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Chapter 3

# Loss of Imprinting as an Epigenetic Marker in Bladder Cancer

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Additional information is available at the end of the chapter

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## 1. Introduction

Currently, it is well recognized that epigenetic changes and genetic alterations are involved in the initiation and progression of human cancer. Epigenetics refers to the study of changes in gene expression caused by mechanisms other than classical mutations in the DNA sequence; these changes are potentially reversible but are generally stably maintained during cell division. The most common biological processes resulting from epigenetic mechanisms include X-chromosome inactivation, cellular differentiation, maintenance of cell identity and genomic imprinting.

Genomic imprinting is an epigenetic process of gene regulation in which the parental origin of an allele determines whether the allele will be expressed or repressed [1]. The imprinting is maintained by epigenetic modifications such as DNA methylation and repressive histone marks that are transmitted to the gametes from the parental germ lines to ensure the expression of a gene in a parent-specific manner. In somatic cells, the imprinted pattern is inherited during mitotic division leading to the specific-monoallelic expression of the opposite allele on the homologous chromosome [2]. However, in adult tissues, the patterns of imprinting of a gene may be complex, in which the specific-monoallelic expression is restricted to a limited number of cell types while biallelic transcripts produced from different promoters can be observed in other cells or tissues [3]. Furthermore, the majority of the genes regulated by imprinting are clustered with a long non-coding RNA; the expression of the genes in these clusters is controlled *in cis* by an imprinting control region (ICR) containing a differentially methylated region (DMR) that exhibits parent-specific DNA methylation. Thus, epigenetic modifications lead to the expression from only one of the two chromosome homologues depending on whether they are the maternally or paternally inherited copy of the gene.

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In humans, the appropriate expression of imprinted genes is important for normal development. The loss of genomic imprinting exposes the organism to a greater risk of diseases because the disruption of normal patterns could lead to gain or loss of expression of the alleles and subsequently to imbalances in the amount of the gene product. There are numerous diseases associated with defects of imprinted genes including growth and metabolism disorders; various childhood and adult cancers; and disorders in neurodevelopment, cognition, and behavior as well as certain major psychiatric disorders.

Currently, approximately 80 imprinted genes have been characterized in the mouse genome. Two-thirds of them show conserved imprinting patterns between mice and humans, whereas others show imprinting patterns specific to humans. A large number of genes are also predicted to be imprinted [4].

This chapter will describe the molecular basis of genomic imprinting including epigenetic marks associated with the silencing of imprinted genes, the loss of imprinting as a potential marker of risk and prognostic biomarkers in human cancer with a focus on bladder cancer.

## 2. Imprinted genes: regulation and function

Genomic imprinting has four important principles. First, it must be able to influence gene expression. Second, it must be heritable in somatic lineages such that the memory of the parental origin is propagated into daughter cells. Third, it should be initiated on the paternally and maternally inherited chromosomes during gametogenesis or immediately after fertilization. Finally, imprinting must be erased in the germ line so that parental identity can be established in the gametes for the next generation [5].

Mechanisms responsible for establishing and maintaining imprinting include DNA methylation, chromatin modifications, insulation and the expression of non-coding RNAs (ncRNAs). DNA methylation is a reversible reaction that is catalyzed by DNA methyltransferases, an enzyme family that adds a methyl group to the 5-carbon of a cytosine that is immediately followed by a guanine. In the human cells, the methylation is almost restricted to these CpG dinucleotides, which are largely under-represented in the genome except at genomic regions called CpG islands, some of them associated with gene promoters [6]. In 2004, Kaneda *et al*. [7] demonstrated that a specific DNA methyltransferase, Dnmt3a, is essential for the establishment of both maternal and paternal imprinting. Once imprinting is established in the germ line, it is necessary to maintain the marks after reprogramming and *de novo* methylation that occurs after the pre-implantation of the embryo [8]. In somatic cells, imprinting is maintained and modified during development [9], and tissue-specific imprinting is frequently observed [10].

