UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE BIOLOGIA VEGETAL

Epitranscriptomic deregulation in bladder cancer: Implications for tumour aggressiveness

Catarina Sofia Guimarães Teixeira

Mestrado em Biologia Molecular e Genética

Dissertação orientada por:
Professora Doutora Carmen de Lurdes Fonseca Jerónimo
Professora Doutora Margarida Gama Carvalho

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"Imagination is more important than knowledge. Knowledge is limited.

Imagination encircles the world."

Albert Einstein

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RESUMO

O cancro de bexiga é o nono cancro mais incidente no mundo e a décima terceira principal causa de morte por cancro, de acordo com o estudo GLOBOCAN 2018. A maioria dos casos de cancro de bexiga ($> 90\%$) surge no revestimento interno do trato urinário (urotélio) e são designados por carcinoma de células uroteliais. Os subtipos menos comuns são o carcinoma espinocelular, adenocarcinoma, carcinoma de pequenas células e sarcoma. Adicionalmente, o cancro de bexiga pode ser classificado em não músculo-invasivo e em músculo-invasivo. Este primeiro, é responsável por aproximadamente 75% de todos os cancros de bexiga diagnosticados. Apesar destes tumores, geralmente, não representarem uma ameaça à vida dos doentes, a taxa de recorrência é elevada. Por outro lado, tumores músculo-invasivos, que representam cerca de 25% dos casos, são tumores clinicamente mais agressivos, que podem progredir rapidamente, apresentando capacidade de invadir e metastizar para outros órgãos. Assim, há uma necessidade urgente de compreender os mecanismos de progressão do cancro de bexiga, para assim desenvolver novas estratégias de diagnóstico, bem como abordagens terapêuticas efetivas.

Nos últimos anos, o número de estudos na área da genética, epigenética e epitranscriptómica aumentaram drasticamente, principalmente devido ao rápido aprimoramento das tecnologias de sequenciação de alto rendimento de nova geração.

CH Waddington, definiu originalmente o termo Epigenética como o estudo de "mecanismos causais pelos quais os genes do genótipo produzem efeitos fenotípicos". Ao longo do tempo, esta definição sofreu algumas alterações, sendo, hoje, definida como "o estudo de alterações hereditárias na expressão génica que ocorrem independentemente das alterações na sequência primária de ADN". Recentemente foram identificados novos mecanismos de regulação da expressão génica, ao nível do ácido ribonucleico (ARN), designado por epitranscriptómica. Esta refere-se ao estudo de modificações químicas, reversíveis, que podem ocorrer quer em moléculas de ARN, quer em ARN não codificante (ncARN) e em ARN mensageiro (mARN).

A N6-metiladenosina (m6A), metilação da adenosina na posição nitrogénio-6, é a modificação química interna mais abundante nos mARNs dos seres eucarióticos. Esta modificação ocorre, preferencialmente, na sequência DRACH (onde D indica A/G/U; R indica A/G e H indica A/C/U), sendo especificamente enriquecida próximo do codão *stop*, nas regiões 3' não-traduzidas e em grandes exões internos. O seu potencial na regulação da expressão génica foi recentemente explorado e sabe-se que, esta modificação pode afetar diferentes vias do mARN, como a transcrição, *splicing*, exportação do núcleo e tradução. Nas células de mamíferos, esta modificação é catalisada pelo complexo m6A metiltransferase ("*writters*") e pode ser removida pelas desmetilases ("*erasers*"). Além disso, existem proteínas que se ligam diretamente à modificação m6A, mediando a sua função. Estas são conhecidas como "*readers*". Vários estudos revelaram que a metilação do m6A, bem como das suas proteínas reguladoras, desempenha um papel crucial no processo de tumorigénese em diferentes modelos. No entanto, o envolvimento desta modificação no cancro de bexiga é ainda limitado, sendo, portanto, necessário investigar as suas funções no contexto desta neoplasia.

O objetivo do nosso estudo é descobrir o papel da m6A no cancro de bexiga, a fim de perceber quais os mecanismos moleculares subjacentes à agressividade tumoral.

A seleção das proteínas reguladoras da m6A mais informativas para o nosso projeto foi realizada pela análise *in silico* dos dados de ARN-seq de doentes com cancro de bexiga músculo-invasivo, disponíveis na base de dados do TCGA. Esta análise revelou que as principais proteínas reguladoras da m6A desreguladas eram o METTL3, METTL14, VIRMA, WTAP, que formam um "*writter complex*", sendo o METTL3 a única proteína com atividade catalítica, o ALKBH5 e FTO, que têm a capacidade de desmetilar o m6A, e o YTHDF3 que tem como função "ler" a marca no citoplasma, determinando se o mARN é traduzido ou degradado.

Assim, foi selecionada (n=120) uma série de tecidos de cancro de bexiga primário, sem qualquer tratamento, dos quais 50% são músculo-invasivo e 50% não músculo-invasivo), bem como uma série de tecidos normais. Em seguida, foi realizada quantificação proteica, por imunohistoquímica. Tal serviu para comparar a expressão em tecidos tumorais e tecidos normais, sendo que, dentro dos tecidos tumorais, a expressão diferencial nos músculo-invasivo e não músculo-invasivo também foi avaliada.

A METTL3, METTL14, VIRMA, ALKBH5 e YTHDF3 apresentam uma expressão significativamente menor nos tumores por comparação com tecidos normais. Além disso, a METTL3 e a METTL14, que formam um heterodímero estável, apresentam uma redução de expressão significativa nos tumores músculo-invasivo, quando comparado com os não músculo-invasivos.

Analisámos a correlação da expressão destas proteínas reguladores e da m6A entre si. Para além disso, observámos que a expressão do *reader* (YTHDF3) e de todos os *writters* estudados se correlacionou positivamente com a expressão da m6A. O mesmo foi observado em relação à expressão das *erasers*. Curiosamente, também foram observadas correlações entre a expressão dos *writters* e *erasers*, com exceção da WTAP com ambas as *erasers*, bem como do VIRMA e do FTO. Assim, estes resultados sugerem que a correlação observada entre os *writters* e as *erasers* pode ser explicada através de um feedback compensatório, ou seja, a diminuição dos escritores vai diminuir consequentemente a m6A, levando a uma diminuição das *erasers* que desmetilam esta modicação.

A metilação desta modificação no ARN e a expressão das suas proteínas reguladoras, foi avaliada em linhas celulares de bexiga através do ensaio colorimétrico e Western blot, respetivamente. Uma linha celular normal, SVHUC-1, e sete linhas celulares tumorais foram usadas. Relativamente à modificação da m6A, não foram observadas diferenças significativas nas diferentes linhas celulares. Em relação às proteínas reguladoras, também não se observaram diferenças significativas para a expressão destas entre as linhas celulares testadas, com exceção do METTL14 que apresentou níveis bastante variáveis, sendo a linha UMUC3, aquela com valores mais elevados desta proteína.

Nesta sequência, com o objetivo de induzir *in vitro* a ablação da expressão do METTL14, foi realizado o *knockdown* deste gene na linha celular UMUC3, recorrendo ao sistema CRISPR-Cas9. Para avaliar a eficiência desta técnica, realizámos Western blot, onde foi comparada a expressão do METTL14 na linha celular com *knockdown* e na linha UMUC3 normal.

Após confirmar a eficiência da redução da expressão em pelo menos 50% do METTL14, os níveis da modificação da m6A foram avaliados, a fim de perceber a importância desta proteína no complexo. Posteriormente, foram realizados ensaios fenotípicos para determinar o impacto da diminuição da sua expressão na linha celular de cancro de bexiga.

Os ensaios *in vitro* demonstraram que a redução da expressão do METTL14 promoveu uma redução de 50% na modificação da m6A. Destes, os ensaios de viabilidade e proliferação celular, demonstram que a redução de METTL14 aumenta significativamente a viabilidade e proliferação das células de cancro de bexiga. Além disso, verificámos igualmente, que havia um aumento na capacidade de invasão e migração, comparativamente com as linhas controlo. Em sentido inverso, o *knockdown* do METTL14 traduziu-se numa redução na taxa de apoptose das células tranfectadas.

Em resumo, a expressão das proteínas modeladoras da marca m6A encontram-se desregulada no cancro de bexiga. Particularmente, a regulação negativa do complexo METTL3/METTL14 metiltransferase associou-se à progressão do cancro de bexiga não musculo-invasivo para o cancro de bexiga músculo-invasivo.

Estudos anteriores indicam que, embora a METTL14 não possua uma função catalítica, é capaz de formar um heterodímero com a METTL3, sendo necessário para a estabilização e função do complexo. Interações específicas entre o domínio METTL14- MTD14 e o domínio do METTL3- MTD3 são necessárias para a atividade catalítica da METTL3. Na mesma linha, descobrimos que a redução da expressão da METTL14 diminui a atividade do complexo *writter* e, portanto, a capacidade de estabelecer m6A nas moléculas de ARN. Estes resultados sugerem que a METTL14 poderá desempenhar

um papel supressor tumoral, estando a redução da sua expressão associada a características celulares tumorais de maior agressividade.

Palavras-chave: Cancro de bexiga, Epitranscriptomica, N6-metiladenosina, METTL14.

ABSTRACT

N6-methyladenosine (m_6A) modification is the most abundant internal chemical modification of mRNAs in eukaryotes. Several studies revealed that m_6A RNA methylation and the associated regulatory proteins, play crucial roles in the tumorigenesis processes of numerous types of cancers. However, knowledge of the mechanistic network between m_6A and bladder cancer (BC) is limited and therefore it is necessary to investigate the functions of this modification.

The aim of our study is to uncover the role of this modification in BC in order to understand the mechanisms associated with tumour aggressiveness.

In silico analysis of TCGA data disclosed altered expression of the major m_6A regulatory proteins, prompting subsequent validation. M_6A, METTL3, METTL14, VIRMA, WTAP, ALKBH5, FTO and YTHDF3 protein expression were evaluated in a series of primary BC (n=120) and normal bladder (n=40) tissues. M_6A RNA methylation and respective regulatory proteins expression were also assessed in bladder cell lines. METTL14 knockdown was performed in UMUC3 cell line using CRISPR-Cas9 system in order to study its relevance in bladder carcinogenesis.

METTL3, METTL14, VIRMA, ALKBH5 and YTHDF3 showed significantly lower expression levels in tumour compared to normal tissues. Moreover, METTL3 and METTL14 (heterodimeric catalytic core) showed a significant reduction in muscle invasive (MIBC) comparing with non-muscle bladder cancer (NMIBC). No differences were apparent for m_6A regulatory proteins' expression among the tested cell lines, except for METTL14, that presented heterogenous levels of this writer in the different cells comparing with normal cell line. The *in vitro* METTL14 downregulation promoted a 50% reduction in m_6A modification. METTL14 knockdown showed increased cell viability/proliferation, invasion and migration capacity, whereas decreased apoptosis was found in the same cells compared with the control.

Our results suggest that METTL14 might have an important role in bladder cancer, and its expression associated with bladder cancer aggressiveness.

Keywords: Bladder cancer, Epitranscriptomic, N6-methyladenosine, METTL14.

