gene expression during spontaneous sleep and extended wakefulness in the mouse cerebral cortex, a neuronal target for processes that control sleep; and the hypothalamus, an important site of sleep regulatory processes. To identify the differentially expressed genes (DEGs), we applied the online tool GEO2R and found out upregulated and downregulated genes.

We performed a GO term enrichment and functional classification by DAVID to investigate the biological and functional roles of these DEGs. In cortex, we observed downregulation of cell adhesion molecules (CAMs) after 3h and 6h of sleep deprivation. Neuroactive ligand-receptor interaction pathway was downregulated after 9h and 12 h of sleep deprivation. Galactose metabolism pathway and Viral myocarditis, Asthma and, Allograft rejection pathways were upregulated after 6h and 12h, respectively. According to hypothalamus analysis, downregulation of Retinol metabolism and Complement and coagulation cascades pathways were observed after 3h and, neurotrophin signaling pathway, Pathways in cancer and signaling pathway were the top three pathways downregulated after 6h of sleep deprivation. Calcium signaling pathway was downregulated after 9h and, colorectal cancer and Melanogenesis pathways were found after 12h. Phosphatidylinositol signaling system and oxidative phosphorylation pathways were found active after 3h and 6h, respectively. Active pathways found after 9h of sleep deprivation were pathways in cancer, maturity onset diabetes of the young, intestinal immune network for IgA production and natural killer cell mediated cytotoxicity. Finally, PPAR signaling pathway and cytokine-cytokine receptor interaction were active after 12h of sleep deprivation.

To analyze the functional classification and to facilitate the high-throughput analysis of these DEGs, a protein classification analysis according to family and subfamily of the identified DEGs was performed by the PANTHER classification system. According to the study, after 3h, 6h, 9h and 12h of sleep deprivation in both hypothalamus and cortex samples, we identified that binding (GO:0005488), catalytic activity (GO:0003824) and molecular function regulator

(GO:0098772) are the top three abundant protein classes.

Data from hepatic tissue of *Cry1*, 2 double knockout temporally restricted feeding mice showed Fatty acid biosynthesis and insulin signaling pathways were downregulated when compared to wildtype mice. Drug and linoleic acid metabolisms were upregulated (GSE13062). Downregulation of cell cycle, Pathways in cancer, axon guidance, and Wnt signaling was observed in clock mutant mice. Cry deficient showed downregulation of cell cycle and neuroactive ligand receptor, while Pathways in cancer was upregulated when compared to wild type mice (GSE454). Expression data from mice suprachiasmatic nucleus after 30 minutes light pulse (GSE6904) showed downregulation of ECM-receptor interaction and upregulation of Mapk signaling, Focal adhesion and Wnt signaling pathways (Supplementary Table 1-16).

Overview of the type 2 diabetes and cancer transcriptomic analysis

We previously characterized the molecular signaling events in the diabetes by using datasets containing the total mRNA of normal/diabetic pairs of pancreatic islets, blood and skeletal muscle tissues (Pereira et al., 2019). We applied the online tool GEO2R and found out upregulated and downregulated genes in isolated human pancreatic islets, liver, blood dataset, and skeletal muscle. By using Entrez Gene and KEGG pathway analysis, we identified 214 circadian rhythm-related genes. Venn diagram was performed to show the overlap between DEG genes identified from the meta-analysis and those from the circadian rhythm-related genes. In diabetic samples, abundant circadian rhythm expression variations were observed indicating that different gene expression patterns may exist in diabetic tissues (Figure 1).

We obtain the gene expression data of specimens across 6 cancer types from TCGA and preprocessed the data of each cancer type with standard methods. These cancer types include Bladder Urothelial Cancer (BLCA), breast invasive carcinoma (BRCA), Colon Adenocarcinoma (COAD), Liver Hepatocellular carcinoma (LIHC), Pancreas adenocarcinoma (PAAD) and Rectum Adenocarcinoma (READ). We conducted a systematic and integrative cancer analysis to explore cancer type-specific circadian rhythm subnetworks. Specifically, to construct a cancer network, we first determine DEGs by comparing expression level of tumors to normal samples. In tumors abundant circadian rhythm expression variations were observed indicating that different gene expression patterns may exist in cancer tissues. *Klf10* was downregulated in BLCA, BRCA, LIHC and READ; *Ntrk3* and *Igf1* were downregulated in BLCA, BRCA, COAD, LIHC and READ; Similarly, *Klf9* and *Usp2* downregulation were found in BLCA, BRCA, COAD and READ. *Ezh2*, *Cdk1*, *Top2a*, *Agrp*, *Aanat* and *Ren* were

upregulated in BLCA, BRCA, COAD and READ, while *Arntl2* was upregulated in BLCA, BRCA, COAD and READ. *Klf10* was also found to be downregulated in blood samples from type 2 diabetic patients. Similarly, *Ntrk3* and *Igf1* were downregulated in blood and skeletal samples and, skeletal and liver samples, respectively. *Usp2* was observed to be downregulated in blood samples. *Ezh2* was upregulated in blood samples, while *Arntl2* and *Agrp* was upregulated in skeletal muscle (Figure 1, Supplemetary Table 17-19, respectively).

Survival analysis showed that *Igf1* and *Usp2* low expression was associated with poor survival in BLCA, LIHC, AND BRCA and high expression of *Arntl2* was associated with poor survival in LIHC and BRCA samples (Supplementary Figure 1). We found a positive correlation between *Klf10, Ntrk3, Igf1* and *Usp2* in almost all cancer samples. The DEG analyses are supported by immunohistochemistry analysis (Supplementary Figure 2-6, Figure 2-5).

IGF1 and ARNTL2 have been shown to play a role in immune modulation. Then, we investigate the association of their expression with immune cells in cancer. We found that their mRNA expressions were negatively associated with tumor purity and positively associated with infiltration of B Cells, CD8+ T cells, CD4+ T cells, Macrophages, Neutrophils and Dendritic cells (Supplementary Figure 7 and 8, respectively).

DISCUSSION

Over the last decade, substantial research has been undertaken to understand the multiple biological processes directed by endogenous clock genes that lead to type 2 DM and carcinogenesis. Integration of the genome-wide biological data with biomolecular networks is required to make a clear conclusion on mechanisms for signature of those diseases (Buttar et al., 2005; Prabhakar et al., 2014). In our previous study associating type 2 DM and cancer, we observed association of type 2 DM and renin-angiotensin biomarkers with breast, bladder, liver, pancreas, colon and rectum cancer and also a significant potential to be considered as systems-level biomarkers that may be used for screening or therapeutic purposes (Pereira et al., 2019). We are now analyzing those samples according to circadian rhythm-related genes.

The aim of our study is to identify the link between type 2 DM, circadian rhythm-related genes and cancer by analyzing animal-based research of sleep deprivation, type 2 DM samples and, BLCA, BRCA, COAD, LIHC, PAAD and READ cancer samples. We performed several analytical methods to determine the underlying molecular mechanisms. First, we detected downregulated and upregulated DEGs in mouse cortex and hypothalamus samples. The authors determine the changes by comparing expression in sleeping animals euthanized at different times during the lights on period, to that in animals sleep deprived and euthanized at the same diurnal time. In summary, upregulated genes active genes associated with oxidative phosphorylation, cancer and diabetes, mainly in hypothalamus specimens. In cortex, we observed mainly downregulation of immune system.

In mammals, light information is perceived by the retina and transmitted to the suprachiasmatic nuclei through the retinohypothalamic tract. The projections of the suprachiasmatic nuclei, have at least four neuronal targets: endocrine neurons, autonomic neurons of the paraventricular nucleus of the hypothalamus, other hypothalamic structures, and areas outside the hypothalamus (Bozek et al., 2009; de Oliveira et al., 2019). These efferent pathways are able to synchronize peripheral clocks, controlling various physiological functions, such as hormone releasing, food behavior and temperature fluctuations. It has been shown that shift work promotes sleep disturbances (Zhu and Zee, 2012). These disturbing external signals induce loss of coherence between the central oscillator and the peripherals and can lead to diseases that characterize the internal desynchronization: insomnia, cardiovascular disorders, obesity, depression, diabetes, dysregulation of metabolic rhythms and endocrine and even cancer. Several reports have revealed that clock genes are found to be deregulated in several cancer types. In comparison with surrounding non-cancerous cells, breast cancer cells reveal

disturbances in the expression of the clock genes attributable to methylation, which is associated with decreased gene expression (Basudhar et al., 2019; Soták et al., 2013; Zhu and Zee, 2012).

We first propose that sleeping disturbance in animal models could promote type 2 DM and cancer development, by hypothalamus and cortex gene expression deregulation. Next, DEGs were combined with 214 circadian rhythm related genes to type 2 DM and cancer samples. We observed that several common genes deregulated in both diseases. *Ntkr3*, *Igf1* and *Usp2* were both downregulated in type 2 DM and cancer samples, while *Arntl2* and *Agrp* were upregulated. It seems that the changes in mRNA are contributing to the phenotypic changes in type 2 DM, resulting in phenotypic changes associated with the malignant transformation. Taking those genes to perform a survival analysis, we found only *Igf1, Usp2* and *Arntl2* genes associated with patient outcomes. While *Igf1* and *Usp2* downregulation had a negative impact, *Arntl2* upregulation was associated with poor survival both in BLCA and BRCA cancer samples.

IGF1 is a mitogen for a variety of cells and exerts this action through the MAP kinase signaling pathway by increasing DNA synthesis and stimulating the expression of cyclin D1, which accelerates progression of the cell cycle from the G1 to S phase. The circulating IGF1 and IGF2 bind to IGF1 receptors (IGF1R) and trigger a signal transduction cascade (Shi et al., 2014). This signaling is very critical for the processes of oncogenesis. IGF1R activity is mediated by the Ras and AKT pathways and results in upregulation of cyclin D1 and its CDK4 linker, leading to retinoblastoma protein phosphorylation, release of the E2F transcription factor and expression of downstream target genes such as cyclin E. Plasma IGF1 level changes during the day, suggesting some circadian control, but molecular mechanisms are not clear. In our study, we observed downregulation of *Igf1* mRNA expression in several cancer types. However, protein analysis showed higher levels of IGF1 in cancer compared to normal samples. A previous study analyzing around-the-clock blood sampling, showed that both healthy and cancer individuals were found to be similarly synchronized to the 24-h sleep-wake schedule. However, growth hormone (GH)-IGF-l axis function, cortisol secretion and IL-2 serum levels were altered in cancer patients. In cancer patients there was an increasing trend and progressive loss of circadian rhythmicity of GH secretion, accompanied by a decreasing of IGF-1 serum levels, upregulation of cortisol secretion and IL-2 serum levels. It has been well established that IGF1 promotes cancer cell proliferation, migration and metastasis. IGF1 has been shown to be controlled by CRY1/2 proteins. *Cry2* mRNA was downregulated in BLCA and BRCA samples in our study. However, we observed higher CRY2 protein expression in cancer samples. This data suggests that CRY2 could control IGF1 levels and these alterations may be related to the

process of carcinogenesis and, could favor cancer progression.

The deubiquitinating enzyme Ubiquitin Specific Protease 2 (USP2) is highly dependent on the circadian cycle, showing biological clock oscillations(Yang et al., 2012). It can be expressed under two isoforms, *Usp2a* and *Usp2b*. *Usp2b* has been shown having oscillations more dependent on circadian regulation. This enzyme has been reported as a stabilizer of *Bmal1* gene (Scoma et al., 2011), and acts on the ubiquitination of *Per1*, a central gene, essential for the production of circadian behavioral rhythms. Animal models have shown that total inhibition of USP2 can compromise the rhythm circadian pathway thereby altering several other genes dependent on these biological oscillations. USP2 directly affects the levels of deubiquitinating proteins such as MDM2, cyclin D1 and CRY1 and their substrates, conferring resistance to apoptosis in mutated cells, acting as an oncogene. Since USP2 is the only enzyme capable of deubiquitinating cyclin D1 in human cells, cyclin D1 is reported as the most effective substrate for USP2.