Although DNA methylation is the most important mechanism for imprinting, it does not appear to be the only mechanism. DMRs are often, but not exclusively, associated with chromatin modifications [11]. The majority of imprinted genes are clustered into megabase-long regions in the genome, which are essential to coordinate their regulation [12]. According

to Barlow [2], more than 80% of the known imprinted genes are clustered into 16 genomic regions that contain two or more genes. The cluster organization reflects the coordinated regulation of the genes in a chromosomal domain [9]. These clusters share a number of features, including a ncRNA that is expressed from the parental allele opposite the protein-coding genes and the ICR [13]. The ICRs exhibit parental-specific epigenetic modifications (DNA methylation and histone modifications) that govern their activity [14].

Chromatin is mainly composed of histone proteins (H2A, H2B, H3, and H4) that are subjected to a variety of post-translational modifications on specific amino acid residues that are located in the histone tails (NH2 terminal regions). These modifications include acetylation, methylation, phosphorylation, sumoylation, ubiquitination and ADP ribosylation [15,16]. In somatic cells, the germline DMRs are marked by allele-specific histone modifications. In both maternal and paternal germ line DMRs, the unmethylated allele is associated with hallmarks of permissive chromatin, such as dimethylation of lysine 4 of histone H3 (H3K4me2) and H3/H4 acetylation [17]. Still, allele-specific DNA methylation at the ICRs in mice is associated with histone H4-lysine-20 and H3-lysine-9 trimethylation [18]. These marks, which also include histone H3-lysine-27 trimethylation (H3K27me3), histone H4-lysine-20 trimethylation (H4K20me3) and histone H3-lysine-9 di/trimethylation (H3K9me2/me3), are frequently associated with heterochromatic regions and a repressed status [19].

In a study conducted by Henckel *et al*. [20] with mid-gestation embryos obtained from Dnmt3L -/- females (DNA methylation at ICRs is not established during oogenesis), they observed a lack of repressive histone modifications suggesting that there is a mechanistic link between DNA and histone methylation at ICRs. It has been suggested that the methylation of the CpG dinucleotides in these control regions can affect the expression of the gene by preventing the binding of insulator proteins to differentially methylated regions. This methylation event precludes the binding of transcription factors to the promoter and changes the chromatin structure by recruiting methyl-CpG binding domain (MBD) proteins that bind to methylated CpGs and recruit other proteins [1]. Thus, the regulation of expression could depend on the local concentration of CpGs within the DMR.

The clusters are regulated by two main imprinting mechanisms. First, imprinting marks in the DMR can act as insulator elements and regulate the expression of imprinted genes, and second, the DMR can serve as a promoter for regulatory non-coding RNAs (ncRNAs). In the first model, the imprinted genes share regulatory elements, and the insulator controls access to these elements.

The *H19*/*IGF2* locus is the well-documented example of this model. Located at 11p15.5 in the human genome, these genes are connected and are expressed in a mutually exclusive manner [21]. In humans and rats, the transcription of *IFG2* and *H19* genes are coordinated by a group of enhancers located downstream to *H19* and a DMR located upstream to this gene [22]. The enhancers, lying between +7 and +9.5 kb from the promoter, include those sites that control expression in endodermal [23] and mesodermal [24] tissues. The second important element in this insulator model is the ICR or DMR. This element resides at -2 Kb to -4 Kb from the *H19* transcriptional start site and is crucial for establishing the molecular imprint of the *H19* gene in the early embryo [25]. This region was shown to block enhancer activity for the *H19* and