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LIST OF ABBREVIATIONS

3ÙTR- 3`untranslated regions
AJCC- American Joint Committee on Cancer
ALKBH5- *alkB homologue 5*
ATCC- American Type Culture Collection
BC- Bladder cancer
BCG- Bacillus Calmette-Guérin
BSA- Bovine serum albumin
BUC- Bladder Urothelial carcinoma
Cis- Carcinoma in situ
DAB- 3,3' – Diaminobenzidine
DAPI- 4',6-diamidino-2-phenylindole
DMSO- Dimethyl Sulfoxide
EDTA- Ethylenediamine tetra acetic acid
EMT- Epithelial-mesenchymal transition
FBS- Fetal Bovine Serum
FFPE- Formalin-fixed and paraffin-embedded
FITC- Fluorescein isothiocyanate
FTO- *Human obesity-associated*
ICC- Immunocytochemistry
IF- Immunofluorescence
IgG- Immunoglobulin G
IHC- Immunohistochemistry
lncRNA- long non-coding RNA
M_6A- N6-Methyladenosine
METTL14- *Methyltransferase-like protein 14*
METTL3- *Methyltransferase-like protein 3*
MIBC- Muscle invasive bladder cancer
MMC- Mitomycin
MMC- Mitomycin C
mRNA- Messenger ribonucleic acids
NAC- Neoadjuvant chemotherapy
ncRNA- non-coding RNA
NMIBC- non-muscle invasive bladder cancer
NUT- Normal urothelial tract
OD- Optical density
PBS- Phosphate buffered saline
PBS- Phosphate-buffered saline
PFA- Paraformaldehyde
PIC- Protein inhibitor cocktail
PVDF- Polyvinylidene fluoride
RBM15/15B- RNA-binding motif proteins 15/15B
RIPA- Radio immune precipitation assay
RNA- Ribonucleic acid
SAM- S-adenosyl-methionine
SDS-PAGE- Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SDS- Sodium dodecyl sulphate

TBS- Tris-buffered saline

TCGA- The Cancer Genome Atlas

TRITC- Tetramethyl rhodamine

TURBT- Transurethral resection of bladder tumour

UCC- Urothelial cell carcinoma

VIRMA- *Virilizer*

WB- Western blot

WHO- World Health Organization

WTAP- *Wilm`s tumour-associated protein*

YTHDF3- *YTH m_6A-binding protein 3*

ZC3H13- Zinc finger CCCH domain-containing

β-ACT- Beta-actin

INTRODUCTION

Bladder cancer (BC) is the tenth most incident cancer worldwide and the thirteenth leading cause of death from cancer, according to GLOBOCAN 2018 study (**Figure 1**) (1). In 2018, 549,000 new cases were diagnosed, and it is expected an increase to 900,700 new cases by 2040 (2, 3). BC has higher incidence and mortality rates in males when compared to females in a 3:1 ratio, approximately. Incidence also correlates positively with age. Furthermore, smoking is the strongest risk factor associated with this disease, with an attributable risk of approximately 50%. Chronic urinary tract infections, exposure to aromatic amines and other carcinogenic substances (such as arsenic in the drinking water) are other common BC drivers (4-6).
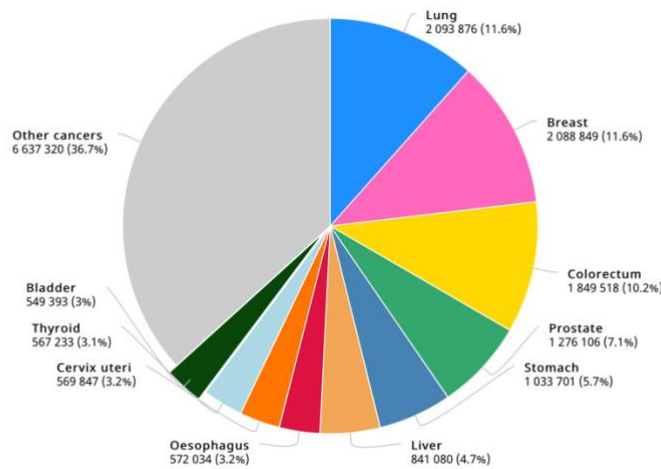
Figure 1. Worldwide Bladder Cancer incidence. Estimated number of new cases in 2018, worldwide, all cancers, both sexes, all ages. Adapted from (1).

Most cases of bladder cancer (>90%) arise from the inner lining of the urinary tract (urothelium) and are commonly designated urothelial cell carcinomas (UCCs). The less-common types are squamous cell carcinoma, adenocarcinoma, small-cell carcinoma and sarcoma (4, 7). Bladder cancer is classified in non-muscle invasive BC (NMIBC) and muscle invasive BC (MIBC). NMIBC accounts for approximately 75% of all diagnosed BC. Although these tumours usually do not represent a survival threat, they frequently recur. Moreover, 10-30% of patients with NMIBC progress to MIBC. The former tumours represent about 25% of the cases are clinically more aggressive tumours that can rapidly progress and metastasize (3, 8). NMIBC and MIBC are staged according with the degree of tumour invasion into the bladder wall. Stage 0 carcinomas, which can be papillary (Ta) or in situ (Tis), and are restricted to the urothelium, and stage 1 (T1) carcinomas, which invade the lamina propria, are grouped as NMIBC. Tumours which invade the superficial muscle (stage T2a) or deep muscle (stage T2b), the peri-vesical fat layer (stage T3) and adjacent organs (stage T4), are grouped as MIBC (**Figure 2**) (9, 10).
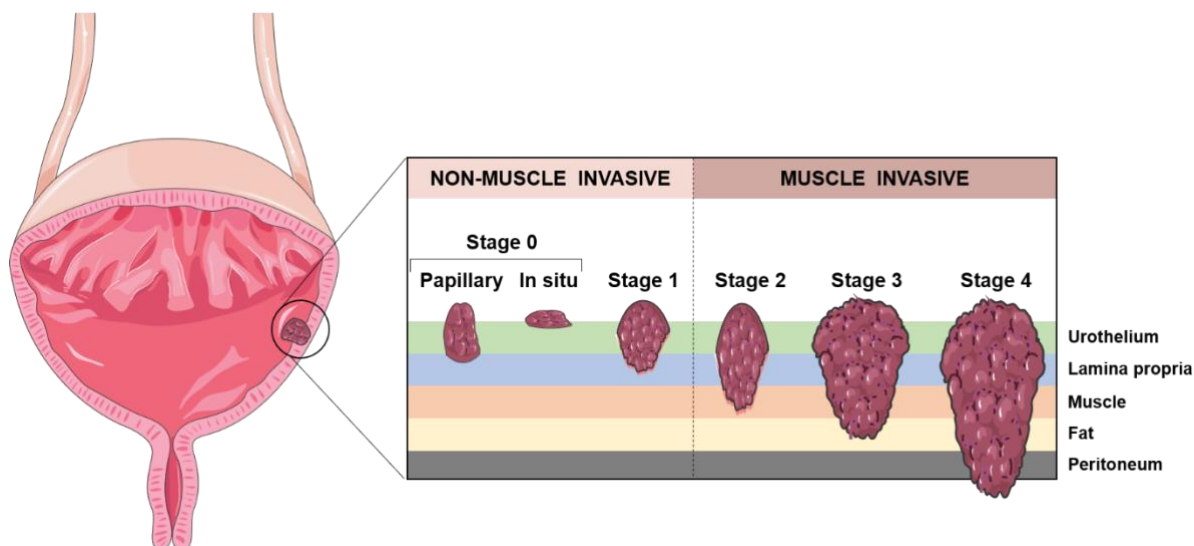
Figure 2. Staging of urothelial carcinoma. Bladder cancer stages are assigned on the basis of tumour invasion through the layers of tissue that constitute the bladder. Adapted from (11).

At the molecular level, several classifications of BC have been, so far, described, and all of them are partially coincident allowing to distinguish two major molecular subtypes: luminal and basal-like BC (10, 12) . The Lund classification was the first defined in urothelial carcinoma including NMIBC and MIBC. Five tumour classes were defined with different cell adhesion gene signatures and also different genetic mutations (11). Two other molecular classification systems have been described: The Cancer Genome Atlas (TCGA) and MD Anderson Cancer Center, defined according to messenger ribonucleic acids (mRNA) expression profile studies, included MIBC alone. The group at the University of North Carolina assembled a large dataset showing that tumours could be divided into only two groups, luminal and basal, which is currently the established classification (13).

The most comprehensive classification is provided by TCGA in which muscle-invasive bladder cancer are characterized by multiple TCGA analytical platforms from studies on mRNA, long non-coding RNA (ncRNA) and microRNA's expression, differential epithelial-mesenchymal transition (EMT) status, carcinoma in situ (Cis) scores, histologic features, and survival. The well-known luminal and basal subtypes of BC were stratified into 5 distinct subtypes: luminal-papillary subtype (35%), luminal-infiltrated subtype (19%), luminal subtype (6%), basal-squamous subtype (35%) and neuronal subtype (5%), was recently recognized by others in an independent cohort (**Figure 3**) (14).
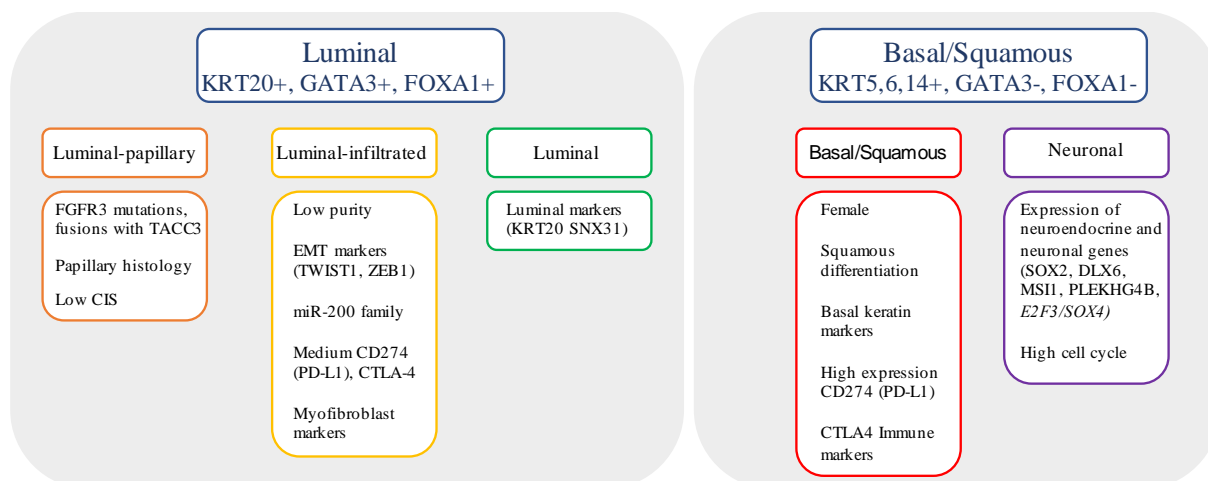
Figure 3. Molecular subtype classification based on RNA subtype classification, pathway information, EMT and CIS signatures, and immune infiltrate analyses. Adapted from (14).

