ROLE OF CLASS I HISTONE DEACETYLASES IN BLADDER CARCINOGENESIS

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RELEVANT ABBREVIATIONS
AUC  Area Under the Curve
BCa  Bladder Cancer
CIS  Carcinoma *In Situ*
CpG  Cytosine–phosphate–Guanine
DNA  Deoxyribonucleic Acid
FBS  Fetal Bovine Serum
HAT  Histone Acetyltransferase
HDAC  Histone Deacetylase
HDMT  Histone Demethylase
HMT  Histone Methyltransferase
HPRT1  Hypoxanthine Phosphoribosyltransferase 1
KW  Kruskall–Wallis test
miRNA  MicroRNA
MSP  Methylation Specific PCR
MTT  3–(4,5-dimethylthiazol–2–yl)–2,5–diphenyltetrazolium–bromide
MW  Mann–Whitney U–test
NB  Normal Bladder Mucosa
NPV  Negative Predictive Value
ON  Overnight
PBS  Phosphate Buffer Solution
PCR  Polymerase Chain Reaction
PPV  Positive Predictive Value
P–S  Penicillin–Streptomycin
qRT–PCR  Quantitative Reverse Transcription–Polymerase Chain Reaction
RNA  Ribonucleic Acid
ROC  Receiver Operative Characteristics
RT  Room Temperature
SDS  Sodium Dodecyl Sulfate
siRNA  Small Interfering RNA
TB  Bladder Tumor
TBST  Tris–Buffered Saline containing 0.1% (v/v) Tween
UCC  Urothelial Cell Carcinoma
**BACKGROUND:** Bladder cancer (BICa) is a worldwide health concern being the second most common tumor of the genitourinary tract and the second most common cause of death in patients with urinary tract malignancies. Due to its aggressiveness, high risk of recurrence and low cure rates of invasive disease, both the early detection of the malignant phases and the prognostic assessment of this neoplasia are extremely important. Epigenetic alterations, such as histone modifications, have been reported to occur earlier than genetic alterations in carcinogenesis. Thus, aberrant patterns of epigenetic modifications might be important biomarkers for cancer diagnosis and prognosis. These modifications are carried out by several chromatin modulators, including histone deacetylases (HDACs), known to play a crucial role in the regulation of several physiological and pathological cellular functions, including developmental processes and tumorigenesis. HDACs comprise a family of 18 genes, which are grouped into four classes depending on amino acid sequence homology in their catalytic domain. The class I comprises HDAC1, HDAC2, HDAC3 and HDAC8, and plays an important role in the regulation of cell differentiation, proliferation, cell cycle progression and apoptosis. Class I HDACs have been described as aberrantly expressed in several human cancers and some of these studies have already demonstrated their participation in different cell functions. Hence, their deregulation may lead to neoplastic transformation. Nevertheless, the role of class I HDACs in bladder carcinogenesis remains elusive.

**AIMS:** The main goal of this thesis was to assess the role of class I HDACs in bladder carcinogenesis. Specifically, we aimed to evaluate the expression levels of each member of class I HDACs in bladder carcinomas, correlate the molecular findings with the clinicopathological data, evaluate the usefulness of their expression levels as tumor biomarkers, and determine the biological role of altered class I HDACs in BICa cells malignant phenotype.

**METHODOLOGY:** To understand whether class I HDACs deregulation might be implicated in BICa and to determine their performance as BICa biomarkers, 127 samples of bladder urothelial carcinoma and 20 normal bladder tissues were analyzed for HDAC1, HDAC2, HDAC3 and HDAC8 mRNA expression using quantitative reverse transcription PCR (qRT-PCR), and protein expression using Western blot. Then, HDAC1 and HDAC2 expression was downregulated using transient transfection in 5637 BICa cell line. Phenotypic assays – cell viability and
apoptosis assay – were performed to assess their impact in the malignant phenotype.

**Results and Discussion:** Both mRNA and protein levels of all class I HDACs were upregulated in bladder tumors compared to normal bladder mucosas, suggesting an oncogenic function of these enzymes. We have found significant associations for **HDAC1** and **HDAC3** expression with tumor grade and stage, as lower expression levels of these HDACs were apparent in less differentiated and more advanced tumors. Therefore, this alteration could be an early event in the non-invasive (papillary) pathway, which is not shared by the invasive pathway of bladder carcinogenesis. Concerning their value as BCa biomarkers, we found that a three gene panel consisting of **HDAC1**, **HDAC3** and **HDAC8** expression accurately discriminates cancerous from normal tissues, with a remarkable sensitivity of 97% and 100% specificity, in tissue samples. Finally, phenotypic assays revealed a significant decrease in cell viability and increased apoptosis in HDAC1 and HDAC2 silenced BCa cells. Based on available literature and in the present data, similar functions for these HDACs might be hypothesized, because similar effects of inactivation of either HDAC1 or HDAC2 were found. Thus, our data support a central role for HDAC1 and HDAC2 in the regulation of cell proliferation and apoptosis of BCa cells.

**Conclusions:** We demonstrated that all class I HDACs are aberrantly overexpressed in BCa tissues and confirmed the oncogenic properties of HDAC1 and HDAC2 in a human BCa cell line. Taken together, our results suggest that HDAC1 and HDAC2 play an important role in BCa development through regulation of genes implicated in cell proliferation and apoptosis. Furthermore, we identified, for the first time, a group of three histone modifiers that might be clinically valuable as BCa biomarkers.
**INTRODUÇÃO:** O carcinoma de bexiga (BCa) é um problema de saúde relevante a nível mundial, sendo o segundo tumor do trato génito-urinário mais frequente e a segunda causa de morte mais comum em pacientes com doenças do trato urinário. Devido à sua agressividade, elevado risco de recorrência e baixas taxas de cura na forma invasiva, tanto a sua detecção precoce como a correta avaliação prognóstica desta neoplasia são extremamente importantes. Tem sido sugerido que durante o processo de carcinogénese as alterações epigenéticas, incluindo as modificações das histonas, precedem as alterações genéticas, podendo constituir biomarcadores tumorais tanto de diagnóstico como de prognóstico. Estas modificações são exercidas por vários moduladores da cromatina, incluindo as histonas desacetilases (HDACs), que são conhecidas por terem um papel crucial na regulação de várias funções fisiológicas e patológicas, entre as quais o processo de transformação neoplásica. As HDACs compreendem uma família de 18 genes, que estão agrupados em quatro classes, consoante a homologia da sequência de aminoácidos dos seus domínios catalíticos. A classe I compreende as HDAC1, 2, 3 e 8, tendo um papel importante na regulação da diferenciação celular, proliferação, progressão do ciclo celular, bem como na apoptose. Os membros desta classe foram descritos como sendo aberrantemente expressos em vários tumores malignos em humanos, e alguns destes estudos já demonstraram a sua participação em diferentes funções celulares. Assim, a sua desregulação tem sido associada ao processo de transformação e progressão neoplásica. Contudo, o papel desta classe de HDACs na carcinogénese vesical permanece largamente por esclarecer.

**Objetivos:** O principal objetivo desta dissertação foi avaliar o papel da classe I das HDACs na carcinogénese vesical. Especificamente, foram avaliados os níveis de expressão de cada membro da classe I das HDACs em carcinomas de bexiga, tendo-se correlacionado as alterações moleculares com os dados clínico-patológicos e avaliado a utilidade dos seus níveis de expressão como biomarcadores tumorais. Adicionalmente, foi ainda avaliado o papel biológico das alterações da classe I das HDACs no fenótipo maligno em linhas celulares de BCa.

**Metodologia:** Cento e vinte sete amostras de carcinomas uroteliais da bexiga e 20 amostras de mucosa de bexiga normal foram analisados relativamente à expressão de mRNA de *HDAC1, HDAC2, HDAC3* e *HDAC8*, utilizando PCR quantitativo (qRT–PCR), e quanto à expressão das respetivas proteínas através de
Western blot. As HDAC1 e HDAC2 foram, então, selecionadas para serem silenciadas pelo método de transfeção transiente na linha celular de BICa 5637, sendo posteriormente efetuados ensaios fenotípicos – viabilidade celular e apoptose – para determinar o seu efeito no fenótipo maligno.

**RESULTADOS E DISCUSSÃO:** Verificámos que os níveis de mRNA e proteína de todas as HDACs da classe I estão aumentados em tumores da bexiga, em comparação com tecido de mucosa normal, sugerindo que estas HDACs possuem uma função oncogénica. Foram encontradas associações significativas entre a expressão de **HDAC1 e HDAC3** e o grau e estádio do tumor, sendo os níveis de expressão destas HDACs mais baixos em tumores menos diferenciados e mais avançados. Estes resultados sugerem que estas alterações ocorrem em estádios precoces da via de carcinogénese vesical superficial (papilar). Adicionalmente, um painel de expressão de três genes, **HDAC1, HDAC3 e HDAC8**, permitiu discriminar, com elevada precisão, tecidos tumorais de tecidos normais, com uma sensibilidade de 97% e uma especificidade de 100%. Finalmente, ensaios fenotípicos revelaram uma diminuição significativa da viabilidade celular e um aumento da apoptose em células silenciadas para a HDAC1 e HDAC2. Tendo em conta a literatura disponível e os nossos resultados, podemos inferir que estas HDACs podem desempenhar funções similares, uma vez que foram observados efeitos semelhantes de inativação da HDAC1 ou HDAC2. Desta forma, os nossos dados apoiam um papel relevante da HDAC1 e HDAC2 na regulação da proliferação e apoptose em células de BICa.

**CONCLUSÃO:** Neste estudo, foi demonstrada uma expressão aberrante da classe I das HDACs em tecidos de BICa, bem como as propriedades oncogénicas da HDAC1 e HDAC2 numa linha celular de BICa. Estes resultados sugerem que a desregulação da classe I das HDACs, particularmente da HDAC1 e HDAC2 desempenha um papel importante no desenvolvimento de BICa através da regulação epigenética de genes envolvidos na proliferação e apoptose. Igualmente, verificámos, pela primeira vez, que a análise da expressão de três modificadores das histonas poderá ser utilizada clinicamente como biomarcador tumoral de BICa.
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INTRODUCTION
**EPIGENETICS**

*Epigenetic mechanisms and their deregulation in cancer*

The term ‘epigenetics’ was firstly used by C.H. Waddington in 1939 to explain why genetic variations sometimes did not lead to phenotypic variations and how genes might interact with their environment to yield a phenotype [1]. Nowadays, the term refers to variability in gene expression, heritable through mitosis and potentially meiosis, without any underlying modification in the actual genetic sequence [2]. This area of science was found to play a major role in physiologic phenomena such as embryogenesis, imprinting, and X chromosome inactivation, and in disease states such as cancer. During the last decade, it became clear that cancer is defined by a variety of epigenetic alterations, which occur in early stages of disease, and parallel genetic mutations [3]. These alterations are ubiquitous and have similar effects to the ones originated by genetic modifications, such as activation of oncogenes and silencing of tumor suppressor genes [4]. Currently, three main epigenetic mechanisms are recognized: DNA methylation, chromatin remodeling, and microRNA regulation.

**DNA methylation**

DNA methylation is probably the most widely studied epigenetic mechanism and plays an important role in gene regulation, since it provides a stable gene silencing [5]. The mechanism of methylation of DNA is well established. The chemical reaction is catalyzed by DNA methyltransferases (DNMTs) and involves the use of S-adenosyl-methionine (SAM) as the methyl donor, to give rise to 5-methylcytosine (mC) [6,7]. In mammals, this covalent modification of DNA occurs almost exclusively at cytosines followed by a guanine nucleotide (CpG dinucleotides), which might be clustered into concentrated DNA stretches called ‘CpG islands’, which occupy around 60% of human gene promoters or are located in regions of large repetitive sequences [8,9]. Unlike the majority of CpG islands across the genome that are methylated, most of the promoters CpG sites remain unmethylated during development and in differentiated tissues [10], except for some imprinted genes, genes on the silenced copy of X chromosome in females, and tissue-specific genes [11,12].

DNA methylation of promoter regions is closely related with gene transcription. This may occur directly through the obstruction of transcriptional activators on methylated regions within or near the promoter, and/or indirectly,
through the recruitment of methylcytosine–binding proteins (MBPs). MBPs recruit large protein complexes which include DNMTs and HDACs, changing chromatin conformation into an inactive configuration, thus repressing gene transcription [11,13–15].

The most common and well studied cancer–related epigenetic alteration is aberrant DNA methylation, which probably initiates tumorigenesis events [5]. The alterations in DNA methylation can include both global and gene–specific hypomethylation and/or gene–specific CpG island promoter hypermethylation. The function of the affected genes and type of epigenetic alteration will predict the outcome of the deregulation. On one hand, promoter hypomethylation may cause oncogenes activation and genetic instability, whilst hypermethylation induces silencing of cancer–related genes with tumor-suppressive properties. On the other hand, instability in genomic repetitive sequences can be caused by a global decrease of genome–wide methylation, particularly at pericentromeric regions, increasing the probability of abnormal DNA recombination, translocations, deletions and chromosomal rearrangements [16,17].