Taking into account that several diseases can be triggered by circadian rhythm dysregulation, among them we can highlight diabetes and cancer (Renehan et al., 2012). The role of *Usp2* expression would be to induce prolonged fasting, occurring at the end of the clear phase of the circadian rhythm, thus stimulating the occurrence of hepatic gluconeogenesis. In addition, USP2 is required to maintain glucose homeostasis and regulate glucose tolerance through glucocorticoid signaling. Both circadian and USP2 deregulations accelerate the process of hepatic gluconeogenesis by increasing glucose secretion. Experiments have shown that a deregulated rhythm changes serum glucose levels, the development associated with glucocorticoid receptors. Excessive activity in glucocorticoid signaling may lead to the development of glucose intolerance. We suggest that changes in circadian rhythm and USP2 levels may be associated with the initiation and progression of type 2 diabetes, leading to cancer development. We also found that *Igf1* and *Usp2* expressions were negatively associated with tumor purity and positively associated with infiltration of B Cells, CD8+ T cells, CD4+ T cells, Macrophages, Neutrophils and Dendritic cells. Besides, TIMER analysis confirms that those genes overexpression are positively associated with macrophage population. The results show that these genes are playing an important role in immune modulation.

Taking all data together, we hypothesized that changes in the expression of circadian rhythm related-genes in type 2 DM and cancer could be responsible for metabolic changes that could lead to the cancer development. Such shifts in tissue metabolism results, at least in part, from profound recruitment of inflammatory cell types, particularly myeloid cells, such as neutrophils and monocytes. Expression of *Igf1* and *Usp2* may be associated with the immune

system in both the diabetes and cancer samples. These disturbances may lead to alterations in immunity by enhancing the infiltration of leukocytes and increased expression of pro-inflammatory cytokines. These diverse cells communicate with each other by means of direct contact or by cytokine and chemokine production and act in autocrine and paracrine manners to control and shape tumor growth and metastasis. Interestingly, we observed lower expression of ARNTL2 protein in cancer samples. This protein plays an important role in circadian rhythm system and it has been shown to be associated with immune escape mechanism. Besides, previous results have demonstrated that *Arntl2*, in association with *Nr1d1* and *Npas2* may function as an oncogene. This indicates that the altered expression levels of those genes could be associated with cancer progression and aggressive metastatic phenotype.

These biological molecules not only represent the association of type 2 DM and circadian rhythm biomarkers with breast, bladder, liver, pancreas, colon and rectum cancer but also have significant potential to be considered as systems-level biomarkers that may be used for screening or therapeutic purposes. Our data stimulate efforts in news studies to achieve the experimental and clinical validation about these biomolecules.

Competing interests

The authors declare that they have no competing interests.

Ethics approval and consent to participate

Not applicable.

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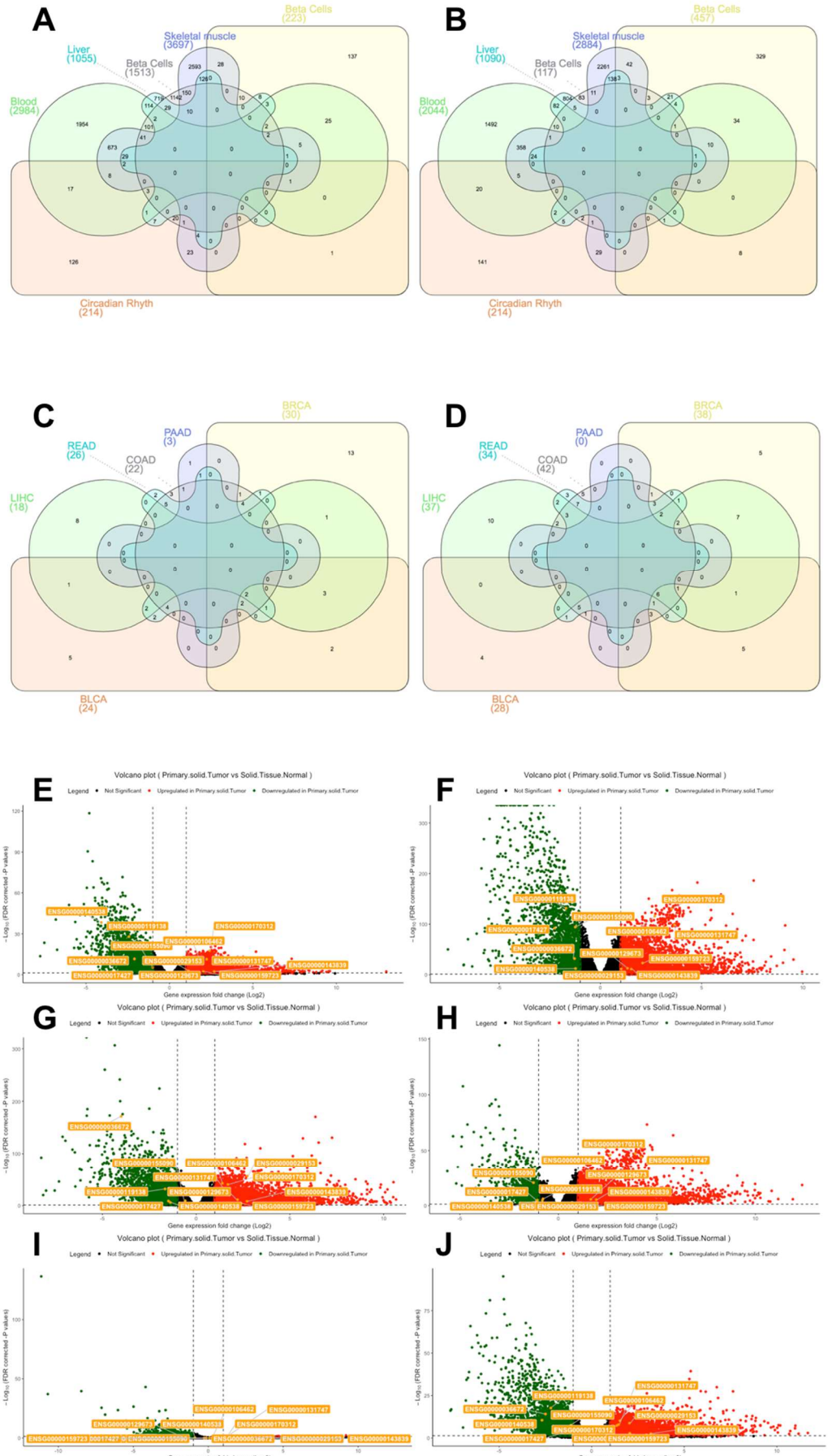
Figure 1 -Venn Diagrams of differentially expressed genes in mRNA expression profiling datasets. We are showing downregulated (A) and upregulated (B) genes in human Beta Cells (GSE20966 and GSE25724), blood (GSE56931 and GSE26168), skeletal muscle (GSE29221), and liver (GSE23343). We are also showing downregulated and upregulated cancer samples (C and D, respectively) combined with clock gene list. The number in each intersecting region represents the number of overlapping genes. Volcano plots of differentially genes expression in breast invasive carcinoma (E), Colon Adenocarcinoma (F)Liver Hepatocellular carcinoma (G), Pancreas adenocarcinoma (H) and Rectum Adenocarcinoma (I). Red: up-regulation; green: down-regulation. *Klf10* (ENSG00000155090) was downregulated in BLCA, BRCA, LIHC and READ; *Ntrk3* (ENSG00000140538) and *Igf1* (ENSG00000017427) were downregulated in BLCA, BRCA, COAD, LIHC and READ; Similarly, *Klf9* (ENSG00000119138) and *Usp2* (ENSG00000036672) downregulation were found in BLCA, BRCA, COAD and READ. *Ezh2* (ENSG00000106462), *Cdk1* (ENSG00000170312), *Top2a* (ENSG00000131747), *Agrp* (ENSG00000159723), *Aanat* (ENSG00000129673) and *Ren* (ENSG00000143839) were upregulated in BLCA, BRCA, COAD and READ, while *Arntl2* (ENSG00000029153) was upregulated in BLCA, BRCA, COAD and READ.


A
Beta Cells (223)
Skeletal muscle (3697)
Liver (1055)
Beta Cells (1513)
Blood (2984)
Circadian Rhyth (214)
B
Beta Cells (457)
Skeletal muscle (2884)
Liver (1090)
Beta Cells (117)
Blood (2044)
Circadian Rhyth (214)
C
BRCA (30)
PAAD (3)
READ (26)
COAD (22)
LIHC (18)
BLCA (24)
D
BRCA (38)
PAAD (0)
READ (34)
COAD (42)
LIHC (37)
BLCA (28)
E
Volcano plot (Primary.solid.Tumor vs Solid.Tissue.Normal)
Legend Not Significant Upregulated in Primary.solid.Tumor Downregulated in Primary.solid.Tumor
Gene expression fold change (Log2)
F
Volcano plot (Primary.solid.Tumor vs Solid.Tissue.Normal)
Legend Not Significant Upregulated in Primary.solid.Tumor Downregulated in Primary.solid.Tumor
Gene expression fold change (Log2)
G
Volcano plot (Primary.solid.Tumor vs Solid.Tissue.Normal)
Legend Not Significant Upregulated in Primary.solid.Tumor Downregulated in Primary.solid.Tumor
Gene expression fold change (Log2)
H
Volcano plot (Primary.solid.Tumor vs Solid.Tissue.Normal)
Legend Not Significant Upregulated in Primary.solid.Tumor Downregulated in Primary.solid.Tumor
Gene expression fold change (Log2)
I
Volcano plot (Primary.solid.Tumor vs Solid.Tissue.Normal)
Legend Not Significant Upregulated in Primary.solid.Tumor Downregulated in Primary.solid.Tumor
Gene expression fold change (Log2)
J
Volcano plot (Primary.solid.Tumor vs Solid.Tissue.Normal)
Legend Not Significant Upregulated in Primary.solid.Tumor Downregulated in Primary.solid.Tumor
Gene expression fold change (Log2)

Fugure 2 - Representative immunohistochemical staining characteristics of ARNTL2 expressions in normal and cancer patients. Data are extracted from The Human Protein Atlas (https://www.proteinatlas.org/).

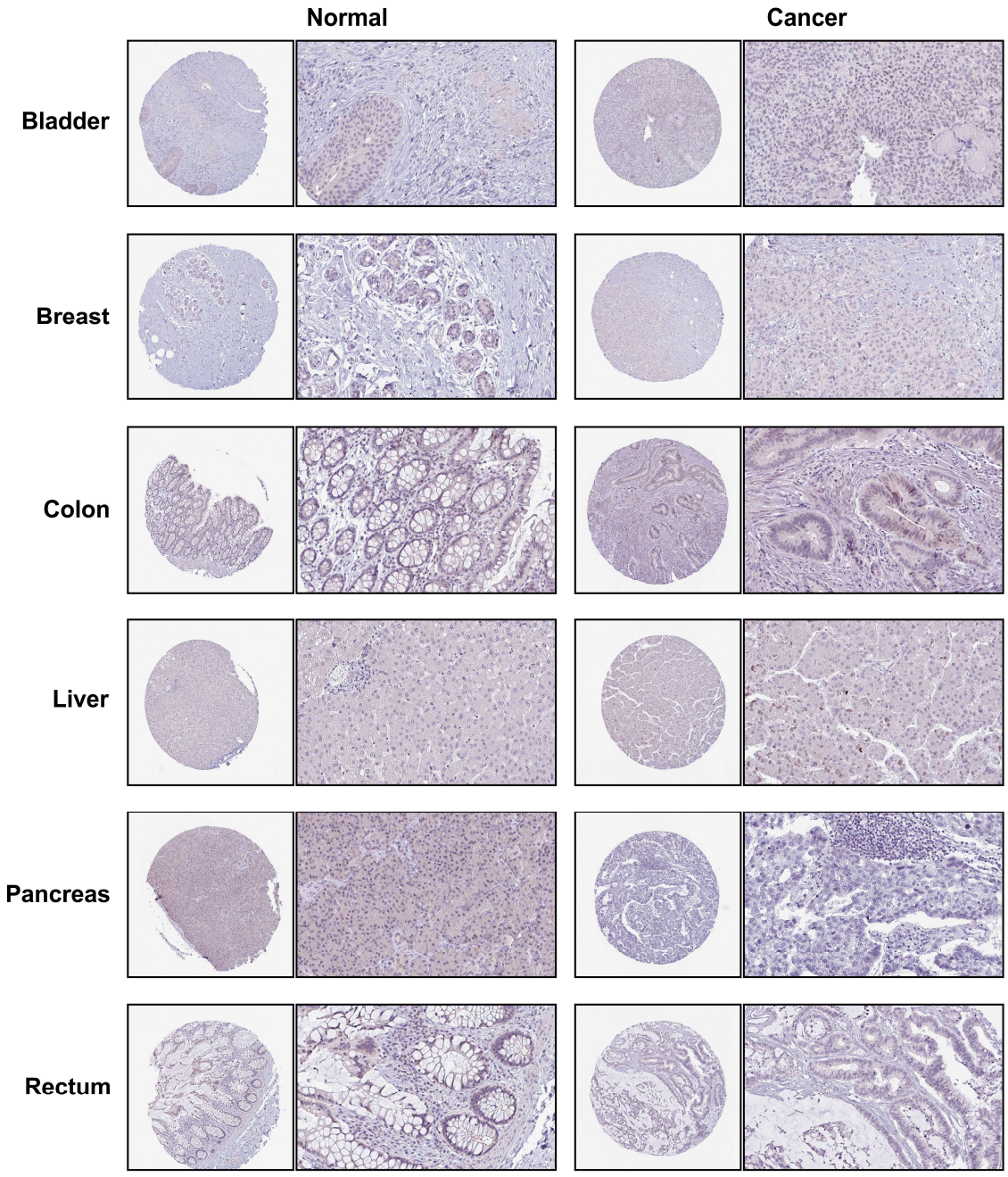


Figure 3 - Representative immunohistochemical staining characteristics of CRY2 expressions in normal and cancer patients. Data are extracted from The Human Protein Atlas (https://www.proteinatlas.org/).