Haematuria (blood cells in urine) is the most common symptom of BC, although it is also associated with various other benign and malignant causes (15). Among benign causes there are trauma, exercise, urinary tract infections, vascular malformations or prostate hyperplasia. Besides BC, malignant causes of haematuria also include kidney and prostate cancer. Because of that, early detection and diagnosis of BC still constitutes a challenge, although urine cytology is performed in high-risk patients for the investigation of the presence of a bladder tumour. Confirmation of presence of tumour cells is performed by cystoscopy (insertion a flexible or rigid scope through the urethra into to bladder), that allows visualization of the inside of the urinary bladder (5). Through this procedure, a biopsy can also be performed, allowing for cytology confirmation. Although, several new tests have been developed, such as bladder tumour associated antigen, the immunocyte assay, nuclear matrixprotein-22 and the Uro Vysion assay, none of them is commonly used in clinical practice due to relatively low sensitivities and/or specificities and the diagnosis is performed by Cytology and/ or histology (4).

The standard treatment for early-stage BC patients is the transurethral resection of bladder tumour (TURBT), that might be followed by intravesical therapy, either with a chemotherapeutic agent (Mitomycin C - MMC) or immunotherapy (bacillus Calmette-Guérin - BCG). Advanced cancers may require removal of the entire bladder (cystectomy), radiation and/or systemic chemotherapy treatments (11, 16). As the recurrence rate for this type of cancer is very high, some patients have to endure various surgeries through time, increasing their morbidity and also the financial costs related with BC treatment.

Therefore, there is an urgent need to understand the mechanisms of bladder cancer progression for the development of new diagnostic and therapeutic strategies. This is of great importance for clinical patient's management. In the last few years, the number of genomic, epigenomic and epitranscriptomic studies have increased dramatically, mostly due to the rapid improvement of high-throughput DNA sequencing technologies.

C. H. Waddington originally defined the term Epigenetics as the study of "causal mechanisms by which genes of the genotype bring about phenotypic effects" (17). However, this initial definition evolved over time, being less focused on the genotype, and more implicated in a wide variety of biological processes. Thus, the current definition of epigenetics is "the study of heritable changes in gene expression that occur regardless of changes in the primary DNA sequence" (18). The main epigenetic molecular mechanisms are grouped into four main groups: DNA methylation, histone post-translational modifications or chromatin remodelling, histone variants, and ncRNAs regulation (19).

Recently, a new layer of gene expression regulation at the RNA level was identified. Epitranscriptomic refers to the study of reversible chemical modifications that may occur in RNA

molecules, including ncRNA and mRNA (20-22). N6-methyladenosine (m6A), methylation of the adenosine base at the nitrogen-6 position, is the most abundant internal chemical modification of mRNAs and in long non-coding RNA (lncRNA) in eukaryotes. Its potential in gene expression regulation has been recently explored (22).

This modification occurs preferentially in DRACH sequence (where D denotes A/G/U; R denotes A/G and H denotes A/C/U) (23, 24). The m6A is specifically enriched near the stop codon, in the 3`untranslated regions (3`-UTR), and within long internal exons, thereby affecting different steps of mRNA's life, such as transcription, splicing, nuclear export and translation (24). In mammalian cells, this modification is catalysed by the m6A methyltransferase complex ("writers") and can be removed by m6A demethylases ("erasers"). There are also proteins that bind directly to the m6A mark and mediate its function, known as "readers" (**Figure 4**) (25-27).
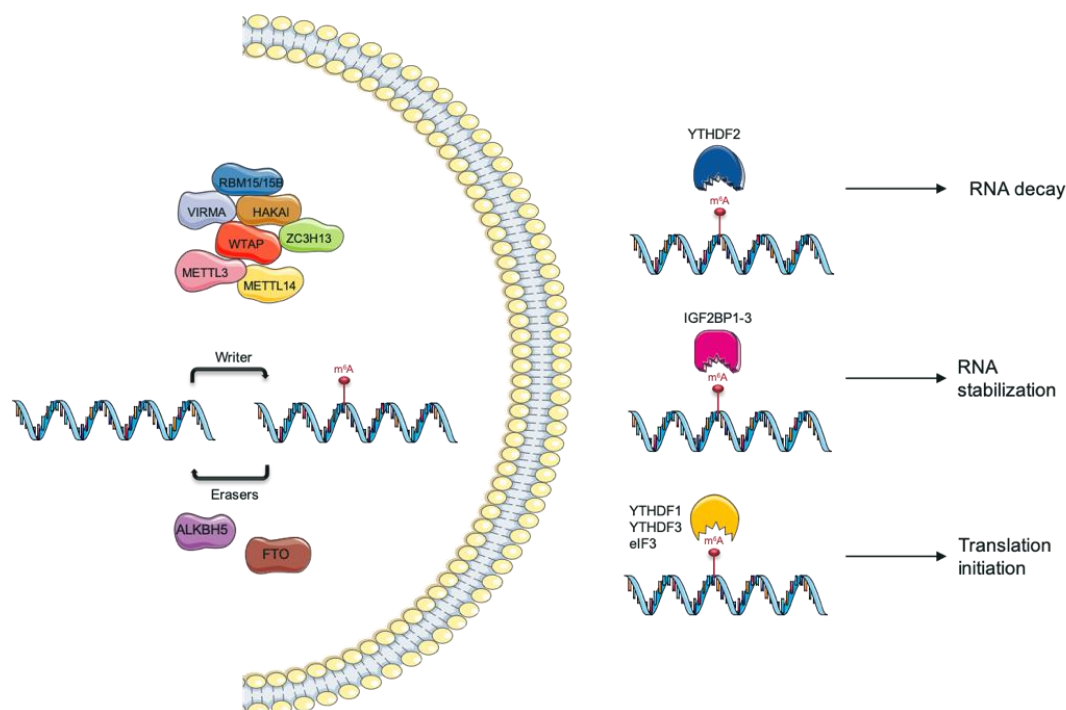
Figure 4. N6-methyladenosine (m6A) modification regulation by "writers", "erasers" and "readers". This modification is a dynamic and reversible process coordinated by a complex consisting of methyltransferases, METTL3/14, WTAP, RBM15/15B, and KIAA1429 (VIRMA); demethylases FTO and ALKBH5 and "reader" proteins that mediate their function. Teixeira, C. *unpublished.*

The m6A modification is catalysed by the methylase complex composed by the methyltransferase-like 3 and 14 proteins (METTL3 and METTL14) and their cofactors: Wilm`s tumour-associated protein (WTAP), Virilizer (KIAA1429/VIRMA), RNA-binding motif proteins 15/15B (RBM15/15B), and zinc finger CCCH domain-containing protein 13 (HAKAI and ZC3H13) (28).

METTL3 and METTL14 constitute a heterodimer that induces m6A synergistically. Although METTL3 was already reported to play a central role in complex stability, data is lacking for METTL14 (28, 29). WTAP is a regulatory subunit required for the nuclear complex, since it positions METTL3 and METTL14 to allow its connection/functionality, and also recruits other factors to the methyltransferase complex (26, 27). VIRMA recruits the methyltransferase core components - METTL3, METTL14 and WTAP - via WTAP in a RNA-independent manner, and rather favours mRNA methylation near the 3`-UTR and stop codon regions (30). The RNA binding protein RBM15 and its

homolog RBM15b, preferentially bind to RNA's U-rich regions, recruiting the m6A complex through interaction with METTL3 in a WTAP-dependent manner (22).

Furthermore, m6A modification can be demethylated by the human obesity-associated protein (FTO), which is conserved among eukaryotes and it is one of the RNA demethylases that belongs to the ALK family. AlkB homologue 5 (ALKBH5) is another demethylase that binds to single stranded nucleic acids and therefore differs from the other members of the ALKB protein family (31, 32). These proteins ensure a balanced equilibrium of m6A modification.

The identified "readers" of m6A are YTH domain family proteins. Specifically, YTH m6A-binding protein 3 (YTHDF3) cooperates with YTHDF1 (promoting translation) and YTHDF2 (promoting mRNA degradation) affecting cytoplasmic metabolism of methylated mRNAs, and translation (33).

Several studies showed that m6A RNA methylation and the associated regulatory proteins are implicated in cancer, including leukaemia, breast, lung, brain, liver, cervical and endometrial cancer. However, knowledge of the mechanistic network between m6A and BC is rather limited, being necessary to further investigate the role of this modification in this malignancy. Hence, the deregulation of the main players: METTL3, METTL14, WTAP, VIRMA, FTO, ALKBH5 and YTHDF3 was also investigated.

Aims

The m$_6$A modification and the associated regulatory proteins (METTL3, METTL14, WTAP, VIRMA, FTO, ALKBH5 and YTHDF3) play critical roles in the pathogenesis of various types of cancers, however, the role of these proteins remains poorly explored in bladder cancer. Thus, studying the role of this modification may uncover mechanisms associated with BC aggressiveness.

Specifically, the following tasks were set:

1. Evaluate m$_6$A, METTL3, METTL14, WTAP, VIRMA, ALKBH5, FTO and YTHDF3 expression in a patient's cohort of BC patients diagnosed with primary tumour prior to any treatment. Proteins' s expression was also evaluated in a series of normal bladder tissues.

2. Compare the immunoexpression findings with clinical and pathological features of bladder cancer patients.

3. Assess m$_6$A RNA methylation expression levels and respective players protein expression and cellular localization in eight bladder cell lines to molecularly characterize and identify the best *in vitro* model for further studies.

4. Modulate the most promising m$_6$A regulator using CRISPR-Cas9 system, in order to study its relevance in bladder carcinogenesis.

MATERIALS AND METHODS

In Silico Analysis

To evaluate the expression of m6A writers, erasers and readers, the online platform cBio-Portal was used for *in silico* analysis. The Cancer Genome Atlas (http://ca ncergenome.nih.gov) database was selected to determine which subunit may play the crucial role of m6A deregulation in BC.

For this, the online platform cBio-Portal for Cancer Genomics was used (34), with the user-defined entry gene set "*METTL3, METTL14, VIRMA, WTAP, ALKBH5, FTO* and *YTHDF3*".

GEPIA web server was used for analysing the RNA sequencing expression data of bladder tumours and normal samples from the TCGA and GTEx projects using a standard processing pipeline: |log2FC| Cut-off =1 ; *p*-value cut-off= 0.01 (35).

Patients and Samples Collection

One hundred twenty (sixty of NMIBCs and others sixty of MIBCs) formalin-fixed and paraffin-embedded tissues were collected from the archives of the department of pathology of Portuguese Oncology Institute of Porto.

These samples are representative of primary bladder urothelial carcinomas (BUCs), without any prior treatment, and diagnosed between 1997 and 2005 at Portuguese Oncology Institute- Porto, Portugal.

Additionally, for control purposes, 40 tissue samples of normal urothelial tract (NUT), originating from nephrectomy specimens of patients with kidney cancer.

This study was approved by the ethics committee (Comissão de Ética para a Saúde) of the Portuguese Oncology Institute of Porto (CES-IPO 372/2017).