**Histone modifications and chromatin remodeling**

Histone modifications influence chromatin structure, which plays a key role in gene regulation and therefore in carcinogenesis. Chromatin is a highly ordered structure consisting of repeats of nucleosomes, which are composed by an octamer of histones (two H2A–H2B dimers and an H3–H4 tetramer), around which 147bp of DNA are wrapped [18,19]. Histones provide physical support to DNA and regulate several cellular mechanisms such as transcription, DNA repair and DNA replication. Histones are highly evolutionary conserved basic proteins, consisting of a globular domain with protruding flexible “tails” through the DNA that enable post–translation biochemical modifications (acetylation, methylation, glycosylation, sumoylation, phosphorylation and ubiquitylation) [20,21]. Depending on which residue is modified and the type and number of modifications, histone alterations produce various structural and translational changes that may have impact in gene activation or repression. From these, acetylation and methylation are the most studied, and the best characterized. Acetylation decreases the affinity between DNA and histones, giving rise to an “open” chromatin conformation, which enables gene transcription. Methylation, on the other hand, may be either associated with transcription activation or repression, according to the specific amino acid residue that is modified [an
activation of the transcription is associated with methylation of lysines 4, 36, and 79 of histone 3 (H3K4me, H3K36me and H3K79me), whereas methylation of lysines 9 and 27 of histone 3 (H3K9me and H3K27me) and of lysine 20 of histone 4 (H4K20me) is associated with silent heterochromatin regions. Histone acetyltransferases (HATs), deacetylases (HDACs), methyltransferases (HMTs) and demethylases (HDMTs) are responsible for the modification of histones and present different substrate specificity [18,20,21].

Genome-wide histone modification maps have been described in malignant cells. Nevertheless, the current knowledge concerning the involvement of histone modifications in tumorigenesis is less clear than DNA methylation modifications. These two different epigenetic phenomena are thought to be inter-dependent [5,22]. Thus, the overall reduction of H4K16ac and H4K20me3 has been associated with hypomethylation of DNA repetitive sequences, whereas enrichment in H3K9me and H3K27me, as well as loss of H3Ac and of H3K4me have been associated with promoter regions of methylation-silenced genes [23]. Accordingly, several expression changes in some histone modulating enzymes (HATs, HDACs, HMTs, HDMTs) have been associated with cancer development and progression [5,19,20,22].

**microRNAs**

MicroRNAs (miRNAs) constitute a class of small (around 22 nucleotides), non-coding RNAs, which are synthesized and processed in the nucleus, shuttled to the cytoplasm, where they complete their maturation process, and are incorporated in the RNA-induced silencing complex (RISC) [24,25]. A large proportion of human gene transcripts are known to be regulated by miRNAs and these display temporal and tissue-specific expression patterns. Interestingly, it has been suggested that miRNAs are not specific, once the same miRNA may regulate multiple mRNAs and the same mRNA may be targeted by multiple miRNAs [26].

In cancer, miRNA expression is frequently altered, acting either as tumor suppressors (via downregulation) or oncogenes (when overexpressed), depending on their specific target genes [25,26]. The altered expression of miRNA may be due to gene amplification, deletion, mutation, chromosomal abnormalities, changes in expression of transcription factors and also due to epigenetic mechanisms [24,27]. Interestingly, on one hand miRNAs are involved in chromatin structure regulation, by post-transcriptional regulation of key
chromatin-modifying enzymes, and on the other hand miRNAs’ coding genes are also regulated by epigenetic events, through DNA methylation or chromatin modifications. Therefore, the deregulation of this crosstalk between the major epigenetic mechanisms might contribute for neoplastic transformation [24,25].

**Histone deacetylases and cancer**

Protein acetylation is one of the most extensively characterized and critical post-translational modifications by which crucial steps of cell functions are regulated at a molecular level [28]. Acetylation of histones N-terminal domains is a dynamic process controlled by the antagonistic actions of two large families of enzymes: the HATs and the HDACs, which maintain the equilibrium of acetyl groups added or removed from lysine residues, respectively [19,29]. HATs are considered as transcription co-activators since they induce the relaxation of chromatin structure, whereas HDACs promote chromatin compaction, and act as co-repressors. Therefore, the final balance between the interplay of these enzymes will determine gene expression regulation in several developmental processes and disease states [30].

Indeed, HDACs play a crucial role in the regulation of several physiological and pathological cellular functions, including developmental processes and tumorigenesis [31]. Although HDACs were initially identified as involved in transcriptional regulation by targeting histones, it is known that they also target non–histone proteins (e.g. transcription factors or cytoskeletal proteins) (*Figure 1*) [32]. One such example is the regulation of several cellular processes, such as cell cycle regulation, DNA repair, apoptosis, proliferation, and differentiation by non–histone targets of HDACs [33].

**Figure 1** – HDACs activity, showing that they could deacetylate both histones and non-histone proteins. (Adapted from [28]).
HDAC inhibitors have been the first approach to study the function of HDACs. The studies focused on their effect on tumor cell proliferation, particularly in the induction of cell cycle arrest and apoptosis. Although very elucidative about the general role of HDACs in the regulation of the cell cycle, the specific role of each individual member of the HDAC family has only recently been addressed genetically. The action of HDACs involves a very complex network of several cellular processes, as shown by recent studies based on deletion and/or overexpression of specific HDACs [34].

To date, 18 HDACs have been identified in humans, which based on sequence similarity and cofactor dependency are divided in two families (Figure 2). The first family has 11 members and includes class I, IIa, IIb and IV HDACs, whose enzymatic activity requires Zn\(^{2+}\). Class I contains HDAC1, 2, 3 and 8, class IIa comprises HDAC4, 5, 7 and 9, class IIb includes HDAC6 and 10, and class IV comprises the unique member HDAC11. The second family of HDACs is composed by seven members, belonging to the class III HDACs or sirtuins (SIRT1–7), which are NAD\(^{+}\) dependent.

![Figure 2](image-url) - Schematic representation of different human HDACs, showing protein domains and cellular distribution. Blue rectangle indicates the conserved HDAC catalytic domains. N, nucleus; C, cytoplasm; Mit, mitochondria. (Adapted from [28]).
Class I HDACs, both from a functional and translational point of view, are a well characterized class of deacetylases, being abundantly expressed in tumor tissues. Concerning class II isoforms, only few information is available on the function and expression. Likewise, very little is known with respect to III HDACs (the sirtuins) and HDAC11 (which has been suggested to constitute a HDAC class on its own) [31].

Class I HDACs, homologous to yeast Rpd3 gene, are nuclear enzymes ubiquitously expressed in mammals. Biochemical purification studies have revealed that, except for HDAC8, they all function as catalytic subunits of several multiprotein complexes. These complexes bind to transcription factors and chromatin in the nucleus regulating transcriptional events, apart from coordinating epigenetic programs. Some developmental processes and tumorigenesis were shown to be regulated by these HDACs [28].

Regarding class II (homologous to yeast Hda1 gene) and class IV HDACs, they are tissue specific and display nuclear or cytoplasmic localization. These HDACs form protein complexes with 14–3–3 proteins, kinases and phosphatases, regulating their subcellular trafficking. In vivo and in vitro studies have revealed that class II HDACs play important developmental roles and HDAC6 is also implicated in neurodegenerative disorders [35,36].

Concerning class III HDACs or sirtuins, homologous to yeast Sir2 gene, these are unrelated to the other classes and localized in the nucleus (SIRT1, 6, 7), cytoplasm (SIRT2) or mitochondria (SIRT3, 4, 5). They are implicated in many biological processes including genome stability and cell cycle regulation, playing critical roles in several metabolic pathways [37]. Some studies have also revealed an important role for these deacetylases in cancer [38].

Class I HDACs

Globally, class I HDACs have been implicated in the repression of transcription. However, the individual deletion of each of these enzymes leads to deregulation of a limited set of genes, indicating a specific role of each member of class I HDACs in the regulation of transcription [39,40].

HDACs have no DNA-binding motif and, therefore, need to interact with other proteins in order to be recruited to their chromatin targets. Except for HDAC8, class I HDACs are found as subunits of several multisubunit complexes and interact with several transcription factors. HDAC1 and HDAC2 present a high sequence homology and have been originated from a common ancestor via gene
duplication. They share 86% amino acid sequence in mice and humans and their C-terminal tail contains tandem casein kinase–2 (CK2) phosphorylation sites [41,42]. HDAC3 shares the same homologous catalytic domain as HDAC1 and HDAC2, but only one CK2 phosphorylation site [43] and HDAC8 has a conserved motif for protein kinase A phosphorylation [44]. HDAC1 and HDAC2 are both found in multiprotein corepressor complexes Sin3, NuRD, and CoREST, which are recruited to chromatin regulatory region by transcription factors and have very diverse, often cell-specific, roles, while HDAC3 is a subunit of the N–CoR/SMRT complex (Figure 3) [34]. At specific developmental stages, class I HDAC-containing complexes may have different subunits in different cell types [45].

Figure 3 – Class I HDACs complexes and their components. (Adapted from [34]).

HDAC1 and HDAC2 form homo- and heterodimers between each other, which presumably allows them to act together or separately. Furthermore, these multiprotein complexes HDAC1–HDAC2 heterodimer versus an HDAC1 or HDAC2 homodimer may have different properties and substrate preferences [46]. These HDACs are commonly co-expressed and are functionally redundant in several adult tissues and cultured cell lines [47,48]. Nonetheless, several studies have reported specific functions for these two homologues during development. Importantly, in several cellular systems, ablation of HDAC1 leads to an increased level of HDAC2 protein and vice versa, although no alteration is observed at the mRNA level. These effects suggest that HDAC1 and HDAC2 proteins most probably share the same regulatory pathways [49].

Class I HDACs were found to be important in the regulation of differentiation, proliferation, cell cycle progression and apoptosis. It has been
demonstrated that HDAC1 and HDAC2 directly control the expression of genes involved in cell cycle regulation such as p21. Additionally, HDAC1, HDAC2, and HDAC3 have been found to play a role in DNA damage response, DNA replication and cell cycle progression [50,51].

Class I HDACs and cancer

Currently, little information is available concerning the role of this class of deacetylases in cancer. However, overexpression of specific HDACs have been observed in various types of cancer and have been correlated with a poor prognosis. Indeed, HDAC1 is overexpressed in gastric, pancreatic, colorectal, prostate, non–small cell lung and hepatocellular cancers, and has been correlated with a poor prognosis [52–57]. Regarding HDAC2, it has been reported to be mutated in colon cancer [58] and overexpressed in several tumors, namely, esophageal, prostate, gastric, colorectal and oral cancers [55,56,59–61]. Similarly, HDAC3 expression has been linked with a worse prognosis in gastric, non–small cell lung, prostate, colorectal cancers and chronic lymphocytic leukemia [55,56,61–63]. HDAC8 is overexpressed in neuroblastoma and also correlates with a poor outcome [64].

Concerning class I HDACs role in cancer cell lines, they all seem to have an oncogenic function. Indeed, it has been observed that HDAC1 knockdown in colon, breast, and in osteosarcoma causes cell cycle arrest, growth inhibition and apoptosis [56,65], and in neuroblastoma sensitizes cells for chemotherapy [66]. Moreover, HDAC1 knockdown in cervical cancer cells resulted in inhibition of proliferation and induction of autophagy [67,68]. Similarly, HDAC2 knockdown in cervical cancer cells results in increased differentiation and apoptosis [69], as well as in breast cancer cells, through an increased p53 activity [70], and in colon cancer cells and in neuroblastoma cells, inducing growth arrest and apoptosis, respectively [64]. Additionally, HDAC3 knockdown in hematological malignancies induces the expression of differentiation genes and disrupts cell cycle [71,72]. Likewise, HDAC8 knockdown in neuroblastoma cells induces differentiation, cell cycle arrest and inhibits clonogenic growth [64] and in lung, colon and cervical cancer cells reduces proliferation [44].
**BLADDER CANCER**

**Anatomical and Histological Features of the Bladder**

The urinary bladder, a hollow viscus with strong muscular walls, is characterized by its capability of expansion. It is a temporary reservoir for urine and varies in size, shape, position, and relationships according to its content and the state of neighboring viscera [73]. When empty, it is somewhat tetrahedral and has a base (fundus), neck, apex, a superior and two inferolateral surfaces (*Figure 4*) and lies entirely in the lesser pelvis. As it distends, it expands anterosuperiorly into the abdominal cavity [74].

![Bladder Diagram](image)

*Figure 4 – Urinary bladder of an adult demonstrating the pelvic location and the surfaces of the bladder. (Adapted from [73]).*

The normal bladder epithelium, named urothelium, consists of transitional cell lining in up to seven cell layers thick. Deep into the urothelium is the subepithelial connective tissue (lamina propria or submucosa), which contains irregularly arranged fibroblasts and collagen, as well as some loosely arranged muscle fibers (*muscularis mucosa*). The muscularis propria (detrusor muscle) is adjacent to the lamina propria and is surrounded by perivesical fat [75].