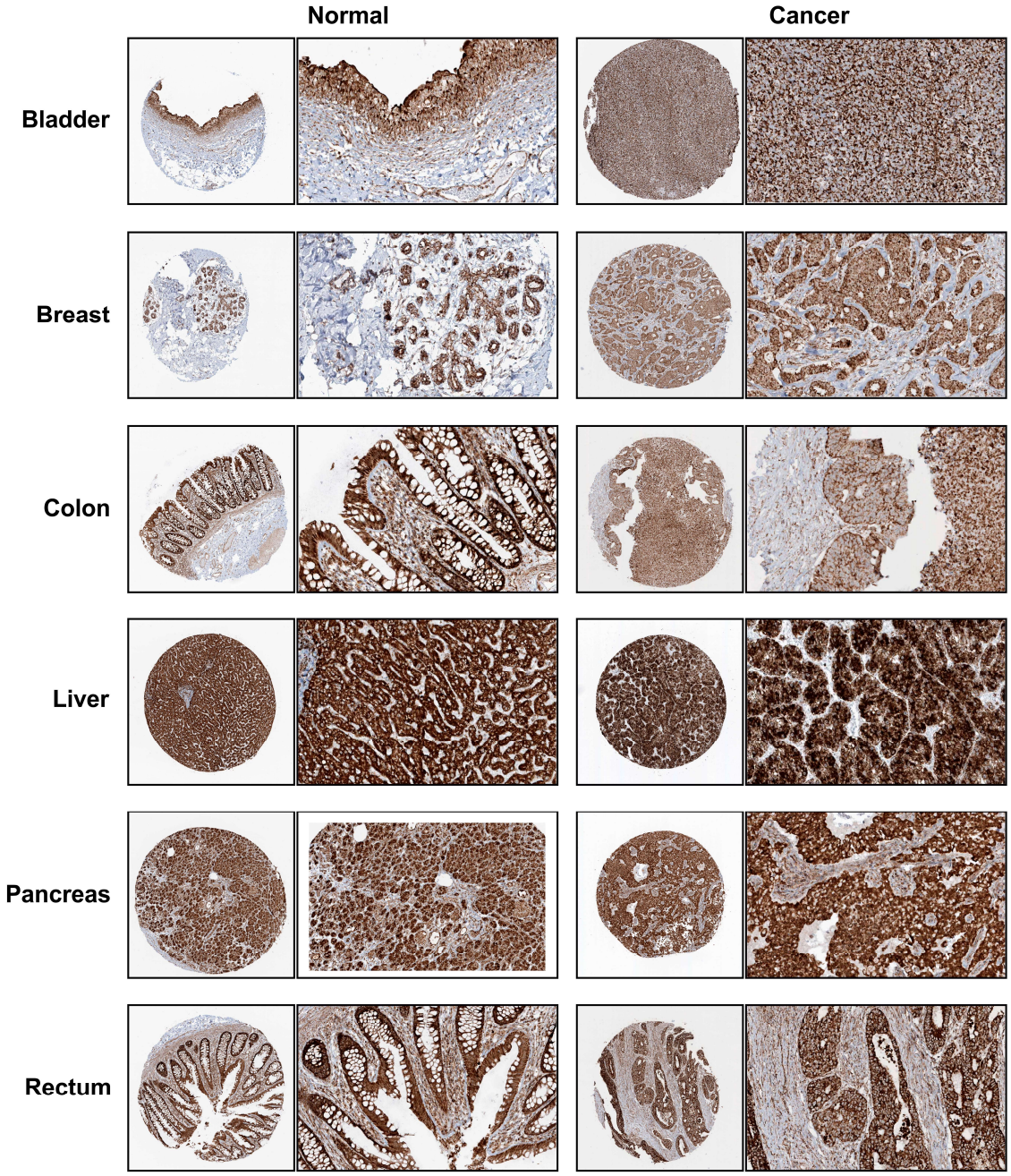


Figure 4 - Representative immunohistochemical staining characteristics of IGF1 expressions in normal and cancer patients. Data are extracted from The Human Protein Atlas (https://www.proteinatlas.org/).

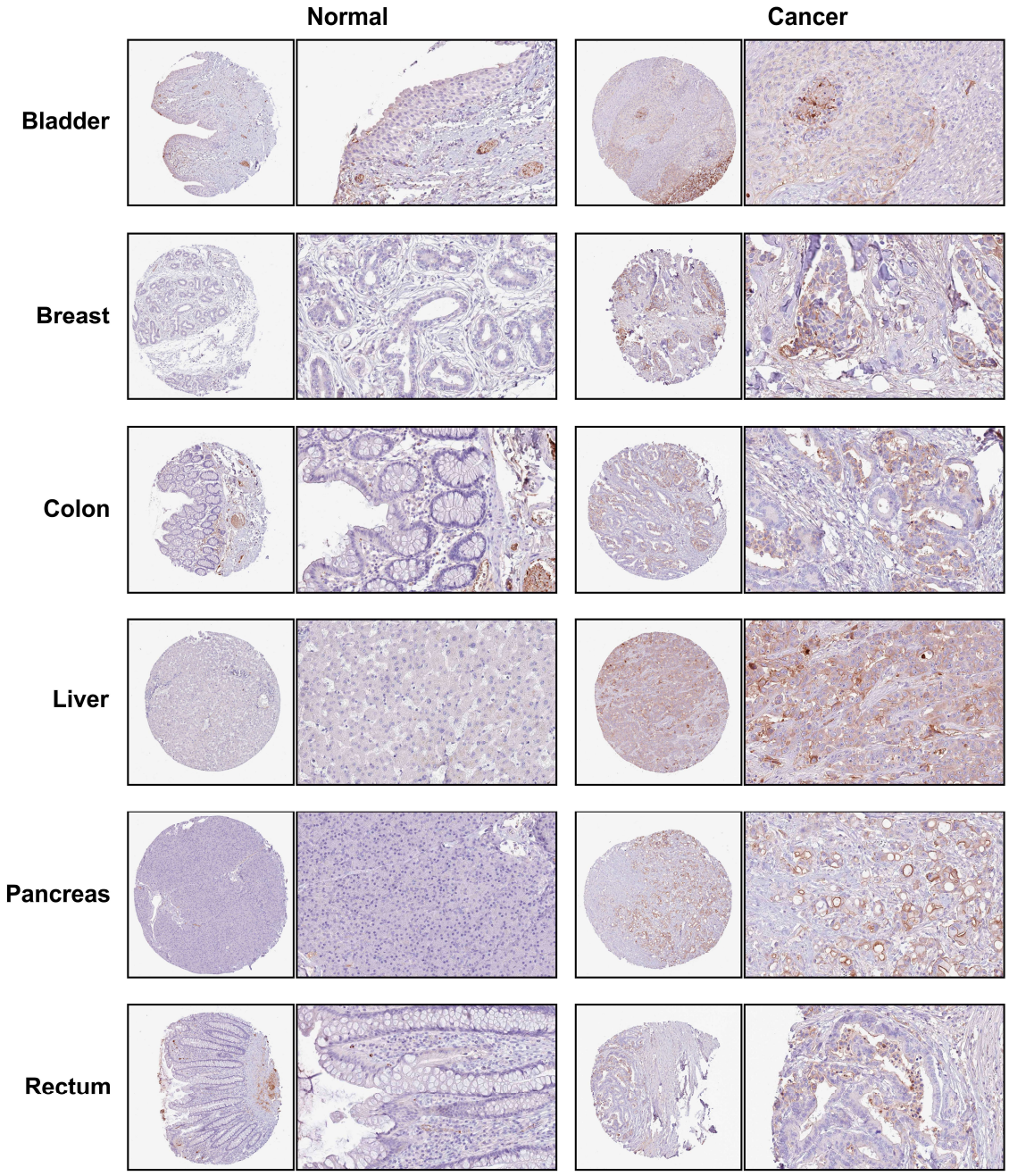
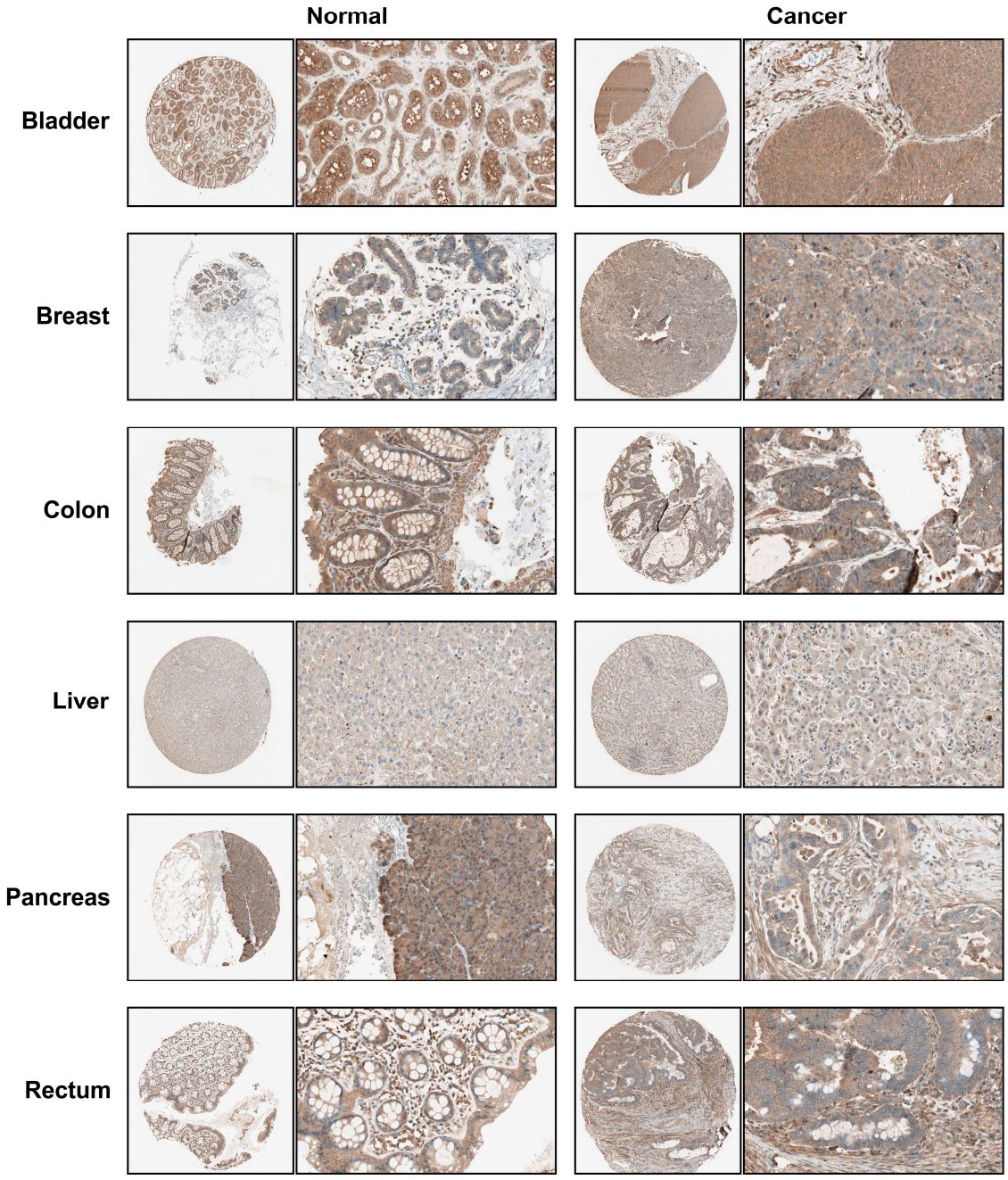
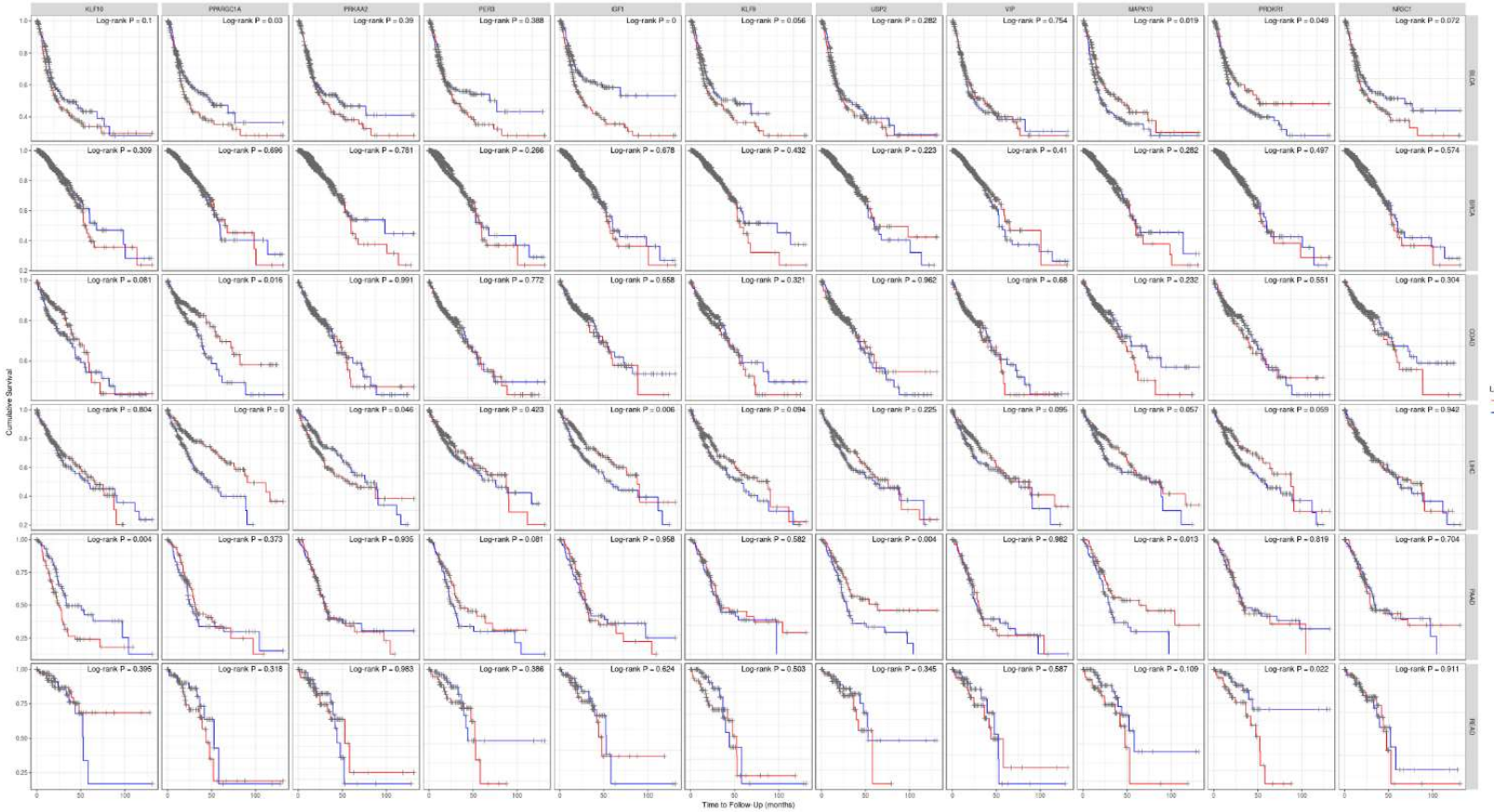