Clinical files and pathology reports were reviewed. All histological slides (of primary tumours) were reviewed by a pathologist and tumours were reclassified in light of the most recent 2016 *World Health Organization (WHO) Classification of Tumours of the Urinary System and Male Genital Organs* (36). Staging was performed according to the 8th edition of the *American Joint Committee on Cancer* (AJCC) staging manual (37).

Immunohistochemical analysis

Immunohistochemistry (IHC) analysis for m6A modification, m6A writers METTL3, METTL14, VIRMA and WTAP, erasers ALKBH5 and FTO, and reader YTHDF3 was performed using the NovolinkTM Max Polymer Detection System (Leica Biosystems, Germany). Four μm thick sections were cut and placed in coated slides. In short, after sections deparaffinization (through xylene) and hydration (through a graded alcohol series), antigen retrieval was accomplished by microwave at 800 W or water bath at 95ºC in specific buffer, depending on the protein studied (**Table 1**). Then, endogenous peroxidase activity was inhibited with freshly prepared 0.6% hydrogen peroxide solution for 20 minutes, and the unspecific bindings were blocked with horse serum (1:50 dilution) for 20 minutes, at room temperature (RT) in a humidified chamber. Incubation of the primary antibody was performed overnight at 4ºC, at a dilution dependent on the specific antibody (**Table 1**). Afterwards, slides were washed in tris-buffered saline (TBS) solution with 0.1% Tween 20, and incubated with post primary, followed by polymer (30 minutes each). Slides were therefore washed, developed with 3,3`-Diaminobenzidine tetrahydrocloride chromogen (DAB, Sigma-AldrichTM, Germany) and counterstained with haematoxylin (Leica Biosytems Richmand, USA). Finally, after dehydration and

diaphanization, slides were mounted in Entellan® (Merck-Millipore, Germany). Appropriate positive controls were used for each antibody and can be consulted in **Table 1**.

The semi-quantitative analysis of immunoexpression was performed by an experienced pathologist and categorized according intensity (between 0-3) and percentage (between 0-100%, and later categorized between 0-3). The score for intensity was performed as score 0 (absent immunoexpression), score 1+ (immunoexpression less intense that in control tissue), score 2+ (immunoexpression similar to control tissue), score 3+ (immunoexpression more intense that in control tissue). The score for percentage was classified in: score 0 (<1% of immunoreactive cells), score 1+ (<50% of immunoreactive cells), score 2+ (50-75% of immunoreactive cells) and score 3+ (75-100% of immunoreactive cells). The final staining score was calculated by multiplying intensity and percentage score, resulting in a score value ranging from 0 to 9+. Pictures were taken in a microscope Olympus BX41 with digital camera Olympus U-TV0.63XC using Cell A software.

Table 1. Primary antibodies used in Immunohistochemistry

Antibodies	Vendor	Catalog number	Positive control	Antigen retrieval	Primary antibody dilution
Anti-METTL3 [EPR18810]	Abcam, Cambrige, United Kingdom	ab195352	Normal testicle	EDTA buffer microwave (1mM, pH=8)	1:500 overnight 4ºC
Anti- METTL14 [CL4252]	Abcam, Cambrige, United Kingdom	ab220031	Normal testicle	Citrate buffer microwave (10mM, pH=6)	1:750 overnight 4ºC
Anti- WTAP [EPR18744]	Abcam, Cambrige, United Kingdom	ab195380	Normal testicle	EDTA buffer microwave (1mM, pH=8)	1:500 overnight 4ºC
Anti-VIRMA/ Virilizer	Cell Signaling Technology, United States, USA	88358	Normal testicle	Citrate buffer microwave (10mM, pH=6)	1:200 overnight 4ºC
Anti-FTO [5-2H10]	Abcam, Cambrige, United Kingdom	ab92821	Normal testicle	Citrate buffer microwave (10mM, pH=6)	1:500 overnight 4ºC
Anti- ALKBH5	Proteintech Europe, United Kingdom	16837-1-AP	Normal testicle	Citrate buffer microwave (10mM, pH=6)	1:500 overnight 4ºC
Anti- YTHDF3	Abcam, Cambrige, United Kingdom	ab103328	Breast cancer	EDTA buffer water bath (1mM, pH=8)	1:100 overnight 4ºC
Anti-N6-methyladenosine (m6A)	Abcam, Cambrige, United Kingdom	ab190886	Normal brain	EDTA buffer water bath (1mM, pH=8)	1:750 overnight 4ºC

Bladder Cancer Cell Lines Studies

Seven bladder cancer, MGHU3, RT112, 5637, J82, T24, UMUC3, TCCSUP, and one bladder cell line SV-HUC1 from American Type Culture Collection (ATCC), were used for m_6A players' characterization (**Table 2**).

All culture media were supplemented with 10% Fetal Bovine Serum (FBS, Biochrom, MERK, Germany) and 1% penicillin/streptomycin (GIBCO®, Invitrogen, USA). Cells were maintained at 37ºC with 5% CO_2 and routinely tested for *Mycoplasma sp.* contamination using a PCR-based universal mycoplasma detection kit (PCR Mycoplasm Detection Set, Clontech Laboratories, Oxford, UK).

To perform cellular assays, sub-confluent cells were detaching with trypsin at 37ºC (Gibco, Invitrogen). Trypsin was inactivated with media 10% FBS, collected and centrifuged at 1,200 rpm during 5 minutes. Cells were resuspended in fresh medium and 10 µl of cell suspension were collected for cell counting with 10 µl of Trypan Blue in the Neubauer chamber. Cell density was then calculated to the different assays.

Table 2. Clinicopathologic characterization of bladder cell lines

Cell Line	Type	Grade[a]	Stage[a]	Subtype[a]	Gender	Media
SV-HUC1	normal	n/a	n.a.	n.a.	male	F12-K
MGHU3	tumour	G1	pTa/T1	luminal	male	DMEM
RT112	tumour	G2	pTa	luminal	female	RPMI
5637	tumour	G2	pTx	mixed	male	RPMI
J82	tumour	G3	pT3	basal	male	MEM
T24	tumour	G3	pTa	basal	female	RPMI
UMUC3	tumour	G3	pT2- T4	basal	male	DMEM
TCCSUP	tumour	G4	pTx	basal	female	MEM

n.a.- not applicable
a- according to (38)

Protein Extraction and Quantification

Total protein was extracted from cells, in triplicates, using the radioimmunoprecipitation assay buffer (RIPA) (Santa Cruz Biotechnology Inc., USA) complemented with 10% of protein inhibitor cocktail (PIC). After 15 minutes in the ice, the samples were centrifuged at 13,000 rpm during 30 minutes at 4ºC and the supernatant was collected.

Subsequently, quantified using a Pierce BCA Protein Assay Kit (Thermo Scientific Inc., USA), according to manufacturer's instructions.

Western Blot

Briefly, 30 µg of protein from each cell line was resuspended in loading buffer, denatured at 95ºC for 5 minutes, and loaded in 8% polyacrylamide gel where they were separated by size through sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) at 120 V for RT. Then, proteins were transferred to 0.2 µm polyvinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories Inc., Hercules, CA, USA) using 25 mM Tris-base/glycine buffer and a Trans-Blot Turbo Transfer system (Bio-Rad) at 25 V and 1.3 mA for 15 minutes. After that, membranes were blocked with 5% bovine serum albumin (BSA; Santa Cruz, USA) or 5% dry milk in TBS with 0.1% Tween (TBS-T, pH=7.6), and then incubated with primary antibody (**Table 3**). After incubation, membranes were incubated with secondary antibody coupled with horseradish peroxidase (Bio-Rad, USA), for 1 hour at RT. To ascertain equal loading of protein, the membranes were incubated with an endogenous control antibody. Quantification was performed using band densitometry analysis from the ImageJ software (version 1.6.1, National Institutes of Health), by comparing the specific protein band intensity with the loading control beta-actin (β-ACT).

Table 3. Primary antibodies used in Western blot and Immunofluorescence

Antibodies	Vendor	Catalog number	Western-blot dilution	Primary antibody: incubation time	Secondary antibody specie	Blocked	IF dilution
Anti-METTL3	Abcam, Cambrige, United Kingdom	ab195352	1:1000	1h at RT	Anti-rabbit	BSA 5%	1:250
Anti-METTL14	Abcam, Cambrige, United Kingdom	ab220031	1:1000	1h at RT	Anti-mouse	BSA 5%	1:50
Anti-WTAP	Abcam, Cambrige, United Kingdom	ab195380	1:1000	1h at RT	Anti-rabbit	BSA 5%	1:100
Anti-VIRMA / Virilizer	Cell Signaling Technology, United States, USA	88358	n.a.	n.a.	n.a.	n.a.	1:100
Anti-FTO	Abcam, Cambrige, United Kingdom	ab92821	1:1000	1h at RT	Anti-mouse	Milk 5%	1:100
Anti-ALKBH5	Proteintech Europe, United Kingdom	16837-1-AP	1:1000	1h at RT	Anti-rabbit	Milk 5%	1:500
Anti-YTHDF3	Abcam, Cambrige, United Kingdom	ab103328	n.a.	n.a.	n.a.	n.a.	1:250
β-Actin	Sigma-Aldrich	A1978	1:10,000	1h at RT	Anti-mouse	BSA 5%	n.a.

n.a. – not applicable; RT – room temperature

Immunofluorescence analysis

M6A, METTL3, METTL14, VIRMA, WTAP, ALKBH5, FTO and YTHDF3 protein expression and cellular localization assessed by immunofluorescence (IF). Cells were seeded in coverslips in 24-well plates at 25,000 cells/well previously optimized concentration and allowed to adhere at 37ºC, 5% CO_2 overnight. In the next day, cells were fixed 4% paraformaldehyde (PFA) for 10 minutes and permeabilized with 0.25% Triton X-100 solution in phosphate-buffer saline (PBS) for 15 minutes. After that, cells were blocked with 5% BSA for 30 minutes, followed by primary antibody incubation at specific dilution (**Table 3**), overnight at RT.

Following primary antibody, cells were incubated with secondary antibody anti-rabbit immunoglobulin G (IgG) (Alexa Fluor™ 488 goat, A11008; Invitrogen, USA) or anti-mouse IgG-fluorescein isothiocyanate (FTIC goat SLB4878, Sigma-Aldrich™) for 1 hour, at RT (**Table 4**). Then, nuclear stained was performed with 4',6- diamidino-2-phenylindole (DAPI) (AR1176, BOSTER Biological Technologies, China) in mounting medium. Pictures were taken in fluorescence microscope Olympus IX51 with a digital camera Olympus XM10 using CellSens software (Olympus, Japan) (400x magnification).

M6A Quantification

RNA was extracted from cell lines by TripleXtractor (GRiSP®, Portugal) according to manufacturer's recommend protocol. To detect m6A levels, m6A RNA Methylation Quantification Kit (ab185912; Abcam) was used as the recommended. In this assay, the m6A is detected using capture and detection antibodies. The detected signal is enhanced and then quantified using colorimetric methodology by reading the absorbance in a microplate spectrophotometer. The amount of m6A is proportional to the optical density (OD) intensity measured.