The most superficial cells of the transitional epithelium are responsible for the osmotic barrier between urine and tissue fluids. In these cells, the plasma membrane in contact with urine is specialized, with thick plates separated by strips of a thinner membrane. When the bladder is empty, transitional cells are all bunched together and when the bladder is full, they are stretched out into a single layer [76].
Epidemiology of Bladder Cancer

BICa is a worldwide health concern, being the 11th most common cancer in both genders, accounting for 386,300 new cases and 150,200 deaths per year [77,78]. It is the second most common tumor of the genitourinary tract and the second leading cause of cancer death in patients with urinary tract malignancies [78,79]. In Europe, the estimated number of newly diagnosed cases in 2008 was 133,696, accounting for 4.2% of all cancers (excluding non–melanoma skin cancer) (Figure 5). In Portugal, BICa accounts for approximately 4.5% of all cancer types, with 1,935 estimated incident cases in 2008 (Figure 5) [77].

Europe:

Portugal:

Figure 5 – Incidence of different types of cancer in Europe and Portugal (number of newly diagnosed cases and proportion of each cancer comparing with all types of cancer in both genders). (Adapted from [77]).
Cancer incidence and death rates are quite variable among racial and ethnic groups. BiCa is more common in Caucasians than in Africans, Americans, Hispanics, and Asians [80]. The incidence of BiCa also varies between geographic regions, with peak incidences in the United States, Egypt, Tunisia, Iraq and Europe, whereas it is uncommon in Africa, South Asia and Central America (Figure 6). Indeed, there is a 14-fold variation in incidence internationally [78].

![Figure 6 - Estimated age-standardized BiCa incidence rate per 100,000 inhabitants, across the world. (Adapted from [77]).](image)

The trends in BiCa mortality rates are easier to interpret than trends in incidence rates because trends in mortality are less affected by differences in reporting of low-grade tumors [78]. In the United States, mortality rates have stabilized in males and decreased in females from 1997 through 2006 [81]. In several western European countries, mortality by BiCa has shown downward trends over the last 2 decades of approximately 16% in men and 12% in women, but is still increasing in some eastern European countries [82,83]. The existence of disparities in mortality rates by cancer site can be due to differences in cancer exposure to underlying risk factors, access to high-quality regular screening and timely diagnosis and treatment.

The actual prevalence of BiCa constitutes a major economic burden on global health care systems. Measured on the basis of cumulative per patient cost from diagnosis until death, BiCa is the most expensive cancer to treat, accounting for $3.7 billion of direct costs in the United States [84].
The incidence rates are strongly related with gender and age, being three times more frequent in males and in the 7th decade of life [85]. BICa stands as the 4th leading malignant condition among men, representing 7% of all cancer sites. However, among women, BICa is not considered in the ten leading cancer types (Figure 7). In the USA, it has been estimated that, for the current year, the probability of developing BICa would be 1 out of every 27 men and 1 out of 102 women [79].

<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostate</td>
<td>241,740</td>
<td>226,870</td>
</tr>
<tr>
<td>Lung &amp; bronchus</td>
<td>116,470</td>
<td>109,690</td>
</tr>
<tr>
<td>Colon &amp; rectum</td>
<td>73,420</td>
<td>70,040</td>
</tr>
<tr>
<td>Urinary bladder</td>
<td>55,600</td>
<td>47,130</td>
</tr>
<tr>
<td>Melanoma of the skin</td>
<td>44,250</td>
<td>43,210</td>
</tr>
<tr>
<td>Kidney &amp; renal pelvis</td>
<td>40,250</td>
<td>32,000</td>
</tr>
<tr>
<td>Non-Hodgkin lymphoma</td>
<td>38,160</td>
<td>31,970</td>
</tr>
<tr>
<td>Oral cavity &amp; pharynx</td>
<td>28,540</td>
<td>24,520</td>
</tr>
<tr>
<td>Leukemia</td>
<td>26,830</td>
<td>22,280</td>
</tr>
<tr>
<td>Pancreas</td>
<td>22,090</td>
<td>21,830</td>
</tr>
<tr>
<td>All Sites</td>
<td>848,170</td>
<td>799,740</td>
</tr>
</tbody>
</table>

Figure 7 - Estimated new cases of cancer in USA by gender, 2012. (Adapted from [79]).

**Risk Factors**

Although there are numerous known potential risk factors for BICa, the causes for this disease are not well understood. Indeed, several lifestyle choices, occupations, dietary factors, drugs, family history and genetic alterations have been associated with increased BICa risk. However, among these factors, only a few are clearly established, namely male gender, aging and heavy smoking habits [86].

**Gender, Age and Ethnicity**

Men are nearly 3–4 times more likely to develop BICa than women, but they are only twice as likely to die of BICa [87,88]. Several reports suggest that women are diagnosed at more advanced stages of the disease and, in general, have poorer survival than men. Furthermore, when compared to men, women can be under different exposure effects and might have different susceptibility to develop BICa [89]. In men, the increased incidence has been attributed to environmental and dietary factors, innate gender characteristics (e.g., anatomic differences), urination habits, and hormonal factors [90]. The risk of BICa
increases with advancing age, peaking between the age of 50 and 70 years, being more common in Caucasians [80,87].

_Tobacco smoke_

The most well-established risk factor for BICa is tobacco smoking, causing 50%–65% of BICa male cases and 20%–30% of female cases. It has been estimated that smoking is responsible for about 34% of BICa deaths in males and 13% in females [91]. The relative risk of BICa development in smokers is 2–4 times higher than in non-smokers, and it increases with increasing duration of smoking and the number of cigarettes smoked [92]. Cigarette smoking seems to have the same effect in both genders, and in different ethnic groups [93]. Former smokers generally have a lower risk of developing BICa than do current smokers, and the relative risk declines with increasing time after quitting [94]. Much of the risk associated with smoking likely results from aromatic amines and other carcinogens present in the cigarette smoke, that may form highly reactive species and DNA adducts [95]. Defects in genes that repair DNA damage might lead to further genetic deregulations and alterations in cellular homeostasis [86].

_Occupational and environmental exposure_

Occupational exposure is the second most important risk factor for BICa. Work related cases account for 20%–25% of all BICa cases in several series [89]. The incidence of BICa has increased with industrialization and the disease may develop many years after exposure. However, it is difficult to prove that exposure to such chemicals definitely caused BICa [86]. The first association between chemical exposure and BICa development was observed in 1985 by Rehn, who reported high rates of BICa among men employed in the aniline dye industry [96]. Subsequent research among dyestuffs workers identified the aromatic amines benzidine and 2-naphthylamine as bladder carcinogens [97]. These aniline dyes and aromatic amines form DNA adducts and reduce the DNA-repair capacity of the cell, thereby making cells more susceptible to DNA damage [98,99]. A number of other occupations have been associated with increased risk of BICa, including rubber manufacturing, painting, leather industry and asphalt paving [100].

The source of the drinking water may also be important. Several studies showed that use of drinking water containing chlorination by-products or contaminated by arsenic may increase the risk to develop BICa [101,102]. Arsenic
exposure has been associated with genetic alterations related to regulation of the cell-cycle and gene transcription [86].

**Drugs**

Heavy consumption of phenacetin–containing analgesics has been linked to an increased risk of BICa in a dose–dependent manner [103]. Phenacetin has been shown to cause single–strand DNA breaks, thereby exerting a genotoxic effect. The cytostatic agent cyclophosphamide is an alkylating agent used for the treatment of lymphoproliferative diseases and other nonneoplastic diseases that also increases the risk of BICa via the same carcinogenic mechanism as phenacetin [104].

**Chronic infections**

Chronic urinary tract infection had been related to BICa, particularly with invasive squamous cell carcinoma. In Egypt and parts of the Middle East, this type of carcinoma is the most common type of BICa. These are Schistosoma haematobium–endemic areas, and chronic infection with this parasite produces toxins, inflammation, and N–nitrosamines, resulting in DNA damage [105]. Other urinary tract infections and urinary tract stones may cause chronic irritation of the bladder epithelium, and may thus increase BICa risk [106].

**Histopathological classification**

Among bladder tumors, urothelial (transitional cell) carcinoma is the most frequent histological subtype, whereas about only 10% of the bladder tumors are non–urothelial (e.g., squamous cell carcinomas or adenocarcinomas) [107]. Generally, urothelial carcinomas are divided in two major groups, the noninvasive tumors (mainly with a papillary architecture and a distinct set of molecular alterations), which are more prevalent and less aggressive, and the invasive tumors, that although less common are substantially more aggressive and therefore associated with higher mortality rates [86]. Although noninvasive tumors have a relatively low risk for progression compared with invasive disease, this risk is dependent on the grade of the lesion [108].

- Urothelial carcinoma
  - Papillary carcinoma

Currently, the noninvasive papillary carcinomas are categorized in two groups – low–grade and high–grade tumors – with different risk of recurrence
and progression to invasive disease. In low-grade papillary carcinoma, there is an orderly appearance of the cells within the epithelium, with relatively mild changes in cytology and architecture. Changes in polarity and nuclear size, shape and chromatin texture make up a small but distinct atypia [106]. The recurrence rate is high, but only few patients progress to invasive disease (10%), with very low mortality due to BICa (5%). Besides showing a high recurrence rate, high grade papillary carcinoma also presents a much higher progression (15%-40%) and mortality risk (20%) [89]. The appearance of these tumors is mostly disordered. Cells show irregularity by lots of clustering and disorganization of the epithelium. Clumped chromatin, prominent nucleoli and atypical forms of mitosis are observed [109].

✓ Carcinoma in situ (CIS)

CIS accounts for less than 1%-3% of urothelial carcinomas and can be found in 45%-65% of invasive urothelial tumors and in 5%-19% of noninvasive urothelial tumors [110]. It is a flat lesion of the urothelium, characterized by architectural disorder, presence of cells with large and irregular nuclei with frequent mitotic activity. This lesion is usually high grade and is accepted as a direct precursor of invasive urothelial carcinoma [109,111].

✓ Invasive carcinoma

Invasive (or infiltrating) urothelial carcinoma is defined as an urothelial tumor that invades beyond the basement membrane. In lamina propria invasion, urothelium nests, clusters, or single cells can be observed. At the border of invasive cells, eosinophilic cytoplasm may be found. Although it is rather difficult to evaluate the depth of invasion, it is either focal or extensive [106,111].

Clinical and pathological staging

Stage is the most important factor in predicting prognosis of BICa and comprises information about the extension of the disease (local, regional and systemic). The most widely used staging system is the International Union Against Cancer (UICC)/American Joint Committee on Cancer (AJCC) tumor–node–metastases (TNM) staging system (Table 1) [89,108]. In this classification, T represents the extent of the primary tumor (Figure 8), N refers to the lymph node status and M to distant metastasis. The basic principle is that depth of bladder wall invasion inversely correlates with survival.
Table 1 – Overview of the UICC TNM/pTNM staging system for BlCa. (Adapted from [108]).

<table>
<thead>
<tr>
<th>Primary tumor (T)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TX</td>
<td>Primary tumor cannot be assessed</td>
</tr>
<tr>
<td>T0</td>
<td>No evidence of primary tumor</td>
</tr>
<tr>
<td>TA</td>
<td>Noninvasive papillary carcinoma</td>
</tr>
<tr>
<td>Tis</td>
<td>Carcinoma in situ: “flat tumor”</td>
</tr>
<tr>
<td>T1</td>
<td>Tumor invades subepithelial connective tissue</td>
</tr>
<tr>
<td>T2</td>
<td>Tumor invades muscularis propria</td>
</tr>
<tr>
<td>pT2a</td>
<td>Tumor invades superficial muscularis propria (inner half)</td>
</tr>
<tr>
<td>pT2b</td>
<td>Tumor invades deep muscularis propria (outer half)</td>
</tr>
<tr>
<td>T3</td>
<td>Tumor invades perivesical tissue</td>
</tr>
<tr>
<td>pT3a</td>
<td>Microscopically</td>
</tr>
<tr>
<td>pT3b</td>
<td>Macroscopically (extravesical mass)</td>
</tr>
<tr>
<td>T4</td>
<td>Tumor invades any of the following: prostatic stroma, seminal vesicles, uterus, vagina, pelvic wall, abdominal wall</td>
</tr>
<tr>
<td>T4a</td>
<td>Tumor invades prostatic stroma, uterus, vagina</td>
</tr>
<tr>
<td>T4B</td>
<td>Tumor invades pelvic wall, abdominal wall</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Regional Lymph Nodes (N)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>NX</td>
<td>Lymph nodes cannot be assessed</td>
</tr>
<tr>
<td>N0</td>
<td>No lymph node metastasis</td>
</tr>
<tr>
<td>N1</td>
<td>Single regional lymph node metastasis in the true pelvis (hypogastric, obturator, external iliac, or presacral lymph node)</td>
</tr>
<tr>
<td>N2</td>
<td>Multiple regional lymph node metastasis in the true pelvis (hypogastric, obturator, external iliac, or presacral lymph node metastasis)</td>
</tr>
<tr>
<td>N3</td>
<td>Lymph node metastasis to the common iliac lymph nodes</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Distant Metastasis (M)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>M0</td>
<td>No distant metastasis</td>
</tr>
<tr>
<td>M1</td>
<td>Distant metastasis</td>
</tr>
</tbody>
</table>

The staging of the tumor is at first given by clinical assessment. Clinical staging (cTNM) includes physical examination (biopsy or transurethral resection), appropriate imaging techniques for extravesical extension of the primary tumor and lymph node evaluation. When indicated, evaluation for distant metastasis by imaging of the chest, biochemical and isotopic studies to detect common metastatic sites could also be performed. However, an adequate and more complete staging is given by pathologic staging (pTNM), where microscopic examination and confirmation of extent are required, whether in biopsy material or surgical specimens of cystectomy and lymph node dissection [108].
Figure 8 - Extent of primary BlCa. (Adapted from [108]).