Figure 5 - Representative immunohistochemical staining characteristics of USP2 expressions in normal and cancer patients. Data are extracted from The Human Protein Atlas (https://www.proteinatlas.org/).

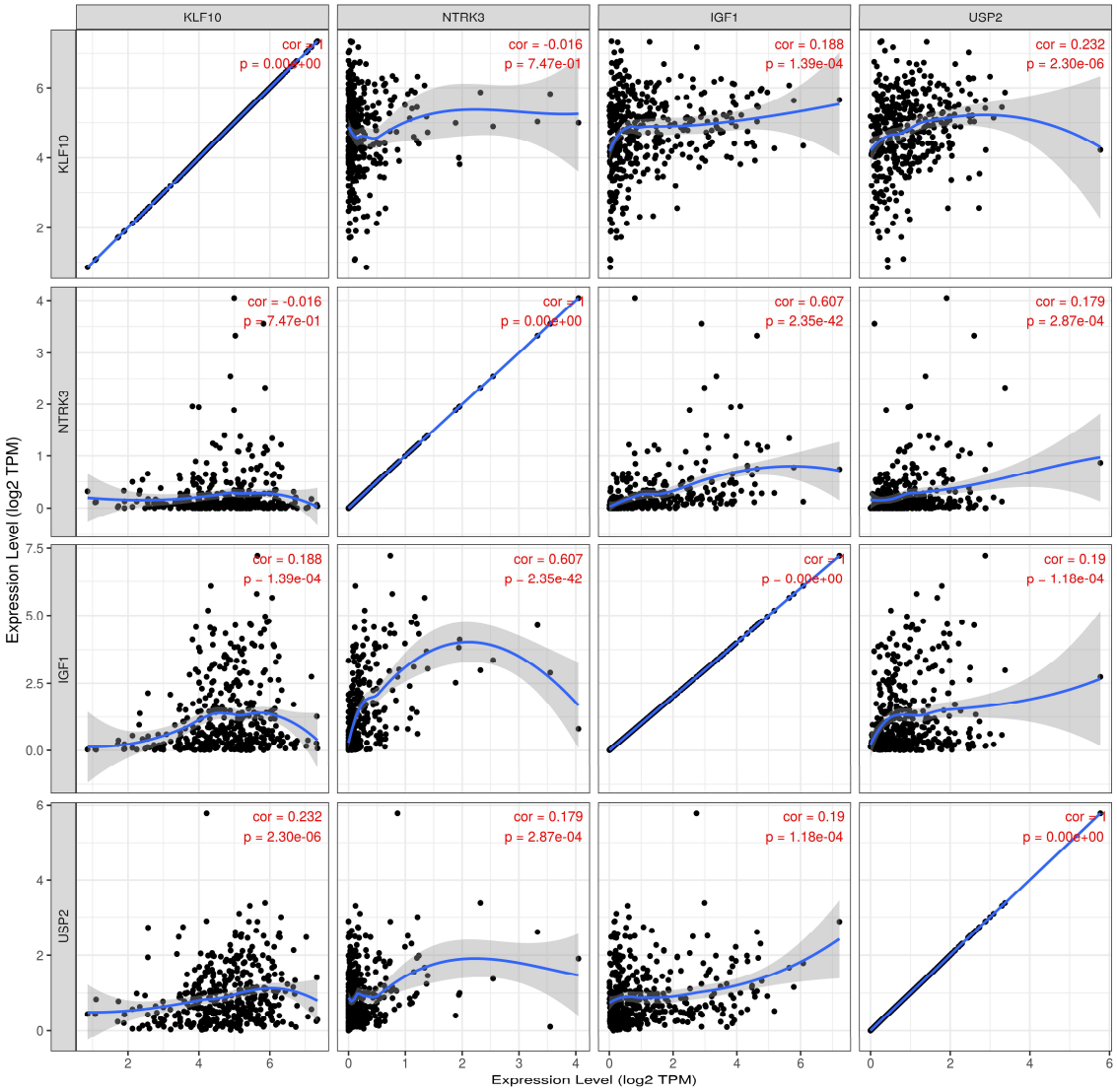


Supplementary Figure 1 - Kaplan-Meier analysis of *Klf10, Ppargc1a, Prkaa2, Per3, Igf1, Klf9, Usp2, Vip, Mapk10, Prokr1* and *nr3c1* in Bladder Urothelial Cancer (BLCA), breast invasive carcinoma (BRCA), Colon Adenocarcinoma (COAD), Liver Hepatocellular carcinoma (LIHC), Pancreas adenocarcinoma (PAAD) and Rectum Adenocarcinoma (READ). Data are extracted from TIMER web server. $P < 0,05$ was considered statistically significant.