METTL14 Gene Knockdown

After protein analysis by Western blot, UMUC3 cell line was chosen to perform METTL14 gene knockdown by plasmids carrying the CRISPR-Cas9 system containing a guide RNA sequence targeting METTL14 (GenScript, Piscataway, NJ). For plasmid transfection, Lipofectamine® 3000 reagent (Invitrogen, USA) was used according to manufacture instructions, followed by selection of cells which incorporated the CRISPR-Cas9 system with 1µg of puromycin for each 1mL of DMEM cell culture medium (Sigma).

After selection, cells were expanded, and total protein was extracted in order to confirm METTL14 protein downregulation. UMUC3 will type cells were used as controls for Western blot analysis.

Cell Viability and Proliferation Assays

The effects on cell viability was assessed by MTT assay (Sigma-Aldrich, Germany). Viable cells incorporate MTT solution and the mitochondrial enzyme succinate-dehydrogenase convert them into a purple coloured formazan product.

Cell proliferation was measured by using the Cell Proliferation ELISA BrdU assay (Roche Applied Sciences), based on DNA synthesis analysis. This is a colorimetric immunoassay where 5-bromo-2'-deoxyuridine (BrdU), a pyrimidine analogous, is incorporated in placed of pyrimidine into

the newly synthesized DNA. The quantity of BrdU incorporated into cells is directly correlated to the number of proliferating cells.

Cells were plated into 96 well plates in complete DMEM Medium at density of 6,000 cells/well and incubated overnight, at 37ºC in 5% CO_2. Then, cells were maintained with DMEM 1% FBS, from 0 hours until 48 hours.

In MTT assay, the culture medium was removed, and cells were incubated during 1 hour at 37ₒC with 5 µg/mL MTT solution in complete culture medium. After that, formazan crystals formed were dissolved in dimethyl sulfoxide (DMSO) and spectrophotometric measurement was done at 540nm (reference wavelength: 655 nm) in a microplate reader (Fluostar Omega, BMG Labtech, Germany). DMSO was used as blanks to correct the OD values, and OD obtained for 48 hours was normalized for the 0 hours' time point.

For BrdU assay, cells were incubated with 20 µM BrdU labelling solution for 12 hours. After removing labelling medium, cells were fixed for 30 minutes at room temperature with FixDenat solution. This solution induces DNA denaturation necessary to allow conjugated antibody binding to the incorporated BrdU. Then, FixDenat was removed, and anti-BrdU-POD antibody (dilution 1:100) was added to detect the incorporated BrdU in DNA. After 90 minutes, at room temperature, the antibody was removed, and cells were rinsed 3 times with 1X PBS. The immune complex formed was detected by adding 100 µl/well of substrate solution and incubated for 5-10 minutes, until colour development. Then, the reaction was stopped with 1 M H_2SO_4 added to each well, and the reaction product was quantified in a microplate reader (Fluostar Omega, BMG Labtech, Germany) by measuring absorbance at 450 nm (reference wavelength: 690 nm). The OD values obtained for 48 hours was normalized for the 0 hours` time point.

The results represent the mean of three independent experiments, each one in triplicate, and were analysed using a GraphPad Software.

Apoptosis evaluation

In order to complement cell behaviour studies, apoptosis assay were performed. Briefly, $4x10_4$ cell/well of cells were seeded on 24-well plates and incubated during 48 hours at 37ºC and 5% CO_2. After 48 hours, the Cell-APOPercentageᴛᴍ apoptosis assay kit (Biocolor, United Kingdom) were performed according the manufacturing instructions. Then, cells were incubated with 300 µl/well of APOPercentageᴛᴍ dye solution at ratio 1:20 respectively, during 30 minutes at 37ºC. Next, cells were washed with PBS 1X and detached from well plate with TrypLeᴛᴍ Express (GBICO, Invitrogen, USA) at 37ºC. After that, APOPercentageᴛᴍ dye release reagent was added and plate were vigorously agitated during 15 minutes, following colorimetric measurement at 550 nm with 620 nm reference filter (Fluostar Omega, BMG Labtech, Offenburg, Germany). The H_2O_2 was used as a positive control. The OD obtained for apoptosis assay was normalized for the OD obtained by viability assay at the same time point.

Wound Healing Assay

The wound-healing assay is one of the earliest developed methods to study directional cell migration *in vitro* and, mimics cell migration during wound healing *in vivo*.

Cells were seeded (6-well plate) in complete DMEM Medium at an optimal density to obtain at least 95% of confluence in the next 24 hours, and incubated at 37ºC, 5% CO_2. In the next day, the culture medium was removed, and two "wounds" were made by manual scratching with a 200 µL pipette tip for each condition. Then, the wells were washed with 1X PBS in order to remove detached cells in

wound and incubated with complete cell medium (DMEM 1% FBS). The wound areas were photographed in two specific sites at 40x magnification using an Olympus IX51 inverted microscope equipped with an Olympus XM10 Digital Camera System. The photos were taken at regular time points (between 0 and 48 hours) until wound closure. The relative migration distance (5 measures by wound) was calculated with the following formula: relative migration distance (%) = (A–B)/C x100, where A is the width of cell wound at 0 hours incubation, B is the width of cell wound after specific hours of incubation, and C is the width mean of cell wound for 0 hours of incubation. For relative migration distance, the results were analysed using the beWound - Cell Migration Tool (Version 1.5) (developed by A.H.J. Moreira, S. Queirós and J.L. Vilaça, Biomedical Engineering Solutions Research Group, Life and Health Sciences Research Institute - University of Minho; available at http://www.besurg.com/sites/default/files/beWoundApp.zip). At least three independent experiments were performed.

Invasion Assay

Cell invasion was evaluated by using 24-well BD BioCoat Matrigel Invasion Chambers, with 8μm pore size membranes (BD BioSciences, USA). These invasion chambers allow to compare the *in vitro* cells invasive behaviour in specific conditions. Invasive cells are able to detach themselves and invade through the Matrigel Matrix. On the order hand, the thin layer of Matrigel Matrix mimics a basal membrane that occludes the pores of the membrane, blocking non-invasive cells from migrating through the membrane.

After BD Matrigel Chambers rehydration between 30 minutes and 2 hours with serum free culture media at 37ºC, cells at a density of 30,000 cells/insert were seeded in complete culture medium supplemented with 1% FBS and incubated during 24 hours at 37ºC in 5% CO_2. Then, the non-invading cells were removed by with swab and invaded cells were methanol fixed, DAPI stained and counted in a fluorescence microscope Olympus IX51 with a digital camera Olympus XM10 using CellSens software. The invasion was calculated as % of cell invasion normalized for the control condition.

Statistical Analysis

Statistical analysis was performed using the GraphPad Prim 7.0 software (GraphPad Software Inc., Chicago IL, USA) and IBM® SPSS® Statistic software version 23 (IBM-SPSS Inc., La Jolla, CA; USA). Significance level was set at $p<0.05$. Non-parametric Mann-Whitney U test were used to compare two groups. For comparison between three or more groups, non-parametric Kruskal-Wallis test was used, followed by Mann-Whitney U test for pairwise comparisons and Bonferroni's correction, when applicable. Differences in METTL3, METTL14, VIRMA, WTAP, ALKBH5, FTO, YTHDF3 and m6A immunoexpression between normal urothelial tract and BUCs tissues was assessed by Chi-square or Fisher's exact test. Correlation between continuous variables was assessed with Spearman's (rs) non-parametric correlation test.

P-values were considered statistically significant when inferior to 0.05. Significance is shown vs. the respective control and depicted as follows: $*p \leq 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ and $_{ns}p > 0.05$ (non-significant).

RESULTS

In Silico Analysis

We first aimed to characterize the expression of m6A regulatory proteins using available data at the online platform cBio-Portal for Cancer Genomics was used (34), with the user-defined entry gene set "*METTL3, METTL14, VIRMA, WTAP, ALKBH5, FTO* and *YTHDF3*".

The database includes tumours samples of 413 patients with MIBC although node negative. Seventy-four % were male whereas 26% were females with a median age at diagnosis of 61 years (**Figure 5**).
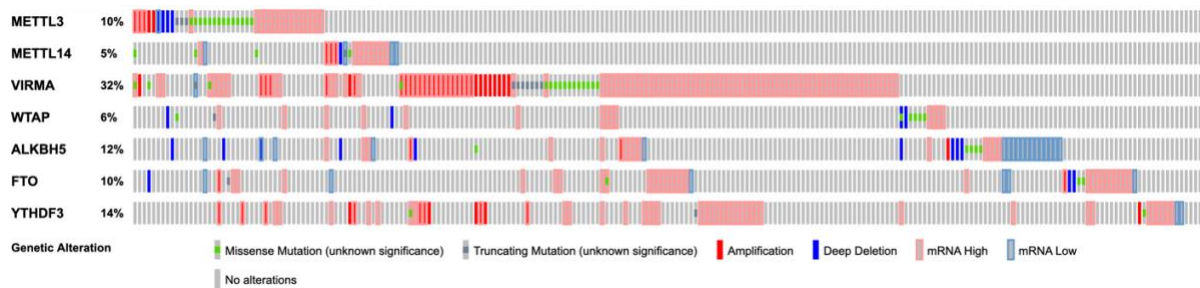
Figure 5. *In silico* **analysis**: frequency of alterations in queried genes in TCGA database.

In silico analysis showed that m6A players displayed different molecular alterations in MIBC. From all the known RNA-modification enzymes related with m6A modifications, VIRMA was the most frequently altered gene in these tumours. Furthermore, only METTL14 mRNA expression was significantly downregulated in bladder cancer compared to the normal bladder tissue (**Figure 6**).

Figure 6. Analysis of expression in normal bladder tissue and BC samples (TCGA data, Red box for tumour tissue, n=404; grey box for normal tissue, n=28). Only METTL14 was downregulated in BC.

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Since, no data is available for NMIBC and validation is lacking for MIBC, these genes were studied in a patient's cohort of IPOPorto.

Characterization of METTL3, METTL14, VIRMA, WTAP, ALKBH5, FTO, YTHDF3 and m6A immunoexpression in primary tumours
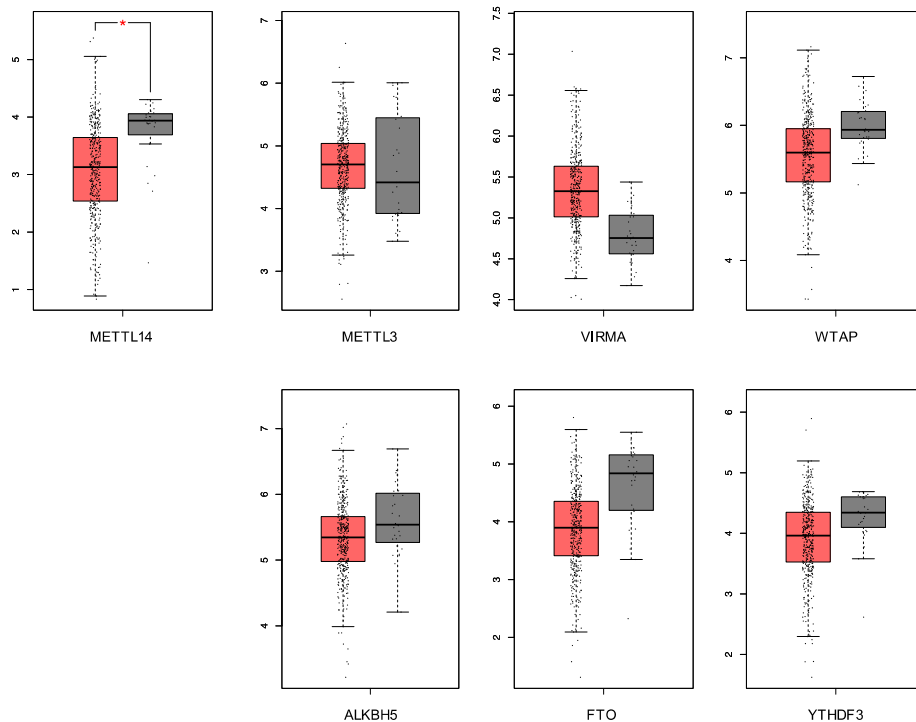
Immunohistochemistry assay was performed to evaluate proteins` expression in IPOPorto bladder tissues. For this, a total of 120 patients, comprising 60 (50%) NMIBC and 60 (50%) MIBC were included in this study. Additionally, normal urothelial obtained from 40 renal cell carcinoma patients submitted to nephrectomy without urothelial cancer was also analysed (**Table 4**).