**Histopathological grading**

Histopathologic examination is required to provide a more accurate classification of the tumor. Tumor grade is an important prognostic indicator for disease recurrence and progression, and refers to how much a tumor resembles the tissue of origin. Hence, the current World Health Organization/International Society of Urologic Pathology (WHO/ISUP) recommended a grading system in which lesions are classified in low or high-grade [108]. According to this classification, urothelial bladder carcinoma can be classified as [112]:

- Intraurothelial neoplasms
  - Dysplasia – low grade
  - Carcinoma in situ (CIS) – high grade
- Papillary neoplasms
  - Papillary neoplasm of low malignant potential
  - Papillary carcinoma, low-grade
  - Papillary carcinoma, high grade
- Invasive neoplasms
  - Lamina propria invasion
  - *Muscularis* (detrusor muscle) invasion
**Bladder cancer development**

Clinical, pathological and molecular evidences suggest that urothelial carcinoma arises and progresses along two distinct pathways, corresponding to two distinct biological and clinical phenotypes: non–muscle–invasive (superficial) and muscle–invasive urothelial carcinoma (*Figure 9*) [113,114]. These two phenotypes arise from different mutations that affect several cell pathways [115]. The muscle–invasive phenotype is a high–risk lesion that carries mutations in tumor suppressor genes such as *RB* [116] and *TP53* [117], and that initially gives origin to a high–grade intraurothelial neoplasia, eventually evolving to a metastatic tumor. The non–muscle–invasive phenotype is a low–grade tumor, which develops from hyperplastic urothelium that consists in a thick urothelium with absent cytologic atypia. Its potential to evolve to neoplasia is rather low. Dysplasia consists of early morphologic manifestations of the alterations that occur between normal urothelium and *in situ* carcinoma [118] and carries a higher malignant potential [113]. Low–grade tumors development associates with oncogene mutations, such as in *HRAS* [119], *FGFR3* [120] and *PIK3CA* [121]. It is possible to observe a loss of heterozygosity of the chromosome 9 in all stages and grades of BICa, and this alteration has been suggested to be a primary genetic event in the development of urothelial carcinoma [122].

![Figure 9 - The divergent molecular pathways of oncogenesis in non–muscle–invasive and muscle–invasive BICa. Abbreviations: CIS, carcinoma in situ; LOH, loss of heterozygosity. (Adapted from [113]).](image-url)
**EPIGENETIC ALTERATIONS IN BLADDER CANCER**

DNA methylation, histone modification and miRNA expression are important epigenetic mechanisms that regulate gene expression. These regulatory mechanisms are altered in BICa, and therefore represent potential biomarkers and therapeutic targets, owing to the reversible nature of their modification.

Numerous tumor suppressor genes involved in bladder carcinogenesis contain CpG islands in their promoters, positioning them as good targets for epigenetic inactivation. Indeed, more than 50 genes undergo aberrant hypermethylation in BICa. These genes are involved in a number of cellular pathways, such as cell-cycle control, cell invasion and cell architecture, DNA damage repair, and cell differentiation [123–125]. Alterations in the expression of these genes contribute to cancer initiation, progression, invasion and metastization. There is a close association between methylation and BICa phenotype and the referred roles are also supported by specific functional in vitro assays [125].

Histone modifications in BICa have been poorly evaluated. H3K27 trimethylation was shown to be associated with gene silencing, acting independently of promoter methylation [126]. EZH2, a frequently overexpressed oncogene in BICa is responsible for this trimethylation [127], leading to the transcriptional silencing of E-cadherin, a tumor suppressor gene [128]. The main focus of research around histone modifications in BICa has been the therapeutic potential of agents to reverse malignant alterations. Of these, histone deacetylase inhibitors (HDACi) are the most evaluated.

Another characteristic of bladder carcinomas is the differential expression of microRNAs. The first study reporting this event was conducted by Gottardo et al. [129] and has shown the upregulation of many miRNAs (such as miR–223, miR–26b, miR–221, miR–103–101, miR–185, miR–23b, miR–203, miR–17–5p, miR–23a and miR–205) in BICa when compared with controls. A recent larger study has shown that there are alterations of miRNA expression in a phenotype–specific manner that can predict BICa disease progression. Moreover, Dyrskjot et al [130], have identified miR–129 upregulation as a poor prognostic factor within BICa, being associated with apoptosis.

As previously mentioned, clinical and molecular observations suggest that BICa is a disease with at least two distinct phenotypes that differ at genetic levels. At epigenetic level, high–grade and low–grade urothelial cell carcinomas (UCCs)
are also distinct. Whilst many gene mutations are mutually exclusive, low- and high-grade UCCs often share epigenetic alterations. High-grade tumors have higher levels of aberrant methylation and upregulation of many miRNAs, when compared with low-grade cancers. In contrast, low-grade UCCs have low levels of aberrant hypermethylation and downregulation of many miRNAs [130–132].

Epigenetic based tumor biomarkers

Tumor development and prognosis of BICa have been closely associated with epigenetic alterations. Due to the fact that those alterations may be detected in specimens (tissue/body fluid), epigenetic markers are a valuable tool for the detection and assessment of prognosis of BICa when compared to conventional methods. Furthermore, epigenetic changes are very interesting from a clinical point of view since they are possible to revert, restoring gene function [133].

Several DNA methylation markers have been identified over the last decades in serum, bladder washes, urine samples, and cancer tissues using a wide variety of molecular biology techniques [123,134]. This identification became pivotal for early and non-invasive BICa detection. Although until the present date no single gene has been found to be methylated in the great majority of bladder tumors, several gene panels have been proposed for BICa detection. Accordingly, a novel panel of three genes – GDF15, TMEFF2, and VIM – has been recently identified using a genome-wide approach, and their combined analysis achieved 94% sensitivity with perfect specificity for BICa detection in urine samples. Importantly, this gene panel discriminates BICa from both healthy individuals and renal or prostate cancer patients [135].

When compared to methylation markers, the clinical value of other epigenetic alterations, such as histone post-translational modifications, chromatin machinery proteins and aberrantly expressed miRNAs for BICa detection and diagnosis seem rather scarce. Few research studies indicate that expression levels of histone modifiers EZH2 and LSD1 are significantly elevated in BICa tissue specimens, compared with normal tissues [136–138]. On the other hand, bladder tumors, even low grade lesions, might be detected in urine by a panel of miRNAs – miR–126 and miR–152 – with 72% sensitivity and 82% specificity [139].

Regarding tumor prognostic value, promoter hypermethylation of CpG islands was found to be strongly associated with tumor development, stage, recurrence, progression, and survival of BICa patients in several studies. In
addition, tumor grade can be predicted by methylation of TERT and EDNRB, whereas tumor stage can be associated with RASSF1A, ARF and APC [140,141]. Both tumor stage and grade were significantly associated with methylation levels of BCL2 [140] and PMF1 [142]. When focusing on disease recurrence and patient survival, DAPK methylation levels were significantly associated with higher tumor recurrence rate [143] while LAMC2 promoter methylation correlated with shorter survival [144] and OPCML promoter methylation was associated with worse disease-specific survival [145].

Recently, associations of miRNAs and histone modifications/modifiers with disease recurrence, pathological stage and poor prognosis are becoming emergent. Phosphorylation at serine 139 of histone H2AX (H2AXS139Ph) was found to be associated with lower tumor recurrence rate [146], whereas non-recurrent and recurrent low malignant potential papillary urothelial neoplasms exhibited distinct levels of H3K9 acetylation [147]. Concerning histone methylation marks, H3K4me1, H4K20me1, H4K20me2 and H4K20me3 were found to be correlated with advanced pathological stages [148]. Finally, increased expression of EZH2 has been suggested as a predictor of aggressive disease and of poor prognosis in BlCa patients [149,150]. Concerning miRNAs, a recent genomic profiling suggested that miR–129, miR–133b, and miR–518c* were associated with worse outcome [130], whereas increased levels of miR–452 and miR–452* were associated with poor prognosis [151].
AIMS OF THE STUDY
Class I HDACs has been reported as frequently overexpressed in several solid tumors, including gastric, pancreatic, colorectal and prostate. However, concerning BICa, no data is known thus far. Therefore, the main goal of this thesis was to assess the role of class I HDACs in bladder carcinogenesis.

Specifically, the aims of this study were to:

- Evaluate the expression levels of each member of class I HDACs in tumor tissues and normal bladder mucosas
- Correlate different expression levels of class I HDACs with BICa clinicopathological data
- Evaluate the usefulness of class I HDACs expression levels as BICa tumor biomarkers
- Determine the biological role of the altered class I HDACs in BICa cell lines malignant phenotype
METHODOLOGY
**Sample Collection**

One hundred and twenty seven bladder tumor (TB) samples were prospectively collected from patients with clinically localized disease. Those patients were diagnosed and treated with transurethral resection or radical cystectomy at the Portuguese Oncology Institute – Porto, Portugal, between 1992 and 2011. The twenty morphological normal bladder mucosa (NB) tissues, used as controls, were obtained from patients with prostate cancer submitted to radical prostatectomy.

After surgery, all tissue specimens were promptly frozen at –80°C and subsequently cut in a cryostat for nucleic acid extraction. The bulk material was routinely fixed in buffered formalin and paraffin–embedded. The corresponding hematoxylin–eosin stained sections were examined by a pathologist to determine tumor type, grade classification, and pathological stage according to the TNM staging system.

Relevant clinical data was collected from patient’s clinical records. Patients for this study were enrolled after informed consent. These studies were approved by the institutional review board (Comissão de Ética) of Portuguese Oncology Institute – Porto, Portugal.

**Quantitative expression analysis**

*RNA extraction and quantification*

Total RNA extraction was performed from the 127 TB and the 20 NB samples using TRIZol® Reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s protocol. Briefly, 1mL of TRIZol® Reagent was added to each tissue sample and this tissue was homogenized using a syringe. The homogenized samples were incubated for 5min at room temperature (RT) to allow the complete dissociation of nucleoprotein complexes. Then, 200μL of chloroform were added, the tubes were vortexed for 15sec and incubated for 3min at RT followed by a centrifugation for 15min at 10,600rpm at 4°C. After centrifugation, it was possible to distinguish a lower red, phenol–chloroform phase, an interphase and an upper colorless aqueous phase (RNA enriched). RNA was collected into a fresh RNase–free tube and placed on ice. After that, 500μL of isopropyl alcohol were added, the tubes were vigorously inverted by hand and placed at RT for 10min to precipitate RNA. Then, tubes were centrifuged for 10min at 10,600rpm at 4°C
and the supernatant was eliminated without disturbing the pellet. Finally, 1mL of 75% (v/v) ethanol was added to wash RNA pellets by vortexing and a centrifugation for 5min at 8,400rpm at 4°C was performed. The supernatant was carefully discarded and the RNA pellets were air-dried for 20 to 30min. RNA pellets were eluted in a variable volume of DEPC–treated water (deionized, nuclease–free water) (MP Biomedicals, LLC, OH, USA), according to pellet size and placed on ice for at least 30min before evaluation of RNA concentration and quality using a NanoDrop ND–1000 spectrophotometer (NanoDrop Technologies, USA). RNA was stored at −80°C until further use.

**cDNA synthesis**

For gene expression analysis, cDNA was synthesized by reverse transcription using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer’s instructions. The following components were added to a RNase–free PCR tube on ice: 1000ng of template RNA (thawed on ice), 1x RT random primers, 1x RT buffer, 1x dNTP mix (4mM), 1µL of multiscribe™ reverse transcriptase, 1µL of RNase inhibitor and DEPC–treated Water (deionized, nuclease–free water) (MP Biomedicals, LLC, OH, USA) to complete a total volume of 20µL. All the components were gently mixed and the mixture was incubated at 25°C for 10min, followed by 37°C for 120min and finally 85°C for 5min in a Veriti® Thermal Cycler (Applied Biosystems, Foster City, CA, USA). Tubes were chilled on ice and 260µL of DEPC–treated Water (MP Biomedicals, LLC, OH, USA) were used to dilute newly synthesized cDNA. All this procedure was also applied to Human Bladder Total RNA (Applied Biosystems, Foster City, CA, USA), that was used as a positive control for qRT–PCR assays described below. In this case, cDNA was diluted in 180µL of DEPC–treated Water (MP Biomedicals, LLC, OH, USA). All samples were stored at −20°C.