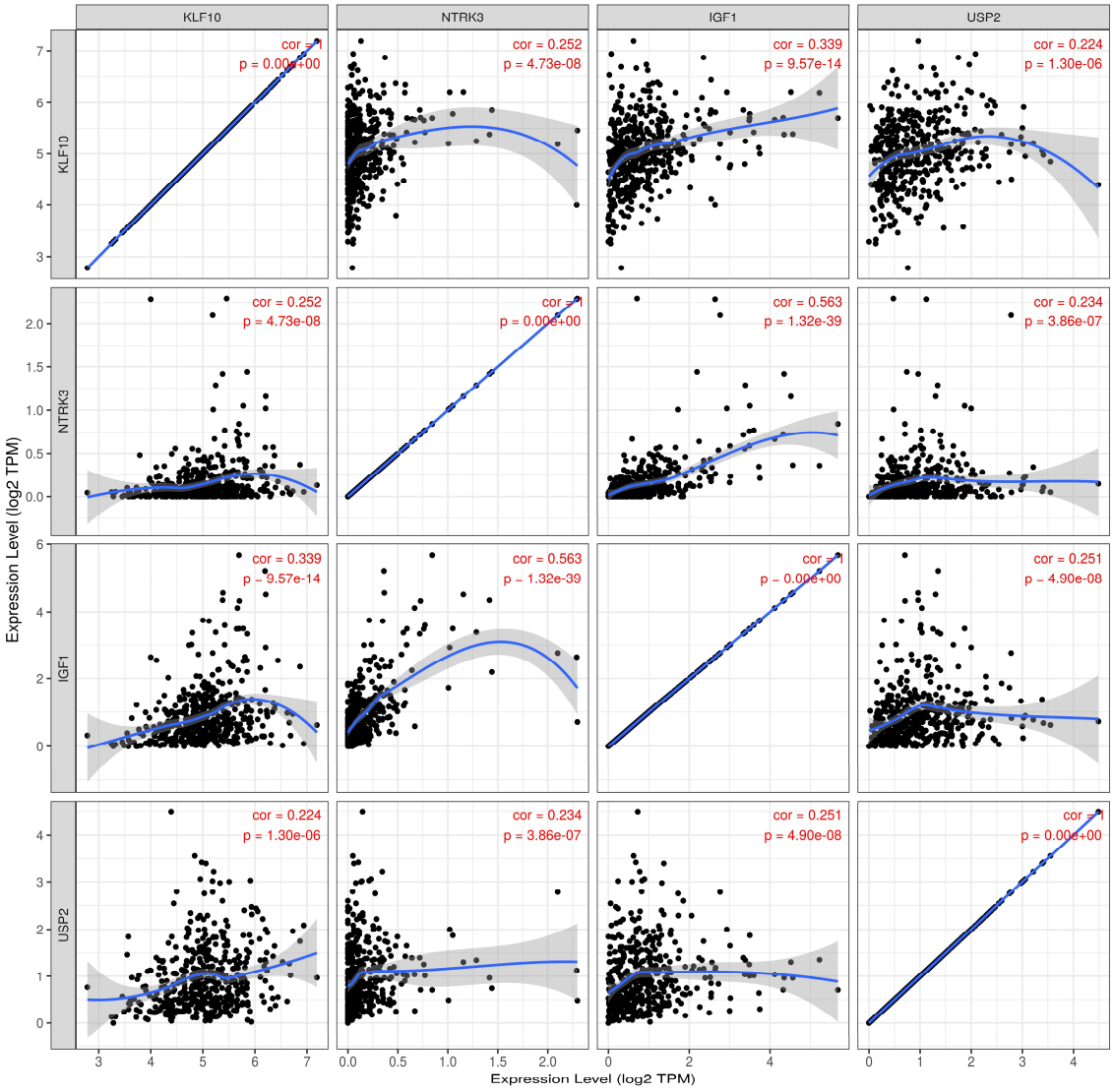


-- High (Top 50%)
--_Low (Bottom 50%

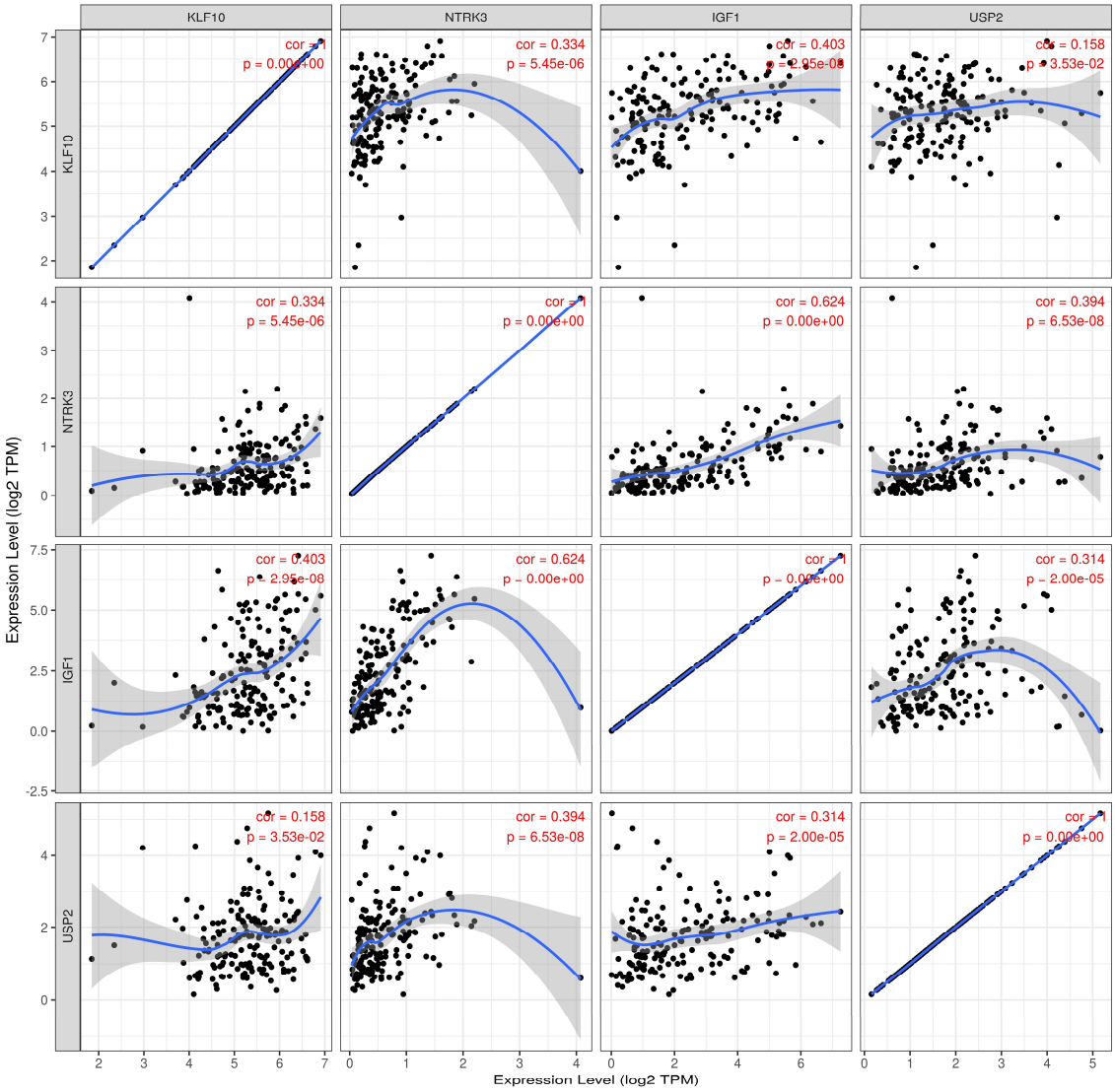
Supplementary Figure 2 - Correlation between *Klf10, Ntrk3, Igf1* and *Usp2* in Bladder Urothelial Cancer (BLCA). Data are extracted from TIMER web server. $P < 0,05$ was considered statistically significant.



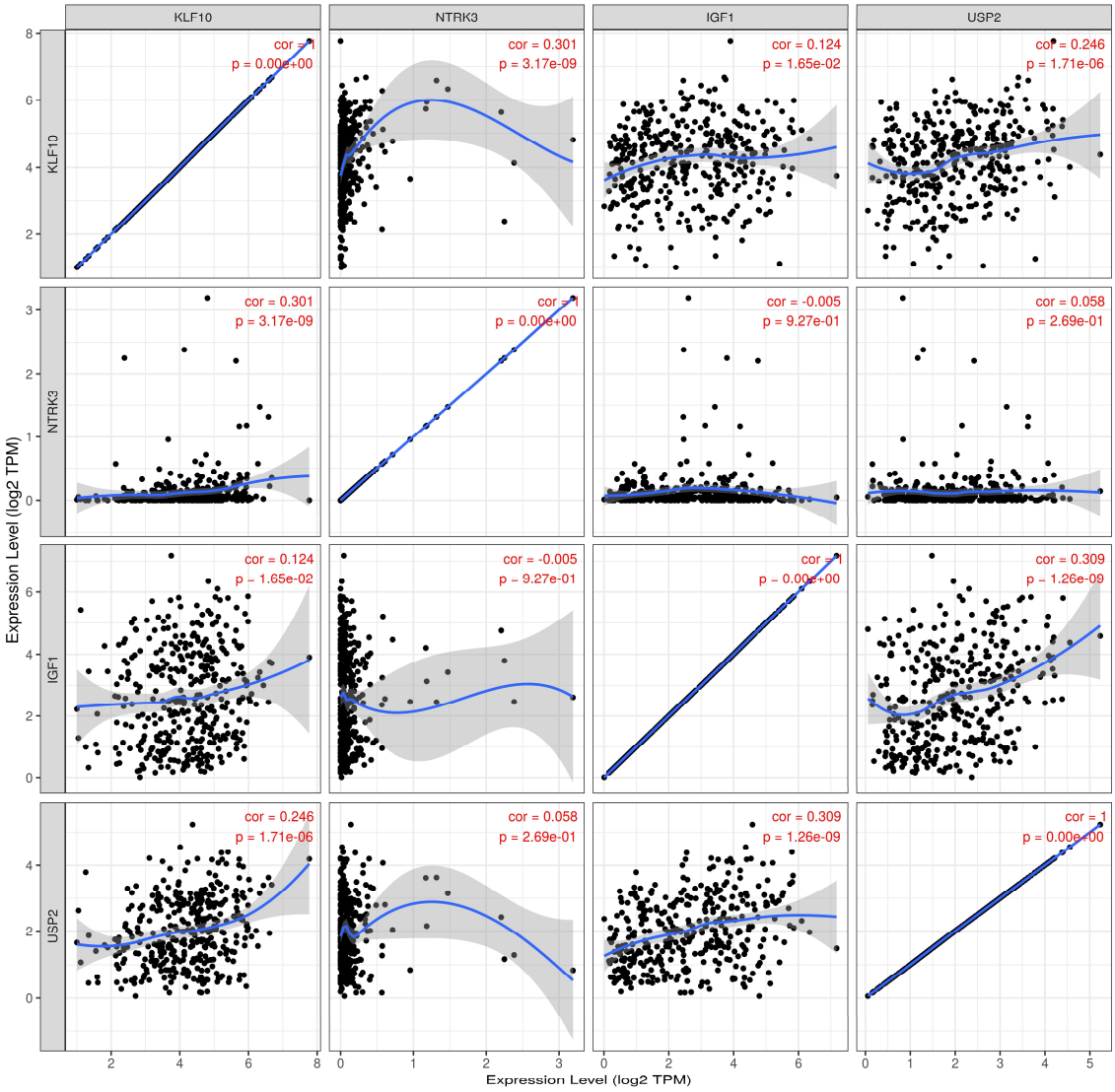
Supplementary Figure 3 - Correlation between *Klf10, Ntrk3, Igf1* and *Usp2* in Colon Adenocarcinoma (COAD). Data are extracted from TIMER web server. $P < 0,05$ was considered statistically significant.



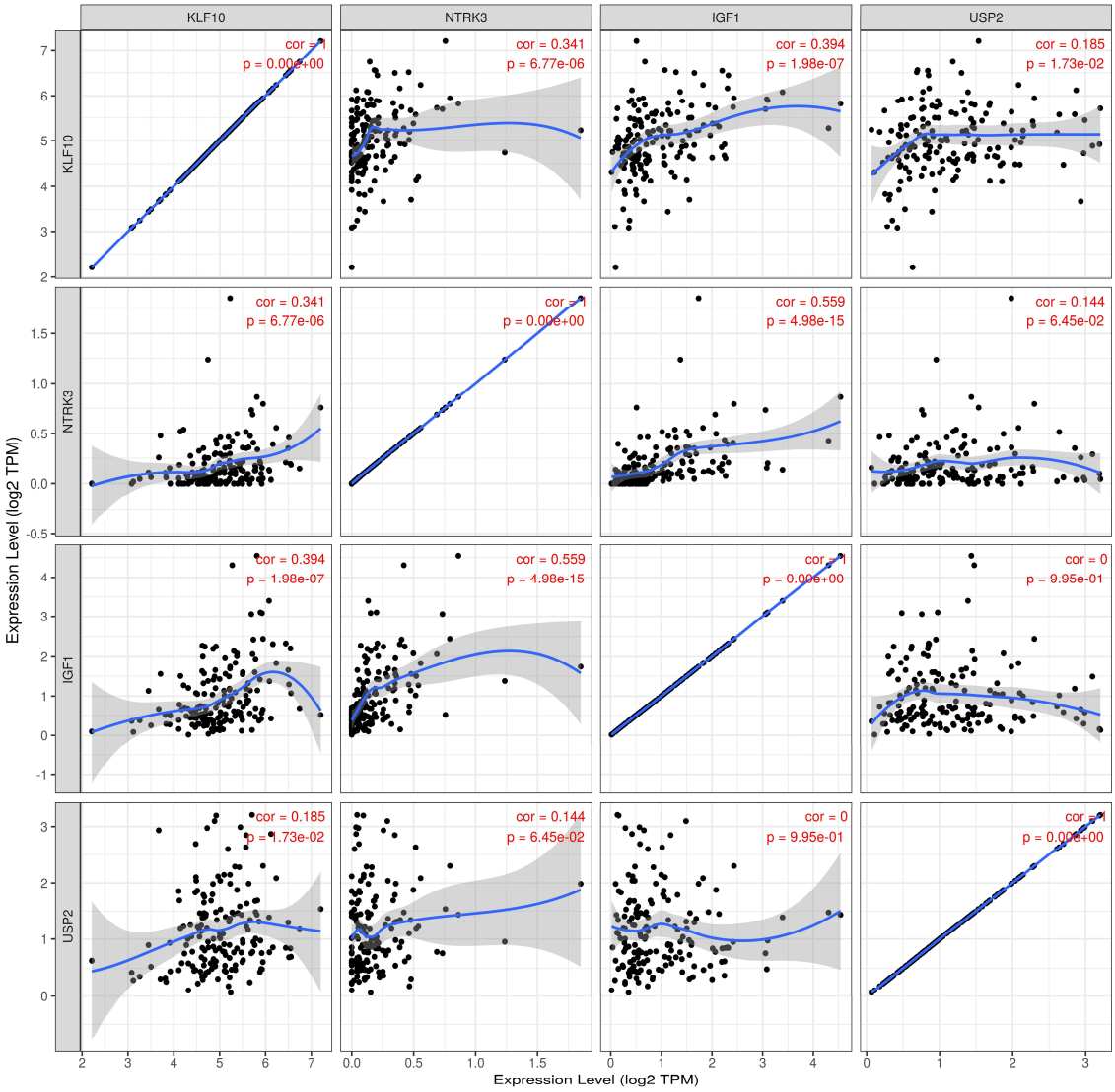
Supplementary Figure 4 - Correlation between *Klf10, Ntrk3, Igf1* and *Usp2* in Pancreas adenocarcinoma (PAAD). Data are extracted from TIMER web server. $P < 0,05$ was considered statistically significant.