Table 4. Clinicopathological parameters of the bladder cancer patients

CLINICOPATHOLOGICAL FEATURES	BUC	NUT
Patients, n	120	40
Gender, n (%)		
Male	93 (77.5%)	25 (62.5%)
Female	27 (22.5%)	15 (37.5%)
Median age, years (range)	69 (43-89)	63 (40-87)
MIBC and NMIBC, n (%)		
Muscle Invasive	60 (50%)	n.a.
Non-Muscle Invasive	60 (50%)	n.a.
Pathological stage, n (%)		
pTa	11 (9.2%)	n.a.
pT1	29 (24.2%)	n.a.
pT2	44 (36.7%)	n.a.
pT3	22 (18.3%)	n.a.
pT4	10 (8.3%)	n.a.
pTx	4 (3.3%)	n.a.

BUC: bladder urothelial carcinomas; NUT: normal urothelial tract

Nuclear expression was observed for all tested proteins, excepting for the "reader" YTHDF3, which exclusively showed a cytoplasmic expression (**Figure 7**).
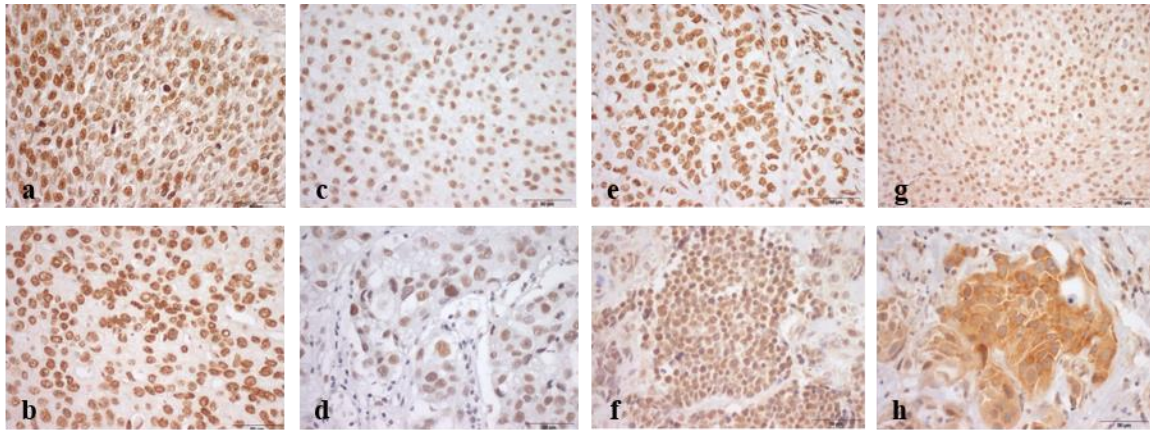
Figure 7. Illustrative images of immunostaining for m6A (**a**), METTL3 (**b**), METTL14 (**c**), VIRMA (**d**); WTAP (**e**), ALKBH5 (**f**), FTO (**g**) and YTHDF3 (**h**) in bladder cancer. **a** Strong (9+) m6A nuclear immunoexpression in bladder cancer; **b** Strong (9+) METTL3 nuclear immunoexpression in bladder cancer; **c** Weak/moderate (6+) METTL14 nuclear immunoexpression in bladder cancer; **d** Weak/moderate (6+) VIRMA nuclear immunoexpression in bladder cancer; **e** Strong (9+) WTAP nuclear immunostaining in bladder cancer; **f** Strong (9+) ALKBH5 nuclear immunostaining in bladder cancer; **g** Strong (9+) FTO nuclear immunostaining in bladder cancer; **h** Strong (9+) YTHDF3 cytoplasmic immunoexpression in bladder cancer.

Overall, METTL3, METTL14, VIRMA, ALKBH5 and YTHDF3 immunoexpression differed significantly between tumour and normal tissues. Specifically, METTL3 (p=0.0030), METTL14 (p=0.0019), VIRMA (p=0.0081), ALKBH5 (p<0.0001) and YTHDF3 (p=0.0107) showed significantly lower expression in bladder cancer compared with normal tissues. Moreover, METTL3 and METTL14 (heterodimeric catalytic core) showed a significant reduction in muscle invasive tumours comparing with NMIBC (p=0.0227 and p=0.0489, respectively) (**Figure 8**). Nonetheless, no significant differences were apparent regarding m6A, WTAP and FTO expression among the three studied groups.
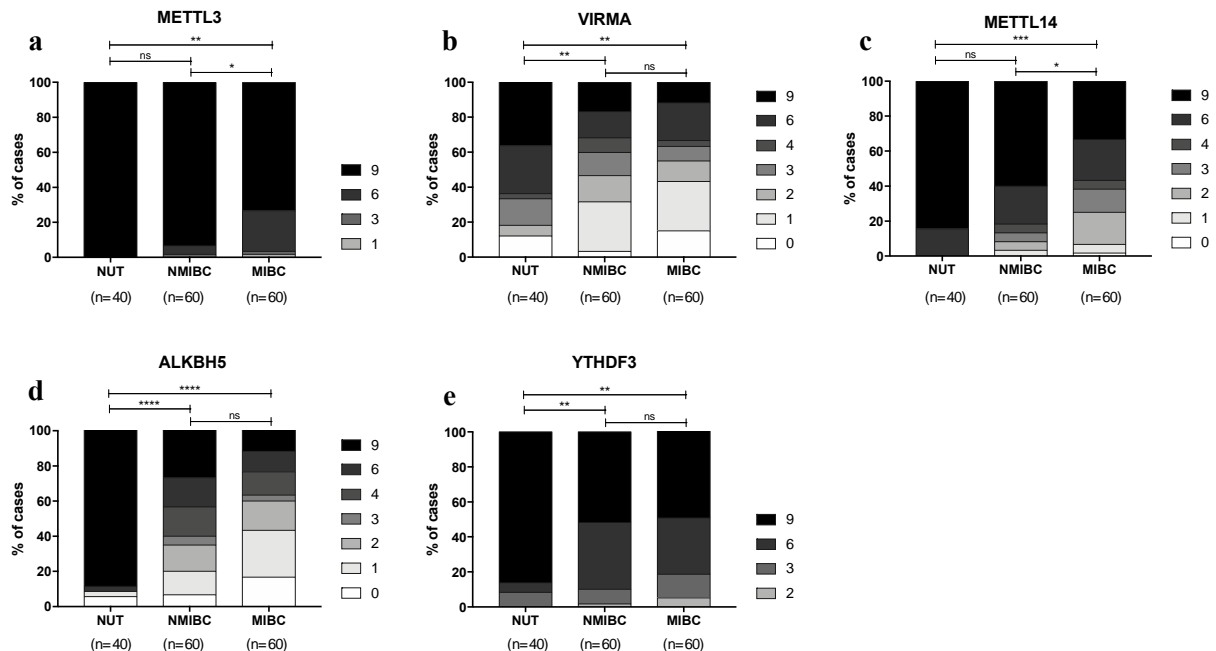
Figure 8. Characterization of METTL3, METTL14, VIRMA, ALKBH5 and YTHDF3 in normal and bladder cancer tissues by immunohistochemistry. a METTL3 (NMIBC *vs* MIBC p=0.023; NUT *vs* NMIBC p=0.005) , **b** VIRMA (NUT *vs* NMIBC p=0.003; NUT *vs* MIBC p=0.006), **c** METTL14 (NMIBC *vs* MIBC p=0.049; NUT *vs* MIBC p=0.003), **d** ALKBH5 (NUT *vs* NMIBC p<0.0001; NUT *vs* MIBC p<0.0001;) and **e** YTHDF3 (NUT *vs* NMIBC p=0.003; NUT *vs* MIBC p=0.006) immunostaining based on h-score (ranges from 0, +1, +2, +3, +4, +6, +9). Qui-square, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns- non signification. NUT- normal urothelial tumour, NMIBC- non muscle-invasive bladder cancer, MIBC- muscle-invasive bladder cancer.

The expression of the reader (YTHDF3) and all studied writers positively correlated with m$_6$A expression in tumour samples. The same was observed regarding ALKBH5 eraser expression. Interestingly, correlations were also observed between writers and erasers' expression, excepting for WTAP and both the erasers, as well as for VIRMA and FTO. Nonetheless, no correlation was found among the erasers' expression (**Table 5**).

Table 5. Correlation between different regulatory proteins. Spearman's rank correlation coefficient (rho)

	m$_6$A	METTL3	METTL14	VIRMA	WTAP	FTO	ALKBH5	YTHDF3
m$_6$A	-	$p= 0.001$ R= 0.488	$p< 0.0001$ R= 0.306	$p= 0.015$ R= 0.223	$p= 0.035$ R= 0.194	$p= 0.081$ R=0.162	$p= 0.014$ R=0.225	$p= 0.003$ R=0.269
METTL3	-	-	$p< 0.0001$ R= 0.435	$p<0.0001$ R= 0.343	$p<0.0001$ R= 0.415	$p= 0.014$ R=0.014	$p= 0.001$ R= 0.294	$p<0.0001$ R= 0.374
METTL14	-	-	-	$p<0.0001$ R= 0.373	$p= 0.008$ R= 0.243	$p= 0.066$ R=0.169	$p<0.0001$ R= 0.458	$p<0.0001$ R= 0.384
VIRMA	-	-	-	-	$p= 0.001$ R= 0.289	$p= 0.309$ R=0.094	$p<0.0001$ R= 0.416	$p= 0.011$ R= 0.237
WTAP	-	-	-	-	-	$p= 0.342$ R=0.089	$p= 0.131$ R= 0.140	$p= 0.043$ R= 0.192
FTO	-	-	-	-	-	-	$p= 0.869$ R=-0.015	$p= 0.063$ R= 0.175
ALKBH5	-	-	-	-	-	-	-	$p= 0.097$ R= 0.156

We evaluation the association between the expression of the m$_6$A regulatory proteins with clinicopathological parameters of the bladder cancer patients.