**Quantitative reverse–transcriptase PCR (qRT–PCR)**

Class I HDACs transcripts were quantified by real time quantitative PCR. The assays were performed using gene expression assays for *HDAC1, HDAC2, HDAC3, HDAC8* and the endogenous control assay *HPRT1*, as shown in Table 2. The *HPRT1* assay was used to normalize cDNA input. The expression assays were performed separately in 96–well plates in a 7000 Real–Time PCR System (Applied Biosystems, Foster City, CA, USA), according to the recommended protocol.
Table 2 – Gene expression assays.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Reference</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDAC1</td>
<td>Hs02621185_s1</td>
<td></td>
</tr>
<tr>
<td>HDAC2</td>
<td>Hs00231032_m1</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Foster City, CA, USA</td>
</tr>
<tr>
<td>HDAC3</td>
<td>Hs00187320_m1</td>
<td></td>
</tr>
<tr>
<td>HDAC8</td>
<td>Hs00218503_m1</td>
<td></td>
</tr>
<tr>
<td>HPRT1</td>
<td>Hs01003267_m1</td>
<td></td>
</tr>
</tbody>
</table>

Briefly, in each well 9μL of previously synthesized cDNA, 1μL of TaqMan® Gene Expression Assay and 10μL of TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) were added. PCR conditions were those predefined by the manufacturer: 50°C for 2min, 95°C for 1 min, 45 cycles at 95°C for 15sec and 60°C for 1min. All cDNA samples were run in triplicate. cDNA synthesized from Human Bladder Total RNA (Applied Biosystems, Foster City, CA, USA), was used to prepare five consecutive cDNA dilutions (dilution factor of 10x) that were used as standards on each plate, allowing the construction of a standard curve for relative quantification and PCR efficiency assessment. Furthermore, multiple water blanks were added to each plate as negative controls.

The results were analyzed using the Sequence Detector Software version 1.2.3 (Applied Biosystems, Foster City, CA, USA). A run was considered valid when the slope of the corresponding standard curve was above -3.60 (corresponding to a PCR efficiency > 90%) and the R² of at least three relevant points exceeded 0,98.

The mean quantity of HDAC1, HDAC2, HDAC3 and HDAC8 expression levels in each sample was normalized against the mean quantity of HPRT1 expression levels for the same sample. This ratio was then multiplied by 1000 for easier tabulation (HDAC Expression Level = (HDAC Mean Quantity / HPRT1 Mean Quantity) x 1000).

**Protein extraction and quantification**

Protein extraction was performed from 3 TB and 3 NB tissues randomly selected, to evaluate the protein levels of each member of class I HDACs, using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s
protocol. Briefly, the extraction was performed as described for RNA extraction until the phase separation. Protein and DNA remained in the interphase and organic phase. To these phases 300µL of 100% ethanol were added and the tubes were vigorously inverted by hand and placed at RT for 3min. Then, tubes were centrifuged for 5min at 4300rpm at 4°C to separate the DNA pellet from the phenol/ethanol supernatant. The supernatant was collected into a fresh tube and 1.5mL of isopropyl was added. The tubes were mixed by inversion and incubated at RT for 10min. Subsequently, to collect the protein pellet, tubes were centrifuged for 10min at 10600rpm at 4°C and the supernatant was discarded. The pellet was resuspended in 1.5mL 0.3M GuHCl in 95% (v/v) ethanol and placed at RT for 20min, followed by a centrifugation for 5min at 8400rpm at 4°C and the supernatant was discarded. This last step was repeated twice. Finally, protein pellet was washed in 2mL 100% ethanol, the tubes were vortexed for 15sec and incubated for 20min at RT followed by a centrifugation for 5min at 8400rpm at 4°C. Supernatant was discarded and the protein pellets were air-dried for 5–10min. To dissolve the pellet, 1% SDS (w/v) and/or 8M urea buffer were added and the tubes were incubated at 50°C. The tubes were centrifuged for 10min at 9700rpm at 4°C, to sediment insoluble material, and the supernatant was transferred into a new tube. All samples were analyzed in a Qubit® 2.0 Fluorometer (Applied Biosystems, Foster City, CA, USA) to assess the concentration of the protein extracted. Protein was stored at −20°C until further use.

**SDS–PAGE and Western Blot**

Protein expression of HDAC1, HDAC2, HDAC3 and HDAC8 was evaluated by Western Blot using specific antibodies. The electrophoretic separation of the proteins was performed on a polyacrylamide gel. The gel was prepared as previously described by Laemmli et al. [152]: 12.5% running gel [12.5% (w/v) acrylamide/bis–acrylamide, 0.375M Tris–HCl, pH=8.8, 0.1% (w/v) SDS, 0.1% (w/v) APS and 0.04% (v/v) TEMED] and 4% stacking gel [4% (w/v) acrylamide/bis–acrylamide, 0.062M Tris–HCl, pH=6.8, 0.1% (w/v) SDS, 0.1% (w/v) APS, 0.25% (v/v) TEMED]. Then, 20µg of protein were resuspended in loading buffer and denatured at 95°C for 5min. The proteins were applied in the running gel for separation and electrophoresis was run in a drive Mini–PROTEAN 3 Electrophoresis System (Bio–Rad, Hercules, CA, USA), in running buffer [0.025M Tris, 0.192M glycine and 0.1% (w/v) SDS, pH=8.3] at 120V for 1h.
Proteins were then transferred to Protran nitrocellulose transfer membranes (Whatman, Dassel, Germany). The system was mounted on a transfer unit Mini–PROTEAN 3 Electrophoresis System (Bio–Rad, Hercules, CA, USA), according to the manufacturer's instructions and the transfer occurred at 50V at 4°C for 1h in transfer buffer [0.025M Tris, 0.192M glycine, 20% (v/v) Methanol]. After electroblotting, the membranes were incubated in blocking solution (5% (w/v) nonfat dry milk in 0.01M Tris-buffered saline containing 0.1% (v/v) Tween–TBS) for 1h at RT with gentle shaking. The blocking solution was then replaced by the antibody solution (diluted in blocking solution) and the membrane was incubated overnight (ON) at 4°C with gentle shaking. We used an antibody specific for HDAC1 (1:1000, Sigma Aldrich), HDAC2 (1:6000, Abcam), HDAC3 (1:6000, Abcam) and HDAC8 (1:1000, Abcam). The membrane was washed with TBST for 10min with gentle agitation, followed by two washes of 5min each. After the washing steps, the membranes were incubated for 1h at RT with a horseradish peroxidase conjugated secondary anti–mouse antibody (Bio–Rad, Hercules, CA, USA) for HDAC1, HDAC2 and HDAC8, and an anti–rabbit (Bio–Rad, Hercules, CA, USA) for HDAC3, both diluted 1:3000 in blocking solution. Then, the membranes were washed with TBST as previously described. The membranes were developed using Immun–Star WesternC Chemiluminescent kit (Bio–Rad, Hercules, CA, USA) and exposed to Amersham Hyperfilm (GE Healthcare). To ascertain equal loading of protein, the membranes were stripped and reprobed with a monoclonal mouse antibody against β–Actin (Sigma–Aldrich®, Germany), diluted 1:8000 in blocking buffer.

Methylation analysis

In Silico Search for CpG Island in Genes Promoters

In order to understand the presence of several downregulated cases for HDAC2 and HDAC8 genes, we performed methylation analysis to check if there was a deregulation of their methylation pattern that could justify the presence of these outliers. In order to do so, we analyzed the promoter region of these genes for the presence of CpG islands. To accomplish that, RefSeqs of the candidate genes were obtained from the UCSC Genome Browser Database\(^1\), including the 2000-bp sequence upstream of the first exon. Then, they were analyzed in silico using CpG Island Searcher software\(^2\). The criteria used to define a CpG island were those set as default in CpG Island Searcher (this algorithm has been described by Takai and Jones [153]): a minimum stretch of 500bp with at least 55% CG content and a ratio of observed/expected CpG of at least 0.65. To consider two different adjacent islands they had to be, at least, 100bp apart.

\(^1\) - http://genome.ucsc.edu/
\(^2\) - http://cpgislands.usc.edu/

DNA extraction

DNA from some clinical samples was extracted by the phenol–chloroform conventional method, as described by Pearson et al. [154]. Tissue digestion was performed by adding 2,700μL of SE solution (75mM NaCl; 25mM EDTA), 300μL of SDS (Sodium Dodecyl Sulfate) 10% and 25μL of proteinase K (20mg/mL) (Sigma–Aldrich\(^\circledast\), Germany) to each tube. The tubes were incubated for 2 to 3 days in a bath at 55°C until total digestion. Proteinase K was added twice a day during this period. After digestion, DNA extraction was performed with 3mL of phenol–chloroform solution at pH 8 (Sigma–Aldrich\(^\circledast\), Germany; Merck, Germany) in Phase Lock Gel Light tubes (5 PRIME, Germany). After centrifuging the tubes for 20min at 4,000rpm, the upper aqueous phase containing DNA was transferred to a new tube. Then, 2 volumes (of original amount of this phase) of absolute ethanol and 1/3 volume of 7.5M ammonium acetate (Sigma–Aldrich\(^\circledast\), Germany) were added to each sample. Tubes were placed ON at -20°C to precipitate DNA, subsequently centrifuged at 4,000rpm for 20min and washed twice with 70% (v/v) ethanol. Pellets were air dried and eluted in sterile distilled water (B.Braun, Melsungen, Germany). DNA concentration and quality were evaluated using a NanoDrop ND–
1000 spectrophotometer (NanoDrop Technologies, USA). Eluted samples were stored at −20°C.

**Sodium bisulfite treatment of DNA**

After its extraction, DNA was submitted to a chemical reaction leading to the conversion of unmethylated cytosines to uracil, whilst methylated cytosines remain as cytosines. Sodium bisulfite modification was performed using the EZ DNA Methylation–Gold™ Kit [Zymo Research, Orange, CA, USA], according to the manufacturer’s instructions. In this protocol, 1µg of DNA (in a total volume of 20µL) obtained from the DNA extraction procedure was submitted to the modification process. Summarizing, 130µL of CT Conversion Reagent were added to DNA. This mix was then incubated in Veriti® Thermal Cycler (Applied Biosystems, Foster City, CA, USA) during 10min at 98°C, followed by 180min at 64°C for DNA denaturation and sodium bisulfite conversion. After the incubation period, DNA was recovered in a Zymo–Spin™ IC Column using 600µL of M–Binding Buffer and centrifuged for 30sec at 10,000rpm. The column was washed with 100µL of M–Wash Buffer and centrifuged once again in the same conditions as in the previous step. The M–Wash Buffer was discarded, and 200µL of M–Desulphonation Buffer were added for a 20min incubation’s period at RT. After discarding the M–Desulphonation Buffer, two more washes were performed with M–Wash Buffer. Finally, the column was placed into a new tube and DNA was eluted by incubation with 30µL of sterile bidistilled water (B.Braun, Melsungen, Germany) for 5min at RT, followed by a centrifugation at 12,000rpm for 30sec. This last step was performed again to obtain a total volume of 60µL. CpGenome™ Universal Methylated DNA (Millipore, CA, USA) and CpGenome™ Universal Unmethylated DNA (Millipore, CA, USA) were also modified and eluted in a final volume of 30µL to be used as controls. Finally, bisulfite modified DNA was stored at −80°C until further use.

**Methylation–Specific PCR (MSP)**

Methylation analysis was performed using the methylation–specific PCR (MSP) method, to test the methylation status of **HDAC2** and **HDAC8** genes in some cases, along with a positive control (fully methylated DNA) and a negative control (fully unmethylated DNA). MSP primers were designed to distinguish methylated from unmethylated sequences of modified DNA. For this purpose, cytosine–rich regions were selected for primer design (all primers must also include non–CpG
cytosines to amplify only modified DNA) [15], using Methyl Primer Express Software v1.0 (Applied Biosystems, Foster City, CA, USA). Beside having non-CpG cytosines, all primers included 2–4 CpG sites in order to discriminate methylated sequences. Preferentially, some of these CpG dinucleotides were near the 3’ end, to increase primer specificity. The sequences of each pair of designed primers are presented in Table 3.

Table 3 – Sequence of forward and reverse primers for HDAC2 and HDAC8 genes.