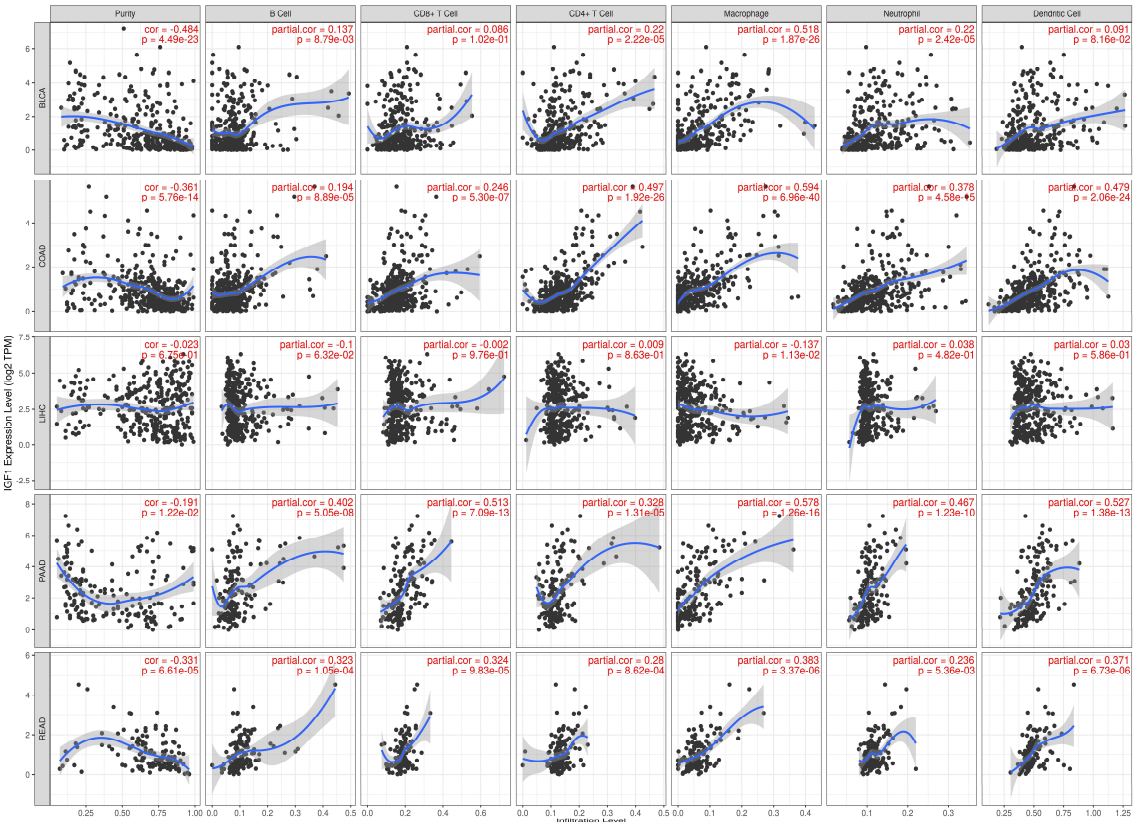
Supplementary Figure 5 - Correlation between *Klf10, Ntrk3, Igf1* and *Usp2* in Liver Hepatocellular carcinoma (LIHC). Data are extracted from TIMER web server. $P < 0,05$ was considered statistically significant.



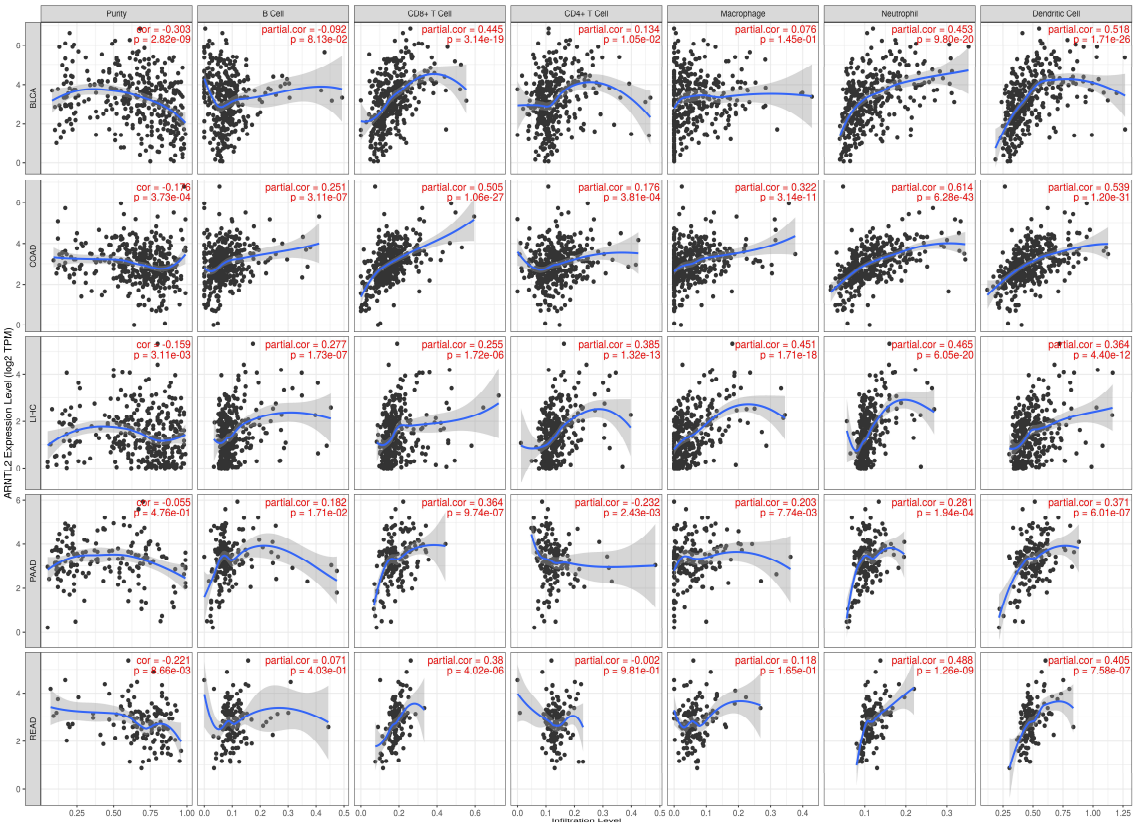
Supplementary Figure 6 - Correlation between *Klf10, Ntrk3, Igf1* and *Usp2* in Rectum Adenocarcinoma (READ). Data are extracted from TIMER web server. $P < 0,05$ was considered statistically significant.



Supplementary Figure 7 - Correlation of IGF1 expression with immune infiltration level in in Bladder Urothelial Cancer (BLCA), breast invasive carcinoma (BRCA), Colon Adenocarcinoma (COAD), Liver Hepatocellular carcinoma (LIHC), Pancreas adenocarcinoma (PAAD) and Rectum Adenocarcinoma (READ). The scatterplots are generated and displayed after inputs are submitted successfully, showing the purity-corrected partial Spearman's correlation and statistical significance. Data are extracted from TIMER web server. $P < 0{,}05$ was considered statistically significant.



Supplementary Figure 8 - Correlation of ARNTL2 expression with immune infiltration level *in* in Bladder Urothelial Cancer (BLCA), breast invasive carcinoma (BRCA), Colon Adenocarcinoma (COAD), Liver Hepatocellular carcinoma (LIHC), Pancreas adenocarcinoma (PAAD) and Rectum Adenocarcinoma (READ). The scatterplots are generated and displayed after inputs are submitted successfully, showing the purity-corrected partial Spearman's correlation and statistical significance. Data are extracted from TIMER web server. $P < 0{,}05$ was considered statistically significant.



Supplementary Table 1 - Functional annotation analysis of downregulated differentially expressed genes in hypothalamus (GSE6514) intersected datasets using the DAVID tool. Mice were euthanized following 3 hrs of total sleep deprivation. $P < 0,05$ was considered statistically significant.

Term	PValue	Genes
mmu00830:Retinol metabolism	0.026941435737683 82	RDH12, ALDH1A2, CYP26B1, RDH16
mmu04610: Complement and coagulation cascades	0.034646624863555325	F13B, C5AR1, FGA, BDKRB2
mmu04310:Wnt signaling pathway	0.053667515105268 47	CSNK1A1, NKD2, PRICKLE1, PPP2CB, FZD3

Supplementary Table 2 - Functional annotation analysis of upregulated differentially expressed genes in hypothalamus (GSE6514) intersected datasets using the DAVID tool. Mice were euthanized following 3 hrs of total sleep deprivation. $P < 0,05$ was considered statistically significant.

Term	PValue	Genes
mmu04070:Phosphatidylinositol signaling system	0.011670372824607887	PRKCA, DGKG, PLCD3, PLCD4, ITPR3
mmu04512:ECM-receptor interaction	0.07485797333737988	ITGA9, TNC, ITGB1, COL4A6
mmu04020:Calcium signaling pathway	0.07500498114905663	PRKCA, EDNRA, ATP2A2, PLCD3, PLCD4, ITPR3
mmu05222: Small cell lung cancer	0.07916201586803795	TRAF1, ITGB1, TRAF4, COL4A6
mmu04510:Focal adhesion	0.0845437456343312	PRKCA, ITGA9, TNC, FLNC, ITGB1, COL4A6
mmu05200:Pathways in câncer	0.08626742006122387	TRAF1, PRKCA, FGF14, NKX3-1, ITGB1, TRAF4, STAT3, COL4A6

Supplementary Table 3 - Functional annotation analysis of downregulated differentially expressed genes in hypothalamus (GSE6514) intersected datasets using the DAVID tool. Mice were euthanized following 6 hrs of total sleep deprivation. $P < 0,05$ was considered statistically significant.