No significant differences were found for any of the tested proteins expression and patients smoking habits, gender and age of diagnosis. Nonetheless, a significant association was found between METTL3 expression and pathological stage. Indeed, decreased METTL3 expression was displayed by advanced disease (**Figure 9**).
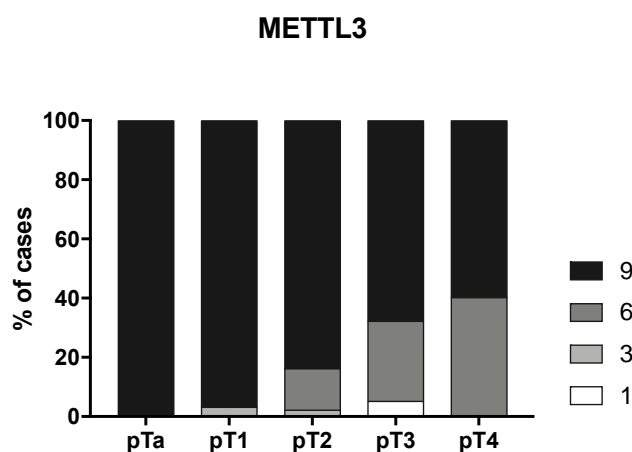
METTL3

Figure 9. Association between METTL3 protein level and pathological stage. Kruskal-Wallis test (p=0.01).

Characterization of METTL3, METTL14, VIRMA, WTAP, ALKBH5, FTO and YTHDF3 protein expression in bladder cell lines

Western blot was performed to evaluate proteins` expression in bladder cell lines.

No differences were apparent for m_6A regulatory proteins' expression among the tested cell lines, except for METTL14 (**Figure 10a**). Specifically, all BC cell lines presented heterogenous levels of this writer comparing with normal cell line. Among the tumour cells, UMUC3 showed the highest METTL14 expression (**Figure 10b**).
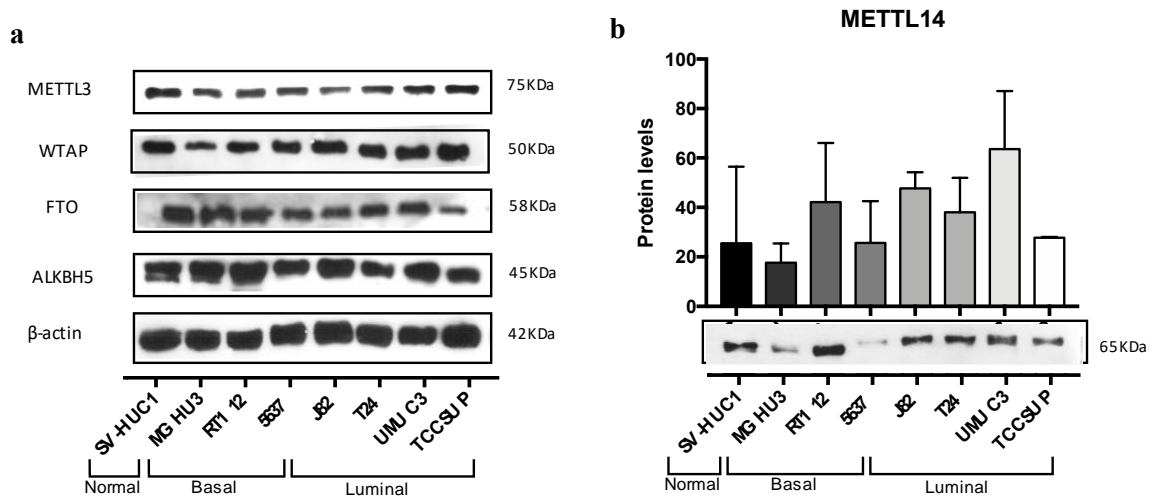
Figure 10. Characterization of regulatory proteins in bladder cancer cell lines a. METTL3, WTAP, FTO and ALKBH5 protein levels in the same cell lines. **b.** The expression of METTL14 protein in 7 bladder cancer cell lines in Western blot. SV-HUC1 cell line was used as control. Kruskal-Wallis test.

Cellular localization of m_6A and regulatory proteins in cell lines

Immunofluorescence was performed in order to verify cellular localization.

Overall, all writers and erasers were located in the nucleus of all cell lines, whereas the reader was found in the cytoplasm (**Figure 11**), which is in accordance with the observations in primary tumours by IHC.
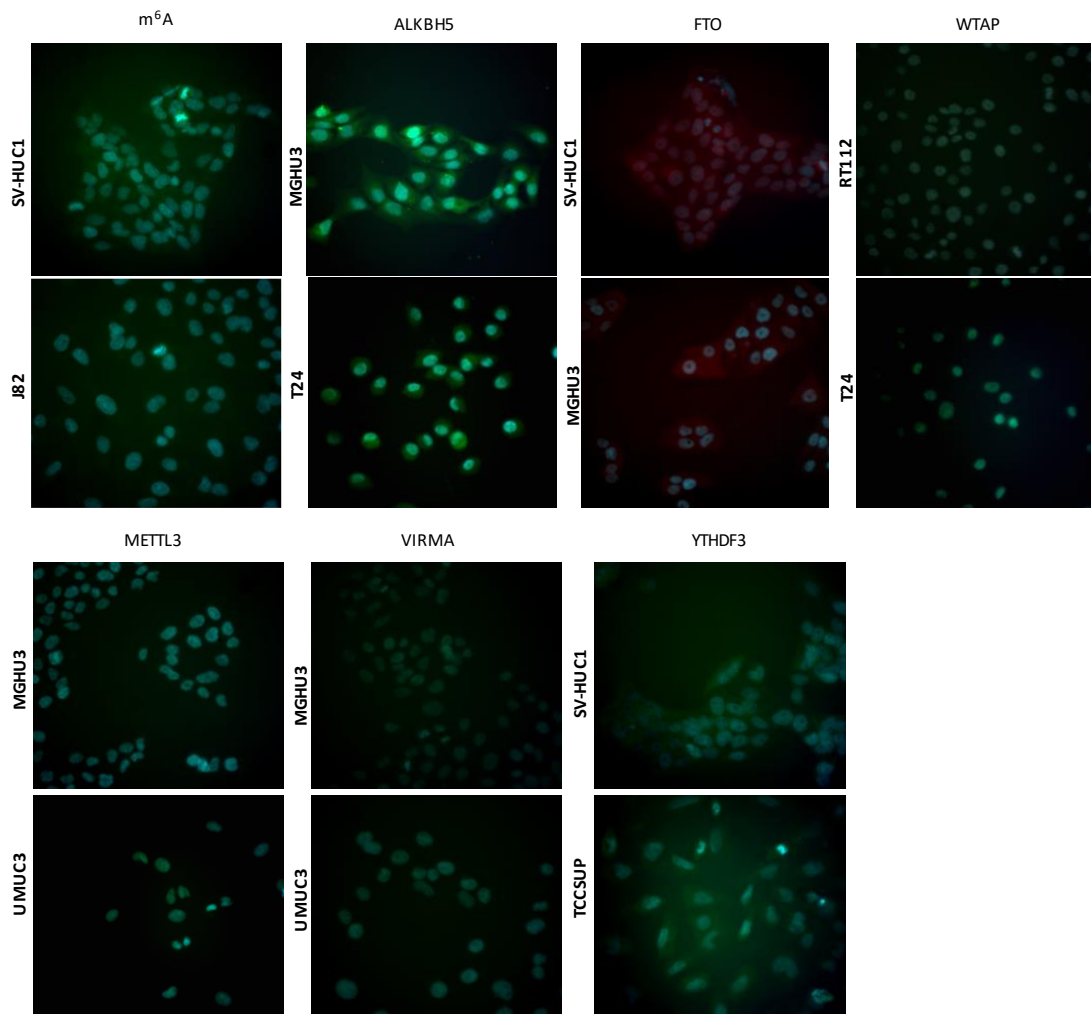
Figure 11. Illustrative images of IF for all tested m6A regulatory proteins. Results are compared to negative control. Photograph taken in microscope Olympus IX51 with a digital camera Olympus XM10 (400x amplification).

RNA m6A methylation quantification in cell lines

The m6A quantification was performed for assess RNA methylation expression levels.

Overall, bladder cancer cells exhibited higher RNA m6A methylation percentage comparing with normal cells, although no statistically significant difference was found. Indeed, the highest % was observed in 5637, T24 and UMUC3, with 47%, 29% and 52%, respectively (**Figure 12**).

Figure 12. Percentage of m6A in mRNA, using the ELISA m6A. Kruskal-Wallis test.

CRISPR-Cas9 System in UMUC3

Effective METTL14 knockdown was achieved in UMUC3 cells, confirmed at protein level. A higher efficiency of METTL14 knockdown was accomplished using 1.5 µL lipofectamine b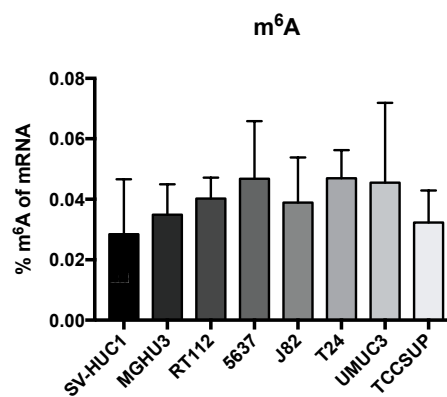y CRISPR-Cas9 system (p=0.05). Specifically, a reduction of about 50% was obtained in UMUC3 cell line (**Figure 13a**), which was paralleled by decreased m6A levels in 73% (p=0.0286) (**Figure 13b**).
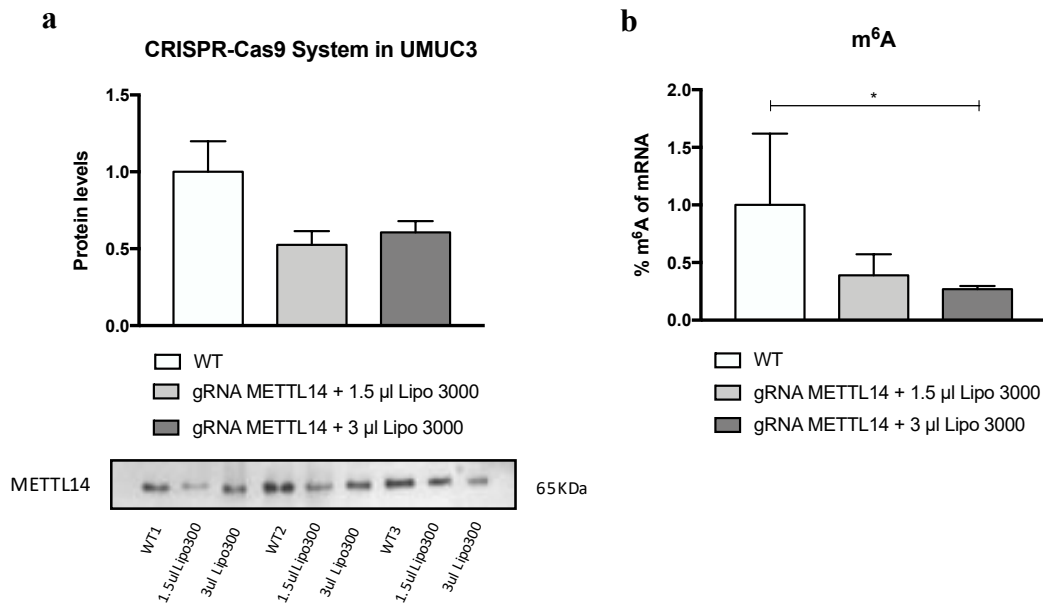

Figure 13. Knockdown of METTL14 on the UMUC3 cell line. a. We observed an efficiency in the protein expression of 48% in the condition of 1.5 µl Lipofectamine 3000 and 40% in the condition of 3 µl Lipofectamine 3000. Kruskal-Wallis test, p=0.05. **b.** Through the colorimetric assay of m6A, we found a reduction in the levels of 62% in the condition of 1.5 µl Lipofectamine 3000 (n.s.) and 73% in the condition of 3 µl Lipofectamine 3000 (p=0.0407). Kruskal-Wallis test: *$p < 0.05$, p=0.0286. Abbreviations: WT- Will type.