<table>
<thead>
<tr>
<th></th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDAC2</td>
<td>5’-CGATGGGTATAGGTCCGC -3’</td>
<td>5’-GCCTCGTAACTTAACGATCT -3’</td>
</tr>
<tr>
<td>HDAC8</td>
<td>5’-GTTGATTAGGGAGTATCGTTCG -3’</td>
<td>5’-AACCGAAACCAACCGACTACC -3’</td>
</tr>
</tbody>
</table>

MSP reaction was performed as follows: 2µL of template modified-DNA (with a concentration of 15ng/µL), 0.2mM of dNTPs mix (Fermentas, Ontario, Canada), 250nM of each primer (forward and reverse), 2µL of 10x DyNAzyme™ II Hot Start Reaction Buffer (Finnzymes, Finland), 0.24µL of DyNAzyme™ II Hot Start (2U/µL) (Finnzymes, Finland) and sterile bidistilled water (B.Braun, Melsungen, Germany) were admixed in a total reaction volume of 20µL. PCR was then performed according to DyNAzyme™ II Hot Start manufacture's conditions: 94°C incubation for 10min (initial denaturation), followed by 35 cycles of 94°C for 30sec, a 30sec incubation at the optimized annealing temperature for each set of primers (60°C for HDAC2 and 67°C for HDAC8), 72°C for another 30sec, and a final extension step at 72°C for 10min. After amplification by PCR reaction, samples were submitted to an electrophoresis in a 2% agarose gel, at 120V, in an Electrophoresis Power Supply EPS 1001 (Amersham Pharmacia Biotech, Piscataway), for 40min. At the end of the run, the gel was observed in an ultraviolet transilluminator [Pharmacia Biotech ImageMaster VDS (Pharmacia Biotech, Bay Area)].
Selection of candidate bladder cancer cell lines from quantitative expression analysis

Cell culture

The five BCa cell lines included in this study were 5637, J82, T24, TCCSUP, and SCaBER [ATCC – American Type Culture Collection, Lockville, MD, USA]. With the exception of SCaBER cell line, which derives from a squamous cell carcinoma, the remaining cell lines are of urothelial type.

All the cell lines were grown in the recommended medium (Table 4) supplemented with 10% Fetal Bovine Serum (FBS) (GIBCO®, Invitrogen, Carlsbad, CA, USA) and 1% Penicillin–Streptomycin (P–S) (GIBCO®, Invitrogen, Carlsbad, CA, USA). Cell lines were maintained at 37°C in a humidified atmosphere containing 5% CO₂. Cell lines were subcultured, using Dissociation Reagent trypLE™ Express (GIBCO®, Invitrogen, Carlsbad, CA, USA) to harvest them as many times as necessary to obtain the desired number of cells/75 cm² culture flasks.

All BCa cell lines were tested for Mycoplasma spp. contamination (PCR Mycoplasma Detection Set, Clontech Laboratories, Oxford, UK).

Table 4 – Characteristics and growth conditions of BCa cell lines.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Bladder cancer type</th>
<th>Growth medium</th>
<th>Growth properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>5637</td>
<td>Transitional cell carcinoma</td>
<td>RPMI+10%FBS+1%P–S</td>
<td>Adherent Fast growth</td>
</tr>
<tr>
<td>TCCSUP</td>
<td>Transitional cell carcinoma</td>
<td>MEM+10%FBS+1%P–S</td>
<td>Adherent Normal growth</td>
</tr>
<tr>
<td>J82</td>
<td>Transitional cell carcinoma</td>
<td>MEM+10%FBS+1%P–S</td>
<td>Adherent Normal growth</td>
</tr>
<tr>
<td>T24</td>
<td>Transitional cell carcinoma</td>
<td>DMEM+10%FBS+1%P–S</td>
<td>Adherent Fast growth</td>
</tr>
<tr>
<td>SCaBER</td>
<td>Squamous cell carcinoma</td>
<td>MEM+10%FBS+1%P–S</td>
<td>Adherent Normal growth</td>
</tr>
</tbody>
</table>

Notes: RPMI 1640 Medium + GlutaMAX™, DMEM – Dulbecco’s Modified Eagle Medium, MEM–Eagle’s Minimum Essential Medium, from Invitrogen GIBCO®, CA, USA

RNA extraction and cDNA synthesis

RNA from all cell lines was extracted using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) and the cDNA used for gene expression analysis was
synthesized by reverse transcription using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), as previously described for tissue samples.

_qRT–PCR_

In order to select the cell line with more potential to be silenced, cDNAs from all the cell lines were analyzed for HDAC1, HDAC2, HDAC3 and HDAC8 expression levels, as previously described for tissue samples. The mean quantity of HDAC expression levels was calculated as previously referred [(HDAC Expression Level = (HDAC Mean Quantity / HPRT1 Mean Quantity) x 1000)].

**HDACs DOWNREGULATION STRATEGY**

_TRANSIENT TRANSFECTION (siRNAs)_

In order to characterize the biological role of HDAC1 and HDAC2 by RNA interference, chemically synthesized small interfering RNAs (siRNAs) were employed. The HDAC1 and HDAC2 sequences were designed and synthesized by Eurofins MWG, Germany and the scrambled control was designed by Applied Biosystems. For both HDAC1 and HDAC2, two siRNA target sequences were applied (Table 5). The transfection assays were performed in the BICa cell line expressing the highest levels of HDAC1 and HDAC2, that is 5637. Both HDAC1 and HDAC2 were silenced independently and also simultaneously.

One day prior to transfection, 5637 cells were seeded under standard conditions in 6–well and 96–well cell culture plates in such a way that they were 30–50% confluent at the time of transfection. A final siRNA concentration of 100nM was transfected using Oligofectamine™ Reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s protocol. Briefly, for each 96–well plate, 1µl of a 20µM stock siRNA was diluted in 16µl of medium without serum (200nM oligo concentration). 0.8µl of Oligofectamine™ Reagent was added to 2.2µl of Opti–MEM® I Medium (GIBCO®, Invitrogen, Carlsbad, CA). The diluted siRNA and Oligofectamine were gently mixed together and incubated at RT for 20min. In the meanwhile, the adherent cells (on cell culture plates) were washed once with Opti–MEM® I Medium and replaced with 80µl of medium without serum. The siRNA–Oligofectamine Mixture was added drop wise to the cells and incubated at 37°C in a CO₂ incubator. After 4h, 50µl of three fold FBS containing medium was added to the cells and incubated for the desired period until analysis.
Table 5 - siRNA sequences for HDAC1 and HDAC2.

<table>
<thead>
<tr>
<th>siRNA HDAC1#1</th>
<th>5’– AAGCAGAUGCAGAGAUUCAAC –3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>siRNA HDAC1#2</td>
<td>5’– CUGUACAUUGACAUUGAUA –3’</td>
</tr>
<tr>
<td>siRNA HDAC2#1</td>
<td>5’– AACAGACGUUAAGGAAGAA –3’</td>
</tr>
<tr>
<td>siRNA HDAC2#2</td>
<td>5’– GGAUUCAUCAUGCUAGAAGA –3’</td>
</tr>
</tbody>
</table>

**RNA extraction and cDNA synthesis**

5637 cells were plated in triplicate in 6-well plates at 500,000 cells per well and incubated at normal conditions for RNA extraction. 72h after transfection, the medium was removed and the cells washed with PBS. RNA was extracted using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) and the cDNA used for gene expression analysis was synthesized by reverse transcription using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), as previously described for tissue samples.

**qRT-PCR analysis**

All cell lines’ replicates from each treatment were analyzed for HDAC1 and HDAC2 transcript levels, as previously described for tissue samples. Relative quantities (RQ) were calculated by the formula $RQ = 2^{\Delta Ct}$, where $\Delta Ct = Ct_{\text{gene test}} - Ct_{\text{endogenous control}}$ and mock sample was used as reference. The standard deviation (SD) was calculated for Ct values of the technical replicates and was used to calculate the RQ_{min} and RQ_{max}: $RQ_{\text{min}} = 2^{\Delta Ct_{\text{SD}}} - 2^{\Delta Ct_{\text{reference}}}$; $RQ_{\text{max}} = 2^{\Delta Ct_{\text{SD}} + \Delta Ct_{\text{reference}}}$, according to the DataAssist™ v2.0 Software User Instructions (Applied Biosystems, Foster City, CA, USA).

**Protein extraction and quantification**

72h after transfection, protein extraction from whole-cell lysates was performed using RIPA (Radio ImmunoPrecipitation Assay) lysis buffer (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Briefly, the growth medium was removed from the wells by aspiration and the cells were washed with 1x PBS (GIBCO®, Invitrogen, Carlsbad, CA, USA) to remove residual medium. Then, an appropriated volume of complete RIPA Buffer (1x RIPA lysis Buffer, 1mM PMSF solution, 100mM sodium orthovanadate solution and 25x protease inhibitor...
cocktail solution) was added to the wells and cells were scrapped to promote cell lysis. The lysates were transferred into a tube and incubated on ice for 15min. After incubation, the tubes were centrifuged for 30min at 13,000rpm at 4°C to pellet the cell debris. The supernatant was collected to a new tube and the protein concentration was assessed by Qubit® 2.0 Fluorometer (Applied Biosystems, Foster City, CA, USA). Protein was stored at -20°C until further use. Three independent experiments were performed for each condition (Scramble, HDAC1, HDAC2 and HDAC1+HDAC2 silencing).

**SDS-PAGE and Western Blot**

All cell lines replicates from each condition were analyzed for HDAC1 and HDAC2 protein expression levels by western blot as previously described for tissue samples. Protein bands intensities were determined using Quantity One software (Bio-Rad, Hercules, CA, USA).

**Phenotypic assays**

**Cell Viability Assay**

The effect of transient transfection on the viability of 5637 cell line was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) assay. 5637 cells were seeded in 96-well plates (Sarstedt, Numbrecht, Germany) at 12,000 cells per well and incubated at 37°C/5%CO₂. Cells were allowed to adhere ON and acquire 30%-50% confluence and then transient transfection was performed for 3 days. Subsequently, cell viability was measured by MTT assay at 24h, 48h and 72h post transfection. Briefly, the medium was removed and cells incubated with 20μl of MTT solution (5mg/ml) (Sigma–Aldrich®, Germany) diluted in culture medium (final concentration 0.5mg/mL) for 3h at 37°C/5%CO₂. At the end of incubation, MTT was removed and 100μl of Dimethyl sulfoxide (DMSO) (Sigma–Aldrich®, Germany) were added to each well to dissolve formazan crystals formed in viable cells. Finally, the plates were subjected to gentle shaking for 15min to achieve complete dissolution of the crystals. The absorbance was measured using a microplate reader (FLUOstar Omega, BMG Labtech, Offenburg, Germany) at a wavelength of 540nm with background subtraction at 630nm. Three independent experiments were performed, using triplicates for each experiment.
Apoptosis Assay

Cell apoptosis was quantified using the APOPercentage apoptosis assay kit (Biocolor Ltd., Belfast, Northern Ireland), according to the manufacturer’s instructions. 5637 cells were seeded in 96-well plates (Sarstedt, Numbrecht, Germany) at 12,000 cells per well and incubated at 37°C/5%CO₂. Cells were allowed to adhere ON and acquire 30%-50% confluence and then transient transfection was performed for 3 days. Subsequently, apoptosis levels were assessed at 72h post transfection. Briefly, the medium of each well was removed and replaced by 100µL of culture medium supplemented with 5% (v/v) APOPercentage dye for approximately 30min at 37°C/5%CO₂. This medium was further discarded and cells were washed with 1x PBS (GibCO®, Invitrogen, Carlsbad, CA) to remove the unbound dye. Then, 100µL of APOPercentage dye releasing reagent, an alkali solution used to disrupt the cell membrane and release intracellular accumulated dye, were added to each well. Plates were subjected to shaking during 10min and the absorbance was determined using a microplate reader (FLUOstar Omega, BMG Labtech, Offenburg, Germany) at a wavelength of 550nm with background subtraction at 620nm. Three independent experiments were performed, using six replicates for each experiment.
**Statistical Analysis**

Differences in quantitative expression levels between TB and NB were assessed by pairwise comparisons, using the nonparametric Mann–Whitney U-test \((MW)\). The relationship between expression ratios and other standard clinicopathological variables such as gender, tumor stage, and grade, were evaluated using the \(MW\) or Kruskall–Wallis (\(KW\)) tests, as appropriated. A Spearman nonparametric correlation test was additionally performed to compare age and expression levels. In order to classify each sample as positive or negative for expression, as empirical cutoff value was established based on the higher transcript levels verified in normal mucosas. Then, the diagnostic performance [specificity, sensitivity, positive predictive value (PPV), negative predictive value (NPV) and the accuracy] of expression levels for each class I HDACs (\(HDAC1\), \(HDAC2\), \(HDAC3\) and \(HDAC8\)) were determined. Furthermore, a receiver operator characteristics (ROC) curve was created by plotting the true–positive rate (sensitivity) against the false–positive rate (1–specificity) and the area under the curve (AUC) was calculated.

In cell lines, differences in transcript and protein levels, cell viability and apoptosis, between treatments, were assessed using a One-Way Analysis of Variance (One-Way ANOVA) test followed by a multiple comparison Dunnett’s test, comparing all groups against the Mock, and Sheffe’s test, comparing all groups with each other. To compare Mock and Scramble controls, \(MW\) was used.