Term	PValue	Genes
mmu04722:Neurotrophin signaling pathway	1,70E-06	IRS4, TRP53, PIK3CD, MAPK10, KIDINS220, IRAK4, NTRK3, IRAK3, CRKL, RPS6KA4, CAMK4, RPS6KA2, SOS1, MAPK14, NTRK2, CAMK2D, MAPK8, IKBKB, CAMK2A, MAP2K7, PIK3R1, AKT3
mmu05200:Pathways in cancer	1,14E-05	BID, FGFR1, XIAP, APC2, STK36, STAT5B, BCL2L1, KIT, CTNNB1, TPM3, WNT1, SOS1, ITGAV, TPR, PIK3R1, AKT3, APC, DVL2, TRP53, BCR, PIK3CD, FZD1, SKP2, ITGA3, MAPK10, CTNNA3, FZD6, WNT7B, FZD10, CRKL, PIAS3, PDGFRA, PDGFRB, MDM2, MAPK8, IKBKB
mmu04310:Wnt signaling pathway	1,56E-05	TRP53, DVL2, TBL1XR1, APC2, BTRC, FZD1, MAPK10, FZD6, CTNNB1, CSNK2A2, MAP3K7, WNT1, FZD10, WNT7B, PLCB4, CCND2, NFAT5, CAMK2D, MAPK8, PRKACB, CAMK2A, APC
mmu04910:Insulin signaling pathway	1,64E-05	IRS4, PHKA1, PIK3CD, PRKAB2, MKNK2, HK1, MAPK10, PRKAR2B, PRKAR2A, PPP1R3C, CRKL, INPP5K, SLC2A4, GCK, SOS1, PRKAR1B, MAPK8, PRKACB, IKBKB, AKT3, PIK3R1
mmu05210:Colorectal cancer	2,10E-05	TRP53, DVL2, APC2, PIK3CD, FZD1, MAPK10, FZD6, CTNNB1, FZD10, SOS1, PDGFRA, PDGFRB, MAPK8, AKT3, PIK3R1, APC
mmu04930:Type II diabetes mellitus	2,37E-05	IRS4, GCK, SLC2A4, PIK3CD, HK1, MAPK8, MAPK10, PRKCE, IKBKB, KCNJ11, PIK3R1, CACNA1B
mmu04210:Apoptosis	2,42E-05	BID, TRP53, XIAP, PIK3CD, BCL2L1, IRAK4, PRKAR2B, IRAK3, PRKAR2A, PRKAR1B, APAF1, PRKACB, IKBKB, AKT3, IL3RA, PIK3R1
mmu04010:MAPK signaling pathway	1,41E-04	FGFR1, MKNK2, PPM1B, MAP3K7, SOS1, PRKACB, MAP2K7, AKT3, TRP53, CACNA2D1, CACNG3, MAPK10, FLNB, STK3, DDIT3, RPS6KA4, CRKL, RASGRF2, RPS6KA2, MAPK14, NTRK2, PDGFRA, MAPK8IP3, PDGFRB, MAPK8, IKBKB, DUSP8, PPP5C, CACNA1B
mmu05217:Basal cell carcinoma	3,59E-04	DVL2, TRP53, WNT1, FZD10, WNT7B, APC2, STK36, FZD1, FZD6, CTNNB1, APC
mmu04916:Melanogenesis	4,48E-04	DVL2, ADCY3, ADCY7, FZD1, KIT, FZD6, CTNNB1, WNT1, FZD10, WNT7B, PLCB4, CAMK2D, GNAS, PRKACB, CAMK2A
mmu04062:Chemokine	0,001953580933429	ADCY3, PARD3, ADCY7, PIK3CD, STAT5B, CX3CL1, ELMO1, CCR9, CCL25, CRKL, PLCB4,

signaling pathway		TIAM1, SOS1, CX3CR1, CCR2, GNG2, PRKACB, IKBKB, AKT3, PIK3R1
mmu04912:GnRH signaling pathway	0,003324676967888	ADCY3, PLD1, ADCY7, MAPK10, PLCB4, MAPK14, SOS1, CAMK2D, MAPK8, GNAS, PRKACB, CAMK2A, MAP2K7
mmu04120:Ubiquitin mediated proteolysis	0,003386646782457	XIAP, UBE4A, BTRC, SKP2, KEAP1, UBE2I, UBE2H, UBE2B, CDC27, MGRN1, PIAS3, UBE2K, NEDD4, MDM2, SMURF1, TRIP12
mmu05222: Small cell lung cancer	0,003421473530349	TRP53, XIAP, PIAS3, ITGAV, PIK3CD, SKP2, ITGA3, BCL2L1, APAF1, IKBKB, PIK3R1, AKT3
mmu04070:Phosphatidylinositol signaling system	0,004162537604266	PLCB4, DGKB, INPP5K, PIK3C2A, DGKG, PIK3CD, PIKFYVE, PIP5K1C, SYNJ2, PIK3R1, PIP4K2C
mmu05213:Endometrial cancer	0,004166742752708	TRP53, APC2, SOS1, PIK3CD, PIK3R1, CTNNA3, AKT3, CTNNB1, APC
mmu05214:Glioma	0,004506661689157	TRP53, SOS1, PIK3CD, CAMK2D, PDGFRA, PDGFRB, MDM2, CAMK2A, PIK3R1, AKT3
mmu05220:Chronic myeloid leukemia	0,004584859553404	TRP53, BCR, CRKL, SOS1, STAT5B, PIK3CD, MDM2, BCL2L1, IKBKB, PIK3R1, AKT3
mmu04520:Adherens junction	0,004584859553404	PTPRB, CSNK2A2, MAP3K7, FGFR1, PARD3, WASF3, SSX2IP, YES1, CTNNA3, CTNNB1, FARP2
mmu04510:Focal adhesion	0,00504125667864	XIAP, TLN2, PIK3CD, PIP5K1C, ITGA3, MAPK10, FLNB, CTNNB1, CRKL, CCND2, ITGA5, SOS1, ITGAV, PDGFRA, PDGFRB, MAPK8, AKT3, PARVB, PIK3R1, PARVA
mmu04914: Progesterone-mediated oocyte maturation	0,010085211969577	ADCY3, ADCY7, RPS6KA2, MAPK14, PIK3CD, MAPK8, MAPK10, PRKACB, CDC27, PIK3R1, AKT3
mmu04620: Toll-like receptor signaling pathway	0,010820214306126	IRAK4, MAP3K7, IFNAR2, TOLLIP, MAPK14, PIK3CD, MAPK8, MAPK10, IKBKB, MAP2K7, PIK3R1, AKT3
mmu04540:Gap junction	0,010918590302327	ADCY3, PLCB4, ADCY7, CSNK1D, SOS1, PDGFRA, PDGFRB, GNAS, GUCY1B3, PRKACB, HTR2C
mmu04012:ErbB signaling pathway	0,011803265357519	CRKL, SOS1, STAT5B, PIK3CD, CAMK2D, MAPK8, MAPK10, MAP2K7, CAMK2A, PIK3R1, AKT3
mmu04810: Regulation of	0,013078796430939	GNA13, FGFR1, APC2, SSH1, PIK3CD, NCKAP1L, PIP5K1C, ITGA3, ITGAM, CRKL, ITGA5, TIAM1,

actin cytoskeleton		SOS1, ITGAV, PIKFYVE, PDGFRA, PDGFRB, PIP4K2C, PIK3R1, APC
mmu05215:Prostate cancer	0,014783942905923	TRP53, FGFR1, SOS1, PIK3CD, PDGFRA, PDGFRB, MDM2, IKBKB, PIK3R1, AKT3, CTNNB1
mmu04020:Calcium signaling pathway	0,015843975717175	SLC8A3, ADCY3, TRPC1, ADCY7, PHKA1, PLCB4, CAMK4, ATP2A2, ATP2A3, PDE1C, CAMK2D, PDGFRA, PDGFRB, GNAS, PRKACB, HTR2C, CAMK2A, CACNA1B
mmu05414:Dilated cardiomyopathy	0,017062036909474	ADCY3, CACNA2D1, ATP2A2, ADCY7, ITGA5, ITGAV, GNAS, ITGA3, CACNG3, PRKACB, TPM3
mmu00562:Inositol phosphate metabolism	0,018032555627985	PLCB4, INPP5K, PIK3C2A, PIK3CD, PIKFYVE, PIP5K1C, SYNJ2, PIP4K2C
mmu02010:ABC transporters	0,024479438608894	ABCB8, ABCB1B, ABCC10, ABCC12, ABCA1, ABCB7, ABCC6
mmu04144:Endocytosis	0,025955943205138	PLD1, PARD3, DNM1L, LDLR, STAM2, PIP5K1C, KIT, RAB11FIP4, RAB11FIP3, TFRC, ACAP3, NEDD4, PIKFYVE, PDGFRA, DNAJC6, MDM2, SMURF1, RAB11FIP1
mmu05212:Pancreatic cancer	0,027838021509868	TRP53, PLD1, PIK3CD, MAPK8, BCL2L1, MAPK10, IKBKB, PIK3R1, AKT3
mmu04660: T cell receptor signaling pathway	0,035403432135523	MAP3K7, BCL10, CD40LG, SOS1, MAPK14, PIK3CD, NFAT5, IKBKB, MAP2K7, PIK3R1, AKT3, DLG1
mmu04130: SNARE interactions in vesicular transport	0,040755055178891	STX17, GOSR2, VAMP3, VAMP2, GOSR1, STX1B
mmu04630: Jak-STAT signaling pathway	0,042967083724939	STAM2, PIK3CD, STAT5B, BCL2L1, IL7R, IFNAR2, PRLR, PIAS3, CCND2, SOS1, IL13RA1, PIK3R1, AKT3, IL3RA
mmu04664: Fc epsilon RI signaling pathway	0,054010549517779	SOS1, MAPK14, PIK3CD, MAPK8, MAPK10, PRKCE, MAP2K7, PIK3R1, AKT3
mmu04666: Fc gamma R-mediated phagocytosis	0,058455974371693	PLD1, CRKL, DNM1L, WASF3, PIK3CD, PIKFYVE, PIP5K1C, PRKCE, PIK3R1, AKT3
mmu05218:Melanoma	0,065976656867559	TRP53, FGFR1, PIK3CD, PDGFRA, PDGFRB, MDM2, PIK3R1, AKT3

mmu05221:Acute myeloid leukemia	0,066302389356434	SOS1, STAT5B, PIK3CD, KIT, IKBKB, PIK3R1, AKT3
mmu05412: Arrhythmogenic right ventricular cardiomyopathy (ARVC)	0,083302611522674	CACNA2D1, ATP2A2, ITGA5, ITGAV, ITGA3, CACNG3, CTNNA3, CTNNB1

Supplementary Table 4 - Functional annotation analysis of upregulated differentially expressed genes in hypothalamus (GSE6514) intersected datasets using the DAVID tool. Mice were euthanized following 6 hrs of total sleep deprivation. $P < 0,05$ was considered statistically significant.

Term	PValue	Genes
mmu00190:Oxidative phosphorylation	4,69E-07	NDUFA4, NDUFA5, ATP5E, ATP5J2, NDUFA3, COX7A2, COX8B, ATP4B, COX6C, UQCR11, UQCRH, COX6A2, ATP5L, ATP5O, ATP5K, UQCRB
mmu05012:Parkinson's disease	8,51E-05	NDUFA4, NDUFA5, ATP5E, COX7A2, NDUFA3, COX8B, COX6C, UQCR11, CASP9, UQCRH, COX6A2, ATP5O, UQCRB
mmu05016:Huntington's disease	1,32E-04	NDUFA4, NDUFA5, ATP5E, NDUFA3, POLR2E, COX7A2, COX8B, COX6C, UQCR11, CASP9, UQCRH, COX6A2, TGM2, ATP5O, UQCRB
mmu05010:Alzheimer's disease	4,53E-04	NDUFA4, NDUFA5, ATP5E, COX7A2, NDUFA3, COX8B, COX6C, UQCR11, CASP9, UQCRH, BACE2, COX6A2, ATP5O, UQCRB
mmu04260:Cardiac muscle contraction	0,002760529458794	FXYD2, UQCR11, COX7A2, COX8B, UQCRH, COX6A2, COX6C, UQCRB
mmu03010:Ribosome	0,065553365078058	RPL41, RPL35, RPL27, RPS27L, RPL37, RPS24
mmu00410:beta-Alanine metabolismo	0,099424521029235	ALDH2, DPYS, AOC3

Supplementary Table 5 - Functional annotation analysis of downregulated differentially expressed genes in hypothalamus (GSE6514) intersected datasets using the DAVID tool. Mice were euthanized following 9 hrs of total sleep deprivation. $P < 0,05$ was considered statistically significant.

Term	PValue	Genes
mmu04020:Calcium signaling pathway	0,045568518049453	EGFR, PLCZ1, P2RX1, CACNA1H, PLCD1, IGH-VJ558
mmu04010:MAPK signaling pathway	0,053398346139521	EGFR, MAP3K7, RPS6KA3, RELB, CACNA1H, MAP3K14, FGF3

Supplementary Table 6 - Functional annotation analysis of upregulated differentially expressed genes in hypothalamus (GSE6514) intersected datasets using the DAVID tool. Mice were euthanized following 9 hrs of total sleep deprivation. $P < 0,05$ was considered statistically significant.