Phenotypic impact of METTL14 in UMUC3 cell line

Phenotypically, increased cell viability and proliferation was observed in METTL14 knockdown UMUC3 cells at the two different time-points, being more evident at 48 hours (p<0.001; p=0.0033, respectively) (**Figure 14a/14b**), whereas decreased apoptosis was found at 48 hours (about 3 times, p=0.0003) (**Figure 14c**).

Additionally, significantly increased cell invasion was displayed by METTL14 knockdown compared to will-type UMUC3 cells (24 hours, p=0.0476) (**Figure 14d**). Notably, METTL14 knockdown significantly increased the migration comparing with will-type UMUC3 cells (48 hours, p<0.001) (**Figure 14e**).

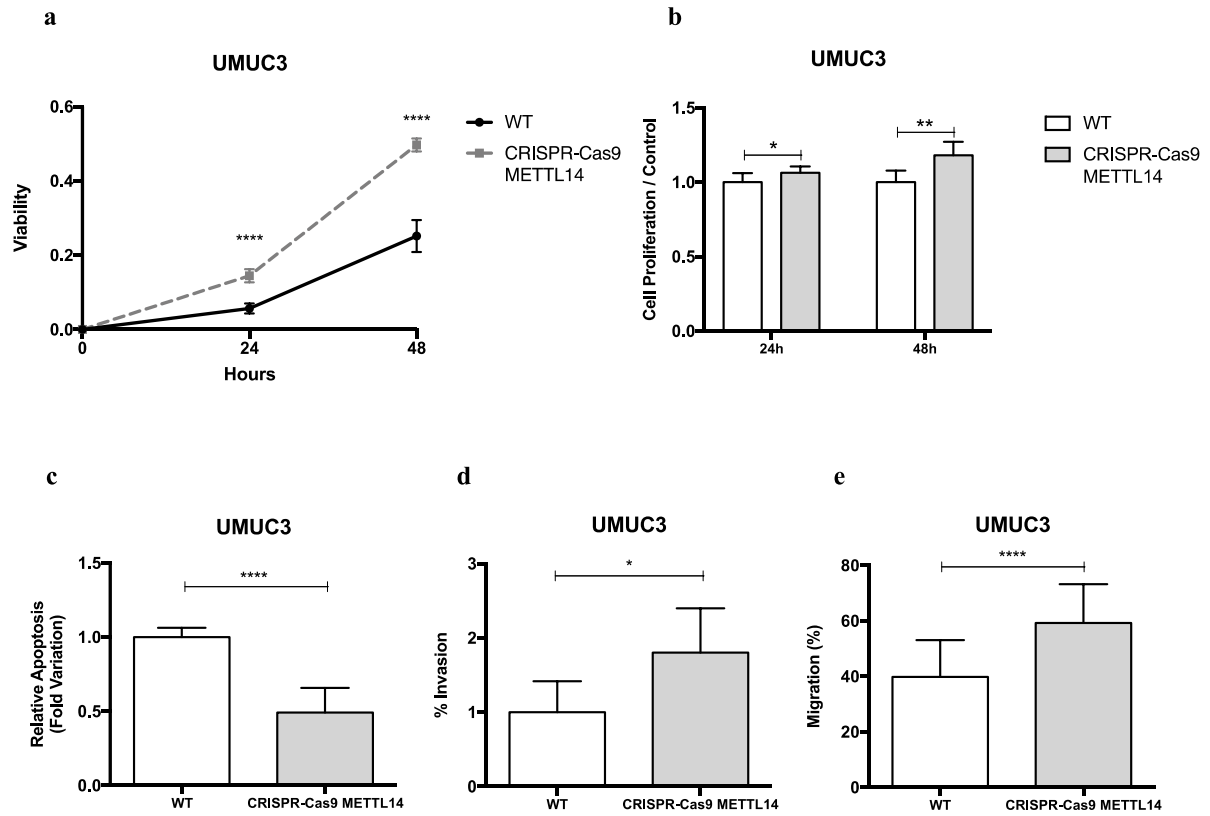**Figure 14. Phenotypic impact METTL14 knockdown in UMUC3 cell line. a.** in cell viability **b.** in BrdU assay **c.** apoptosis levels after 48 hours (mean ± SD, n = 3) **d.** cell invasion after 24 hours **e.** cell migration after 48 hours. Mann–Whitney U-test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$ compared to control (WT: UMUC3). Abbreviations: WT- Will Type.

DISCUSSION

Bladder cancer is one of the most incident urological neoplasms and its treatment remains a major health issue, once survival among patients with advanced disease is rather poor. Hence, the discovery of additional mechanisms underlying disease progression is imperative. M6A RNA methylation was firstly reported in 1974 by Ronald Desrosiers, but only recently its function began to be understood (39). Overall, this modification affects numerous aspects of RNA metabolism and plays a critical role in human disease, including cancer (25). Indeed, although m6A dysregulation have been reported in several tumours, data on bladder cancer is still limited. Herein, we assessed m6A RNA expression deregulation and respective regulatory proteins, also investigating respective role in bladder carcinogenesis.

The selection of more informative m6A players was performed by *in silico* analysis of RNA-seq data from muscle-invasive bladder cancer patients available at the TCGA database. METTL3, METTL14, VIRMA, WTAP (components of the m6A writer complex), ALKBH5, FTO (components of the m6A eraser complex) and YTHDF3 (reader) emerged as the most altered molecules involved in m6A regulatory network in bladder cancer. From the seven players, only METTL14 was found to be differentially expressed between tumours and adjacent normal samples.

The protein expression of all the selected "writers", "erasers" and "reader" was evaluated by IHC analysis in an independent set of bladder tumour and normal tissues. Remarkably, METTL3, METTL14, VIRMA, ALKBH5 and YTHDF3 expression was significantly decreased in bladder cancer compared to normal tissues.

Emerging evidence suggests that, m6A regulatory proteins can function both as oncogenes or tumour suppressors. Namely, METTL3, the most described m6A writer associated with carcinogenesis was found to be downregulated in endometrial carcinoma and glioblastoma, whereas, upregulation was found in leukaemia, pancreatic, breast and lung cancer (25). Moreover, METTL14 was described to be downregulated in hepatocellular carcinoma acting as a tumour suppressor (40), which is in accordance with our findings.

In our hands, the heterodimeric catalytic core METTL3/METTL14 was significantly reduced in MIBC in comparison with NMIBC, suggesting an association between the methyltransferase complex reduction and more aggressive disease. Also, METTL3/METTL14 expression positively correlated with m6A RNA modification, as already reported by others, but for different malignancies (41), reinforcing that writers' downregulation leads to the complex functionality disruption and consequent reduction in global m6A methylation. However, regarding bladder cancer, METTL3 overexpression was also found, but comparing with adjacent normal tissues and not truly normal bladder (3, 23, 42). Nevertheless, these studies used a rather limited number of cases comparing with our patients' cohort. Furthermore, METTL3 downregulation was found in advanced stages, again in line with the potential bladder cancer prognostic indicator already mentioned. Therefore, our findings add a new layer of epigenetic alterations that might contribute to bladder cancer progression.

Although no studies are available for bladder, VIRMA transcript levels were reported to be significantly higher in liver cancer, which counteract our observations in bladder tumours (43).

Conversely, in line with the results also obtained in glioblastoma patients, in our cohort of bladder cancer patients, ALKBH5 expression was highly heterogeneous in bladder tumours (44).

Remarkably, a correlation was observed between writers and erasers expression in tumours, which might be a mechanism to compensate feedback of descending m6A modification, as observed in hepatocellular carcinoma (40).

Indeed, the increase/reduction of m6A regulatory proteins, has already been associated with the increase/decrease of m6A mark in other tumour models. Particularly, in glioblastoma and hepatocellular

carcinoma, METTL14 downregulation associated with m$_6$A levels reduction (40). Indeed, in our set of cell lines, increased m$_6$A modification was found in cancer cells compared with normal cells. Interestingly, METTL14 was the only player differential expressed in the same panel of cells, being upregulated in more aggressive tumour cells, particularly in UMUC3 cell line. Herein, METTL14 knockdown accomplished in UMUC3 cells associated with m$_6$A levels reduction, suggesting the key role of this protein in complex's function.

In summary, m$_6$A-related genes are dysregulated in bladder cancer. Particularly, METTL3/METTL14 methyltransferase complex downregulation is associated with progression of non-invasive bladder cancer to muscle-invasive bladder cancer.

The phenotypic effect of METTL14 knockdown was evaluated in UMUC3 cell line, which resulted in significantly increased cell viability/proliferation, while apoptosis was decreased at 48 hours, contrarily to previous observations in acute myeloid leukaemia cells. Additionally, METTL14 knockdown induced UMUC3 migration and invasion *in vitro*, according to results previously observed by other research team in hepatocellular carcinoma. Hence, our observations support a putative tumour suppressive role in bladder cancer.

Indeed, previous studies, indicate that although METTL14 do not have a catalytic function, it forms a heterodimer with METTL3, required for the complex stabilization and function. Specific interactions between METTL14 methyltransferase domain-MTD14, and METTL3 domain-MTD3, are needed for METTL3 catalytic activity. Hence, METTL3-METTL14 forms a stable methyl capable heterodimer. Wang *et al.* advocate that not only METTL14 structurally supports the METTL3's catalytic cavity, but also has a critical role in substrate RNA recognition (46). In the same line, we found that METTL14 reduction, diminishes the writer complex's activity by reducing m$_6$A RNA modification.

CONCLUSION AND FUTURE PRESPECTIVES

In conclusion, since METTL14 knockdown enhances malignant phenotype, one can suggest that METTL14 may have a tumour suppressive role in bladder cancer. Importantly, this is the first comprehensive study on METTL14 function in BC.

In the near future, we intend to ascertain the interaction of the METTL3-METTL14 heterodimer complex, through immunoprecipitation and Site Directed Mutagenesis assay. Furthermore, the downstream pathways regulated by METTL14 will be also investigated.

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