All tests, except Dunnett’s test, were two-sided and the p–values were considered significant when inferior to 0.05. Statistical analysis was performed using the SPSS Statistics 20.0 Software for Windows. Graphics were built using SPSS Statistics 20.0 Software for Windows (IBM–SPSS Inc., Chicago, IL, USA) or Microsoft® Excel 2007 for Windows.
RESULTS
**Clinical and histopathological data**

We tested tissue samples of bladder carcinoma (n = 127), and mucosa from normal bladder (n = 20). One hundred tumor samples were from male patients, whereas the remaining twenty seven were from females. All patients were Caucasian. Moreover, one hundred and three tumor samples corresponded to primary tumors and only twenty four were tumor recurrences. The median age of the individuals with BlCa was significantly higher than those of controls (MW, P = 0.001). Relevant clinical and histopathological data was collected from patient’s clinical records and are shown in Table 6.

**Table 6 – Clinical and histopathological features of patients with bladder tumor (TB) and normal bladder mucosa (NB).**

<table>
<thead>
<tr>
<th>Clinicopathological features</th>
<th>TB</th>
<th>NB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients, n</td>
<td>127</td>
<td>20</td>
</tr>
<tr>
<td>Median Age, yrs (range)*</td>
<td>72 (35–92)</td>
<td>61 (51–75)</td>
</tr>
<tr>
<td>Gender, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>100 (79)</td>
<td>20 (100)</td>
</tr>
<tr>
<td>Female</td>
<td>27 (21)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Histopathological grade, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Papillary carcinoma, low grade</td>
<td>48 (38)</td>
<td>n.a.</td>
</tr>
<tr>
<td>Papillary carcinoma, high grade</td>
<td>47 (37)</td>
<td>n.a.</td>
</tr>
<tr>
<td>Invasive carcinoma</td>
<td>28 (22)</td>
<td>n.a.</td>
</tr>
<tr>
<td>Pathological stage, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ta</td>
<td>40 (31)</td>
<td>n.a</td>
</tr>
<tr>
<td>T1</td>
<td>53 (42)</td>
<td>n.a</td>
</tr>
<tr>
<td>T2</td>
<td>23 (18)</td>
<td>n.a</td>
</tr>
<tr>
<td>T3</td>
<td>3 (2)</td>
<td>n.a</td>
</tr>
<tr>
<td>T4</td>
<td>4 (3)</td>
<td>n.a</td>
</tr>
</tbody>
</table>

*n.a.* not applicable
**Class I HDACs Expression**

*mRNA Quantitative Expression*

Significant differences were observed between bladder tumor and normal bladder mucosa samples for *HDAC1, HDAC2, HDAC3* and *HDAC8* quantitative transcript levels (*MW, P < 0.001* for all). Indeed, there was a significant overexpression of all class I HDACs in TB when compared with NB (*Figures 10, 11 12 and 13*).

![Figure 10 - Distribution of HDAC1 transcript expression levels in bladder tissues [NB (n=20) and TB (n=127)] (P < 0.001). The represented scale is logarithmic.](image)

Figure 10 – Distribution of *HDAC1* transcript expression levels in bladder tissues [NB (n=20) and TB (n=127)] (P < 0.001). The represented scale is logarithmic.
Figure 11 – Distribution of *HDAC2* transcript expression levels in bladder tissues [NB (n=20) and TB (n=127)] \((P < 0.001)\). The represented scale is logarithmic.

Figure 12 – Distribution of *HDAC3* transcript expression levels in bladder tissues [NB (n=20) and TB (n=127)] \((P < 0.001)\). The represented scale is logarithmic.
Figure 13 – Distribution of *HDAC8* transcript expression levels in bladder tissues [NB (n=20) and TB (n=127)] \( P < 0.001 \). The represented scale is logarithmic.

Surprisingly, some tumor samples (5%) showed *HDAC2* and *HDAC8* significantly downregulated when compared with normal controls (*Figures 11 and 13*). In order to investigate the molecular mechanism behind this underexpression, and since both HDACs have CpG islands at their promoters, we assessed their methylation status by MSP in 6 TBs in which *HDAC2* and *HDAC8* were found to be downregulated. Additionally, for control purposes, 6 TB with upregulated *HDAC2* and *HDAC8* transcript levels and 6 normal bladder mucosas (NB) were also analyzed. However, no methylation was observed for both *HDAC2* and *HDAC8* promoters in any of the analyzed samples (*Figure 14*).

![MSP analysis](image)

Figure 14 – MSP analysis of NB and TB samples for *HDAC2* (A) and *HDAC8* (B). From the right to the left: 1: molecular weight (MW); 2: positive control; 3: negative control; 4–10: NB; 10–16: TBs upregulated; 16–22: TBs downregulated.
Protein expression

Protein extracted from tissue samples was analyzed by Western blot for class I HDACs. Overall, tumor samples displayed higher levels of protein expression of class I HDACs when compared with normal bladder mucosas. This finding is consistent with the results obtained for RNA transcript levels. Differences in HDAC1, HDAC2, HDAC3 and HDAC8 protein levels between bladder tumor and normal bladder mucosa samples are illustrated in Figure 15. A single band was observed in both blots with approximately 60kDa for HDAC1, 55kDa for HDAC2, 49kDa for HDAC3 and 43kDa for HDAC8.

Figure 15 – Protein gel blot analysis of TB and NB tissues for class I HDACs. Blots were incubated with a specific antibody recognizing HDAC1 (A), HDAC2 (B), HDAC3 (C) and HDAC8 (D). Their expression was corrected to the housekeeping gene, actin.
CORRELATION OF TRANSCRIPT LEVELS WITH THE CLINICAL AND PATHOLOGICAL DATA

Concerning the pathological stage and grade, statistically significant differences were observed for *HDAC1* and *HDAC3* \((KW, P < 0.001)\). In fact, these two genes were found to be downregulated in advanced tumors (T2–T4 stages), as well as in invasive carcinoma. Nonetheless, no differences were apparent between papillary low and papillary high grade tumors (Figures 16-19). Regarding *HDAC2* and *HDAC8*, no differences in transcript levels were found among the different pathological stages and grades. Moreover, no significant association was found between class I HDAC transcript levels and patients’ gender \((MW, P > 0.05)\) or age (Spearman’s correlation, \(P > 0.05\)).

![Box plot of HDAC1 relative expression](image)

Figure 16 – Distribution of *HDAC1* transcript expression levels in bladder tissues according to their pathological stage \([NB (n=20), Ta+T1 (n=93), T2+T3+T4 (n=30)] (P < 0.001)\). The represented scale is logarithmic.
Figure 17 – Distribution of HDAC3 transcript expression levels in bladder tissues according to their pathological stage [NB (n=20), Ta+T1 (n=93), T2+T3+T4 (n=30)] (P < 0.001). The represented scale is logarithmic.

Figure 18 – Distribution of HDAC1 transcript expression levels in bladder tissues according to their pathological grade [NB (n=20), low grade papillary carcinoma (n=48), high grade papillary carcinoma (n=47) and invasive carcinoma (n=28)] (P < 0.001; ns - P > 0.05). The represented scale is logarithmic.
Figure 19 - Distribution of \textit{HDAC3} transcript expression levels in bladder tissues according to their pathological grade [NB (n=20), low grade papillary carcinoma (n=48), high grade papillary carcinoma (n=47) and invasive carcinoma (n=28)] ($P < 0.001$; ns - $P > 0.05$). The represented scale is logarithmic.
EVALUATION OF DIAGNOSTIC POTENTIAL OF QUANTITATIVE CLASS I HDAC EXPRESSION IN TISSUE SAMPLES

The diagnostic performance (Table 7) of the four HDACs was assessed using the cutoff values of transcript levels previously determined for each of these genes HDAC1 (1036.75), HDAC2 (547.39), HDAC3 (1297.82) and HDAC8 (1446.28) to discriminate BCa from normal bladder mucosa. The highest sensitivity was observed for HDAC1, whereas the lowest was verified for HDAC3.

Table 7 – Diagnostic performance of class I HDACs as a BCa biomarker.

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity, % (n positive / n total)</th>
<th>Specificity, % (n negative / n total)</th>
<th>PPV, %</th>
<th>NPV, %</th>
<th>Accuracy, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDAC1</td>
<td>92 (117/127)</td>
<td>100 (20/20)</td>
<td>100</td>
<td>67</td>
<td>93</td>
</tr>
<tr>
<td>HDAC2</td>
<td>70 (89/127)</td>
<td>100 (20/20)</td>
<td>100</td>
<td>34</td>
<td>74</td>
</tr>
<tr>
<td>HDAC3</td>
<td>68 (86/127)</td>
<td>100 (20/20)</td>
<td>100</td>
<td>33</td>
<td>72</td>
</tr>
<tr>
<td>HDAC8</td>
<td>69 (87/127)</td>
<td>100 (20/20)</td>
<td>100</td>
<td>33</td>
<td>73</td>
</tr>
</tbody>
</table>

ROC curve analysis (Figure 20) for these epigenetic biomarkers resulted in an area under the curve (AUC) of 0.967 (95% confidence interval (CI), 0.938 – 0.995, P < 0.001) for HDAC1; an AUC of 0.906 (95% CI, 0.856 – 0.956, P < 0.001) for HDAC2, an AUC of 0.934 (95% CI, 0.891 – 0.978, P < 0.001) for HDAC3 and an AUC of 0.880 (95% CI, 0.825 – 0.936, P<0.001) for HDAC8.

![ROC curve - BCa tissues](image)

Figure 20 – Receiver operating characteristics (ROC) curve for each individual gene (HDAC1, HDAC2, HDAC3 and HDAC8) in bladder tissue.
Combining the results of each gene for the 127 tumor samples, we verified that BICa cases might be identified by combining the expression analysis of the several HDACs, thus providing a valuable diagnostic coverage. Among the possible combinations tested for discriminating BICa patients from controls, three gene expression markers – HDAC1, HDAC3 and HDAC8 – demonstrated the best performance in terms of sensitivity and specificity (Table 8). ROC curve analysis for this three–gene panel resulted in an AUC of 0.975, with a 95% CI of 0.951 – 0.998, at significant level of $P < 0.001$ (Figure 21). Interestingly, the same sensitivity and specificity rates were found with the four–gene panel.

Table 8 – Diagnostic performance of class I HDACs panel as a BICa biomarker.

<table>
<thead>
<tr>
<th>Combination</th>
<th>Sensitivity, % (n positive / n total)</th>
<th>Specificity, % (n negative / n total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDAC1/HDAC8</td>
<td>96 (122/127)</td>
<td>100 (20/20)</td>
</tr>
<tr>
<td>HDAC1/HDAC8/HDAC3</td>
<td>97 (123/127)</td>
<td>100 (20/20)</td>
</tr>
<tr>
<td>HDAC1/HDAC8/HDAC3/HDAC2</td>
<td>97 (123/127)</td>
<td>100 (20/20)</td>
</tr>
</tbody>
</table>

Figure 21 – Receiver operating characteristics (ROC) curve for the best combination of three genes in bladder tissue. Reference Line in green; HDAC1, HDAC3 and HDAC8 expression; ROC curve in blue.
**Impact of HDAC1 and HDAC2 silencing on 5637 cell line phenotype**

In order to select a BICa cell line that exhibited the highest class I HDACs transcript levels, and thus might be used as a tumor model to study HDAC1 and HDAC2 role in carcinogenesis, quantitative RT-PCR for all class I HDACs was performed in several BICa cell lines (TCCSUP, 5637, ScaBER, T25 and J82). Among all the cell lines analyzed, 5637 has shown the higher transcript levels of all HDACs and was therefore selected for further studies (*Figure 22*). Furthermore, *HDAC1* and *HDAC2* have the highest mRNA expression levels in all cell lines. Since these two genes are suggested to have redundant functions [49], they were chosen for the downregulation studies.

![Relative expression graph](image)

*Figure 22 – Relative expression of HDAC1, HDAC2, HDAC3 and HDAC8 in 5637, J82, SCABER, T24 and TCCSUP cell lines.*

All the experiments presented below were performed with scramble and mock controls. Since no statistical significant differences (*MW, P > 0.05*) were found between them, all the comparisons were performed against mock control (*Figure 23*).
Figure 23 – 5637 cell line analysis for mock and scramble controls. A) Relative expression of HDAC1 and HDAC2. B) Protein expression levels of HDAC1 and HDAC2. C) Quantification of cell viability by an MTT assay at 0h, 24h, 48h and 72h in culture. D) Quantification of apoptosis by an apoptosis assay at 72h in culture.