Term	PValue	Genes
mmu05200:Pathways in cancer	0,013810995520305	TCF7, CYCT, SKP2, FZD1, FZD3, ZBTB16, CBLC, LAMA3, ITGAV, PAX8, PTCH1, IKBKB, FGF2, MMP1B
mmu04950: Maturity onset diabetes of the young	0,015265675716387	HNF1B, IAPP, HNF4G, PDX1
mmu04672: Intestinal immune network for IgA production	0,023738635669089	CCR9, IGHG, CD80, H2-DMB2, IL2
mmu04650: Natural killer cell mediated cytotoxicity	0,037758267809113	KLRA16, CD48, IGHG, CD244, TNFRSF10B, KLRA7, VAV2
mmu05016:Huntington's disease	0,079202055043023	SLC25A31, NDUFS4, CYCT, CREB1, CYP4A31, NDUFC2, PLCB2, POLR2A
mmu04330:Notch signaling pathway	0,081200303679975	NOTCH2, NOTCH4, NUMB, LFNG
mmu04514: Cell adhesion molecules (CAMs)	0,093775924821431	SELP, SIGLEC1, CD80, ITGAV, NLGN1, CD2, H2-DMB2
mmu04020:Calcium signaling pathway	0,094298620512485	IGHG, SLC25A31, ERBB4, PDE1C, PLCD4, PLCB2, PTAFR, CACNA1A
mmu05222: Small cell lung cancer	0,094733504181432	LAMA3, CYCT, ITGAV, SKP2, IKBKB

Supplementary Table 7 - Functional annotation analysis of downregulated differentially expressed genes in hypothalamus (GSE6514) intersected datasets using the DAVID tool. Mice were euthanized following 12 hrs of total sleep deprivation. $P < 0,05$ was considered statistically significant.

Term	PValue	Genes
mmu05210:Colorectal cancer	0,024514541111056	DVL2, DVL3, MSH2, PDGFRB
mmu04916:Melanogenesis	0,036110899448509	DVL2, WNT1, DVL3, POMC
mmu05217:Basal cell carcinoma	0,060864532183204	DVL2, WNT1, DVL3
mmu04310:Wnt signaling pathway	0,094599923420952	DKK2, DVL2, WNT1, DVL3

Supplementary Table 8 - Functional annotation analysis of upregulated differentially expressed genes in hypothalamus (GSE6514) intersected datasets using the DAVID tool. Mice were euthanized following 12 hrs of total sleep deprivation. $P < 0,05$ was considered statistically significant.

Term	PValue	Genes
mmu03320: PPAR signaling pathway	0,032487926831281	LPL, FABP2, CYP4A14, MMP1B, PCK1
mmu04060: Cytokine-cytokine receptor interaction	0,032708956267818	CSF2, INHBA, TNFRSF9, IL22RA1, IL5, PRLR, MET, TNFRSF14, HGF
mmu04950: Maturity onset diabetes of the young	0,059584308447436	HNF4A, HNF4G, NR5A2

Supplementary Table 9 - Functional annotation analysis of downregulated differentially expressed genes in cortex (GSE6514) intersected datasets using the DAVID tool. Mice were euthanized following 3 hrs of total sleep deprivation. $P < 0,05$ was considered statistically significant.

Term	PValue	Genes
mmu04514: Cell adhesion molecules (CAMs)	0,033264658879409	H2-K1, CLDN3, H2-D1, CD4, VCAN, ITGB1, SELE
mmu00480:Glutathione metabolismo	0,047518951045075	GSTA2, GSTA3, GPX3, MGST1
mmu00562:Inositol phosphate metabolismo	0,052167239572602	PIK3CG, MIOX, PLCD1, PI4KB
mmu04020:Calcium signaling pathway	0,078482240908623	GNA14, P2RX1, ADORA2A, ADRA1B, AVPR1A, PLCD1, GNAS

Supplementary Table 10 - Functional annotation analysis of upregulated differentially expressed genes in cortex (GSE6514) intersected datasets using the DAVID tool. Mice were euthanized following 3 hrs of total sleep deprivation. $P < 0,05$ was considered statistically significant.

Term	PValue	Genes
mmu04010:MAPK signaling pathway	0,069297968818719	MAP4K3, DUSP4, RASGRF2, FGF17, HSPB1, HSPA1B, FLNC

Supplementary Table 11 - Functional annotation analysis of downregulated differentially expressed genes in cortex (GSE6514) intersected datasets using the DAVID tool. Mice were euthanized following 6 hrs of total sleep deprivation. $P < 0,05$ was considered statistically significant.

Term	PValue	Genes
mmu04514: Cell adhesion molecules (CAMs)	0,008627391323092	SDC1, H2-Q10, CD80, H2-D1, CD2, VCAN, MADCAM1, ITGA4, ITGB1
mmu00983:Drug metabolismo	0,012622692734448	CYP3A25, UGT2B5, UPP2, DPYD, UGT2A3
mmu05416:Viral myocarditis	0,032606173670026	BID, H2-Q10, CD80, CYCT, H2-D1, MYH4
mmu00980: Metabolism of xenobiotics by cytochrome P450	0,036119769105627	CYP3A25, UGT2B5, UGT2A3, CYP2C50, MGST2
mmu00140:Steroid hormone biosynthesis	0,053208964993145	CYP3A25, UGT2B5, UGT2A3, CYP19A1
mmu00982:Drug metabolismo	0,053600179465743	CYP3A25, UGT2B5, UGT2A3, CYP2C50, MGST2
mmu04610: Complement and coagulation cascades	0,053600179465743	F11, FGG, FGA, SERPINA1B, C3
mmu00563: Glycosylphosphatidylinositol (GPI)-anchor biosynthesis	0,080829554493124	PIGB, PIGU, PIGN
mmu04672: Intestinal immune network for IgA production	0,082448496750621	CCR9, CD80, MADCAM1, ITGA4
mmu05332: Graft-versus-host disease	0,097250501623575	H2-Q10, CD80, H2-D1, KLRA7

Supplementary Table 12 - Functional annotation analysis of upregulated differentially expressed genes in cortex (GSE6514) intersected datasets using the DAVID tool. Mice were euthanized following 6 hrs of total sleep deprivation. $P < 0,05$ was considered statistically significant.

Term	PValue	Genes
mmu00052:Galactose metabolism	0,034919323507812	G6PC, GLA, HK3

Supplementary Table 13 - Functional annotation analysis of downregulated differentially expressed genes in cortex (GSE6514) intersected datasets using the DAVID tool. Mice were euthanized following 9 hrs of total sleep deprivation. $P < 0,05$ was considered statistically significant.

Term	PValue	Genes
mmu05330:Allograft rejection	0,015132315898876	IGHG, CD80, H2-OB, IGH-VJ558, H2-T3
mmu04650:Natural killer cell mediated cytotoxicity	0,015243320762071	IGHG, H60A, PTPN6, CSF2, CD247, NFAT5, IGH-VJ558
mmu05310:Asthma	0,016751560625554	IGHG, IL3, H2-OB, IGH-VJ558
mmu04080:Neuroactive ligand-receptor interaction	0,027424325573514	P2RX5, ADRB3, P2RY10, P2RY6, ADORA2B, AGTR1B, RXFP1, FPR3, NMBR, GH
mmu05320:Autoimmune thyroid disease	0,030833346182776	IGHG, CD80, H2-OB, IGH-VJ558, H2-T3
mmu04640:Hematopoietic cell lineage	0,049867716005983	IGHG, CSF2, IL3, DNTT, IGH-VJ558
mmu04672:Intestinal immune network for IgA production	0,059503291324067	IGHG, CD80, H2-OB, IGH-VJ558
mmu05416:Viral myocarditis	0,069717111810456	IGHG, CD80, H2-OB, IGH-VJ558, H2-T3
mmu04020:Calcium signaling pathway	0,095935740111107	P2RX5, IGHG, ADRB3, ADORA2B, AGTR1B, CACNA1A, IGH-VJ558

Supplementary Table 14 - Functional annotation analysis of upregulated differentially expressed genes in cortex (GSE6514) intersected datasets using the DAVID tool. Mice were euthanized following 9 hrs of total sleep deprivation. $P < 0,05$ was considered statistically significant.

Term	PValue	Genes
mmu04060:Cytokine-cytokine receptor interaction	0,094813764513206	CCR9, INHBA, TNFRSF25, CXCL15, TNFRSF14

Supplementary Table 15 - Functional annotation analysis of downregulated differentially expressed genes in cortex (GSE6514) intersected datasets using the DAVID tool. Mice were euthanized following 12 hrs of total sleep deprivation. $P < 0,05$ was considered statistically significant.

Term	PValue	Genes
mmu04080:Neuroactive ligand-receptor interaction	0,016453625084603	GALR1, PRLR, TACR3, PRSS2, DRD4, VIPR1, PTAFR, GH

Supplementary Table 16- Functional annotation analysis of upregulated differentially expressed genes in cortex (GSE6514) intersected datasets using the DAVID tool. Mice were euthanized following 12 hrs of total sleep deprivation. $P < 0,05$ was considered statistically significant.

Term	PValue	Genes
mmu05416:Viral myocarditis	0,009693942463592	CYCT, MYH2, H2-D1, H2-AA, H2-DMA, IGH-VJ558
mmu05310:Asthma	0,010540002941723	H2-AA, MS4A2, H2-DMA, IGH-VJ558
mmu05330:Allograft rejection	0,046571027714215	H2-D1, H2-AA, H2-DMA, IGH-VJ558
mmu05320:Autoimmune thyroid disease	0,078534193961941	H2-D1, H2-AA, H2-DMA, IGH-VJ558

5.2 PRODUTO 2

Patente: Privilégio de Inovação. Número do registro: BR10202002668, título: "Painel genético para diagnóstico e prognóstico do câncer de mama", Instituição de registro: INPI - Instituto Nacional da Propriedade Industrial. Depósito: 24/12/2020

5 CONCLUSÕES

Compilando todos os dados, a hipótese aventada foi de que mudanças na expressão de genes relacionados ao ritmo circadiano no diabetes mellitus tipo 2 e no câncer podem ser responsáveis por mudanças metabólicas que podem levar ao desenvolvimento do câncer. Essas mudanças no metabolismo do tecido resultam, pelo menos em parte, do recrutamento profundo de tipos de células inflamatórias, particularmente células mielóides, como neutrófilos e monócitos. A expressão de IGF-1 e USP2 pode estar associada ao sistema imunológico nas amostras de diabetes e câncer, esses distúrbios podem levar a alterações na imunidade ao aumentar a infiltração de leucócitos e aumentar a expressão de citocinas pró-inflamatórias. Essas diversas células comunicam-se entre si por meio de contato direto ou pela produção de citocinas e quimiocinas e agem de maneira autócrina e parácrina para controlar e moldar o crescimento tumoral e metástase. Curiosamente, observamos menor expressão da proteína ARNTL2 em amostras de câncer. Esta proteína desempenha um papel importante no sistema de ritmo circadiano e está associada ao mecanismo de escape imunológico, níveis de expressão alterados desses genes podem estar associados à progressão do câncer e ao fenótipo metastático agressivo.

Essas moléculas biológicas não apenas representam a associação de diabetes mellitus tipo 2 e biomarcadores de ritmo circadiano com câncer de mama, bexiga, fígado, pâncreas, cólon e reto, mas também têm potencial significativo para serem consideradas como biomarcadores em nível de sistema que podem ser usados para triagem ou finalidades terapêuticas, podendo levar a uma medicina de precisão, onde o tratamento é individualizado, garantindo melhores resultados para o paciente, assim como priorizará a fármaco-economia, selecionando quais pacientes serão realmente beneficiados com as drogas, economizando recursos públicos e particulares e evitando que pessoas recebam medicamentos que pouco as beneficiariam e ainda iriam infligir toxicidade decorrente dos mesmos. Nossos dados estimulam esforços em novos estudos para alcançar a validação experimental e clínica sobre essas biomoléculas.

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ANEXO

ANEXO A – Regras para publicação no periódico

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