**mRNA expression**

Silencing was successfully accomplished for both HDAC1 and HDAC2 independently and for HDAC1&2 simultaneously. mRNA expression levels were measured by real time PCR (Figure 24). A decrease of approximately 27% for HDAC1 mRNA expression (Dunnet's test, $P = 0.004$) was observed in the si–HDAC1 cells. Concerning the silenced cells for HDAC2 (si–HDAC2), a decrease of approximately 40% for HDAC2 transcript levels (Dunnet's test, $P = 0.001$) was observed. In si–HDAC1&HDAC2 cells, a decrease of 38% (Dunnet's test, $P < 0.001$) and 28% (Dunnet's test, $P = 0.01$) were observed for HDAC1 and HDAC2 expression, respectively.
Figure 24 - Relative expression of HDAC1 and HDAC2 in Mock, si–HDAC1, si–HDAC2 and si–HDAC1&HDAC2 cells. Results were normalized to the data obtained with the Mock. (* represent statistically significant differences of si–HDAC1, si–HDAC2 and si–HDAC1&HDAC2, comparing to mock: ** $P \leq 0.01$; *** $P \leq 0.001$).

**Protein expression**

Relative quantification of protein levels of HDAC1 (60kDa) and HDAC2 (55kDa) in the silenced and mock cells were assessed by western blot (Figure 25). As for transcript levels, a significant decrease in protein expression was verified upon HDAC1, HDAC2 and HDAC1&2 silencing (Figure 26). Overall, the lowest protein levels were shown by si–HDAC2 cells. In si–HDAC1 cells, a reduction of approximately 31% of protein was observed (Dunnet’s test, $P = 0.005$). Concerning si–HDAC2 cells, a 48% decrease of HDAC2 protein expression (Dunnet’s test, $P < 0.001$) and a 56% increase of HDAC1 protein expression (Dunnet’s test, $P < 0.001$) was found. When silencing simultaneously HDAC1&2, a decrease of 39% (Dunnet’s test, $P = 0.001$) and 38% (Dunnet’s test, $P < 0.001$) was observed for HDAC1 and HDAC2, respectively.

Figure 25 - Protein gel blot analysis of 5637 cell line for HDACs silencing. Blots were incubated with a specific antibody recognizing HDAC1 (A) and HDAC2 (B). Their expression was corrected to the housekeeping gene, actin.
Figure 26 – Protein levels of HDAC1 and HDAC2 in Mock, si-HDAC1, si-HDAC2 and si-HDAC1&HDAC2 cells. Results were normalized to the data obtained with the Mock. (* represent statistically significant differences of si-HDAC1, si-HDAC2 and si-HDAC1&HDAC2, comparing to mock: ** P ≤ 0.01; *** P ≤ 0.001).

**Phenotypic assays**

**Cell viability**

An effective reduction of viable cells was observed for all silenced cells. In fact, HDAC1, HDAC2 and HDAC1&2 silenced cells showed significantly decreased cell viability when compared to the mock cells (Figure 27) for 24h (Dunnet’s test, P = 0.013 for HDAC1, P = 0.046 for HDAC2 and HDAC1&2), 48h (P < 0.001) and 72h (P < 0.001). There were no significant differences for cell viability among HDAC1, HDAC2 and HDAC1&2 silenced 5637 cells (Scheffe’s test, P > 0.05).

Figure 27 – Quantification of cell viability by an MTT assay in Mock, si-HDAC1, si-HDAC2 and si-HDAC1&HDAC2 cells at 0h, 24h, 48h and 72h in culture (* represent statistically significant differences of si-HDAC1, si-HDAC2 and si-HDAC1&HDAC2, comparing to mock: * P < 0.05; *** P ≤ 0.001).
Cell apoptosis

A significant increase in apoptosis was apparent in all tested cell lines at day 3. Indeed, in comparison with mock cells, all 5637-silenced cells showed significantly increased levels of apoptotic cells (Dunnet's test, $P < 0.001$) (Figure 28). No statistical significant differences were found for relative apoptosis among HDAC1, HDAC2 and HDAC1&2 silenced 5637 cells (Scheffe's test, $P > 0.05$).

![Figure 28 - Quantification of apoptosis by APOPercentage assay kit of Mock, si–HDAC1, si–HDAC2 and si–HDAC1&HDAC2 cells at 72h in culture. Results were normalized to the data obtained with the Mock. († represent statistically significant differences of si–HDAC1, si–HDAC2 and si–HDAC1&HDAC2, comparing to mock: *** $P \leq 0.001$).]
Over the past years, increasing evidence has suggested that genes implicated both in cancer initiation and progression are deregulated through the action of HDACs [155,156]. Therefore, several HDAC inhibitors have been developed and tested as anticancer agents in a wide variety of solid and hematological malignancies. Recently, researchers started focusing on the histone acetylation status of human cancers, in general, and, in particular, on the expression of specific HDAC isoforms in different malignancies. Indeed, a number of studies have shown aberrant expression of HDAC family members in several tumors. Importantly, some of these have already been demonstrated to participate in different cell functions, whose deregulation is known to lead to neoplastic transformation [31,157]. Accordingly, aberrant expression of class I HDACs in neoplastic diseases, and, specifically, HDAC1 and HDAC2 depletion, have been shown to cause proliferation inhibition and induction of apoptosis in certain human cancers cells [65,67,69,70]. However, the role of class I HDACs in bladder carcinogenesis remains elusive.

Hence, in order to understand whether class I HDACs deregulation might be implicated in BICa, we firstly evaluated *HDAC1*, *HDAC2*, *HDAC3* and *HDAC8* transcript levels and protein expression in a large set of human BICa tissues. We found that mRNA and protein levels of all class I HDACs were upregulated in bladder tumors in comparison with normal bladder mucosas. These results are in accordance with previous findings in other carcinomas, including colorectal, gastric and prostatic [55,56,61]. Concerning *HDAC1*, our results also corroborate a previous report, in which mRNA expression was also found to be increased in a limited series of urothelial tumors (n=10) [158]. Overall, these results suggest an oncogenic function for these class I HDACs in a relatively wide range of common human neoplasms.

Surprisingly, however, we found a significant downregulation of *HDAC2* and *HDAC8* mRNA levels in 7 and 6 bladder tumors, respectively. In order to explore this finding, and because both *HDAC2* and *HDAC8* have a CpG island within their promoter region, we investigated their respective methylation status. Remarkably, no methylation was observed in any of the analyzed samples. An additional explanation would be a genetic alteration. Indeed, a truncating mutation of the *HDAC2* gene and consequent loss of protein expression has been found in sporadic carcinomas with microsatellite instability, including colon, gastric and endometrial [58]. Since microsatellite instability has been already described for BICa, we could speculate that in cases with *HDAC2*
underexpression, a truncating mutation might be present [159]. Alternatively, a chromosomal deletion might be the cause. Indeed, HDAC2 locus is mapped at 6q21, and 6q deletions have been already demonstrated in BICa [160]. Ultimately, miRNAs regulation of both HDAC2 and HDAC8 cannot be excluded, not only owing to the fact that miRNAs deregulation is clearly implicated in tumorigenesis, but also because miRNAs have been suggested as important epigenetic regulators of HDACs [24,25].

Interestingly, we found statistically significant associations between HDAC1 and HDAC3 expression and tumor grade and stage. Indeed, less differentiated and more advanced tumors displayed lower expression levels of these HDACs. These results indicate that class I HDACs' upregulation is an early event in bladder carcinogenesis. Thus, similarly to DNA methylation alterations, deregulation of deacetylases seems to play a major role in tumor initiation [3]. Moreover, in light of the two accepted pathways of bladder oncogenesis, it is tempting to hypothesize that class I HDACs deregulation is more likely associated with the non–muscle invasive papillary pathway, also characterized by FGFR3, HRAS and PI3K mutations [113,114]. Therefore, our results diverge from previously reported associations of class I HDACs with more aggressive phenotype in other solid tumors [55,61]. However, to the best of our knowledge, there is no published data concerning BICa with which we could compare our findings.

Taking into consideration the time frame of the occurrence of histone deacetylases overexpression in bladder carcinogenesis, we tested the potential of these HDAC family members as potential biomarkers for superficial BICa. Among the various possible gene combinations tested, a three gene panel comprising HDAC1, HDAC3 and HDAC8 demonstrated the best performance for discriminating BICa patients from controls, with a remarkable sensitivity of 97% (123/127) and specificity of 100%, in tissue samples. These results are rather novel, since little data is available on the clinical value of histone post-translational modifications, and herein we firstly identify a group of three histone modifiers with tumor biomarker properties [136–138]. Interestingly, these results compare well with the performance of other epigenetic tumor biomarkers for BICa detection, especially DNA methylation markers [134]. Indeed, our group has established a three gene panel (GDF15, TMEFF2, and VIM) for BICa detection based on DNA methylation, with a sensitivity of 100% for tissue and an impressive 94% for urine samples [135]. Nonetheless, we have been unable to
replicate the promising results of HDACs as BICa detection biomarkers in urine samples. This is due to the low transcript levels of deacetylases in urine, preventing the use of urine for BICa detection purposes. Therefore, our future investigations will focus on the prognostic/predictive value of HDACs expression levels in BICa tissues.

Although class I HDACs relevance in establishing the malignant phenotype has been previously reported for other tumor models, the impact of these HDACs in BICa cells remains to be clarified [157]. Accordingly, we assessed the effect of HDAC1 and HDAC2 silencing in the malignant phenotype of 5637 BICa cell line. We chose this cell line because it is derived from grade II bladder carcinoma, mimicking superficial BICa lesions, and it displays the highest transcript levels of all class I HDACs. Although our ultimate goal was to silence all class I HDACs members, we selected HDAC1 and HDAC2 because they displayed the highest expression levels in all cell lines and have been suggested to play redundant functions in cancer cells, since HDAC1 and HDAC2 are both found in multiprotein corepressor complexes Sin3, NuRD, and CoREST [49]. Silencing was successfully achieved for both HDAC1 and HDAC2, both separately and in combination. Interestingly, HDAC1 protein levels suffered a significant increase when HDAC2 was downregulated. These findings might be explained by the redundancy and compensatory functions already advocated for these two proteins in specific knockdown and knockout studies [49]. Remarkably, and in trend with previous publications, the same effect was not observed in HDAC1 mRNA levels [161–163]. Thus, increased HDAC1 protein levels may be due to translational or post-translational changes in HDAC1 occurring in the absence of HDAC2. Intriguingly, a similar effect was not observed in HDAC2 when HDAC1 was silenced. Specifically, we did not find significant alterations in HDAC2 mRNA or protein levels in the absence of HDAC1. Possibly, this might be due to a higher enzymatic efficiency of HDAC2 when compared to HDAC1. Thus, when HDAC1 was downregulated, vicariation might not require an increase in HDAC2 expression levels.

Finally, a significant decrease in cell viability and increased apoptosis in HDAC1 and HDAC2 silenced cells was found. Likewise, knockdown studies in human cancer cells have revealed individual and overlapping regulatory functions of HDAC1 and HDAC2 in cell proliferation and survival [49]. Based on available literature and in our own results, similar functions for these HDACs might be hypothesized, as similar effects of the inactivation of either HDAC1 or HDAC2
were found. Importantly, the same has been proposed in other cancer models such as breast [65], colon [56] and lung [164]. Interestingly, several reports have suggested that HDAC-mediated repression of genes may cause uncontrolled cell growth, as HDACs repress the transcription of cyclin-dependent kinase inhibitors (CDKIs), such as p21 and p57, allowing for continued proliferation [40,65,155,161]. Our results demonstrated that the effects on proliferation and apoptosis of combined HDAC1 and HDAC2 silencing was similar to that of individual HDAC silencing. This lack of synergy suggests that HDAC1 and HDAC2 are equally important for the appropriate function of the co-repressor complexes in which they are involved. Although this finding contrasts with those reported for some other cell lines [49], they support an important role for class I HDACs, especially HDAC1 and HDAC2, in bladder tumorigenesis.
CONCLUSIONS AND FUTURE PERSPECTIVES
In this study, we demonstrated global overexpression of class I HDACs in BICa tissues, both at mRNA and protein expression level. Importantly, we also verified that lower HDAC1 and HDAC3 expression was present in less differentiated and more advanced tumors. Moreover, we found that expression levels of a three gene panel – HDAC1, HDAC3 and HDAC8 – might accurately discriminate cancerous from normal bladder tissues. Finally, an anti-proliferative and pro-apoptotic effect of HDAC1 and/or HDAC2 silencing was observed in a BICa cell line, further supporting an oncogenic role for those two histone modifiers in bladder carcinogenesis.

These results are preliminary and further studies are required to better support our conclusions. Thus, we plan to obtain clinical follow up data in order to evaluate whether HDACs might be used as biomarkers of prognostic and/or predictive value in BICa. Additional studies will be performed to provide a more complete elucidation of the role of all members of class I HDACs in bladder carcinogenesis. The high sequence similarity between all the class I members might anticipate a significant overlap in function. Nevertheless, these enzymes have already revealed both redundant and specific functions in other systems. Accordingly, we intend to further assess the phenotypic impact of HDAC1, HDAC2, and combined HDAC1 and HDAC2 silencing in 5637 BICa cell lines by assessing invasion and colony formation capabilities. These studies will be then extended to other members of class I HDACs as well as to other BICa cell lines to provide a wider view of the role of these enzymes in bladder carcinogenesis.