*IFG2* genes and contains seven CTCF-binding sites that are required for this activity. When these CTCF-binding sites are methylated, they no longer bind the CTCF insulator protein [26]. CTCF is a ubiquitous, highly conserved transcription factor that plays multiple roles in gene regulation, such as in activation, repression, silencing, chromatin insulation, and long-range chromosome interactions [27]. On the maternal allele, the presence of CTCF blocks the enhancer from interacting with *IFG2* promoters and silences gene expression [28]. In contrast, CTCF does not bind to the methylated, paternally inherited chromosome. As a result, the enhancers are free to interact with the *IFG2* promoter, and the *H19* promoter is repressed [5]. The three-dimensional arrangement of the chromatin fiber created by CTCF-mediated interactions also plays an important role in imprinted gene expression at the *H19*/*IFG2* locus [29]. In 2004, by using the chromosome conformation capture (3C) method in a mouse model, it was demonstrated that the *Igf*2 DMR1 (one of the three DMRs found in mouse, located upstream to the promoter 1 of the *Igf*2 gene) is able to interact with the *H19*-DMR [30]. Another study also suggested that chromosomal looping is involved in the imprinting mechanism and that the CTCF sites can mediate allele-specific chromosome interactions that control the accessibility of the *IFG2* promoter to the shared enhancer [31,32].

The second mechanism regulating the expression of imprinted gene clusters involves a ncRNA. These ncRNAs function to silence large domains of the genome through their interaction with chromatin [33]. At present, several classes of ncRNAs have been identified within imprinted regions, including small nucleolar RNAs (snoRNAs), microRNAs (miRNAs), small interfering RNAs (siRNAs), Piwi-interacting RNAs (piRNAs), antisense ncRNAs and long non-coding RNAs (lncRNAs). While the expression of some plays a functional role in the regulation of genomic imprinting, the function of others remains to be determined [34]. It has been demonstrated that each imprinted cluster expresses at least on ncRNA that display reciprocally imprinted expression patterns relative to the neighboring protein-coding genes and that some of these genes are transcribed in an antisense orientation relative to the protein-coding gene [35]. The most studied and well-understood clusters in this class are the *Ifg2r* and *Kcnq1* clusters. *Ifg*2r and two neighboring genes, *Slc22a2* and *Slc22a3* (solute carrier 22a2 and 22a3), are maternally expressed. This region also harbors one paternally expressed transcript, *Air* (antisense to *Ifg2r* RNA) [36]. *Air* localizes to the silenced *Slc22a3* promoter, recruits the KMT1C lysine methyltransferase and leads to targeted H3K9 methylation and allele-specific gene silencing by chromatin remodeling [37]. Similar to the *Air–Ifg2r* locus, the *Kcnq1* locus contains a series of maternally expressed genes (at least eight) and a unique non-coding paternally expressed gene, *Kcnq1ot1* [34]. This locus is governed by the maternally methylated ICR, KvDMR1, located within an intron of the *Kcnq1* gene. The promoter for the *Kcnq1ot1* gene resides within KvDMR1[14]. According [38], *Kcnq1ot1* is required for epigenetic silencing of neighboring genes upstream and downstream of the *Kcnq1* locus.

The imprinted genes showed that complex regulation and functional consequences are associated with imprinting-induced changes in the expression level. One consequence of genomic imprinting is that viable embryos must receive two haploid genome complements that come from parents of the opposite sex [39]. Generally, the imprinted genes are highly expressed during embryonic development and are down-regulated after birth.

The majority of imprinted genes in mammals has a critical role in the development and function of the placenta [40] and brain [41], have been linked to cancer development and are associated with growth disorders, such as Beckwith-Wiedemann and Silver-Russel syndromes [42], and neurodevelopmental disorders, such as Angelman [43] and Prader-Willi syndromes [44].

## 3. Imprinting and cancer

Loss of imprinting (LOI), defined as the break the methylation patterns of DMRs associated with monoallelic parental-specific expression, is a common event in human cancer [45]. This term includes both the activation of the normally silenced allele and inactivation of the allele that is expressed upon normal imprinting conditions.

Abnormal imprinting of the *IGF2* and *H19* genes in tumors was first described in the Wilms' tumor [46,47]. This tumor is a common solid cancer in children, and loss of imprinting has been described as the most prevalent abnormality in the development of this tumor [48]. Thereafter, loss of imprinting of *IGF2* and *H19* genes has been correlated with several common adult human cancer (Table 1).

Despite these findings, the number of genes demonstrating LOI in human cancer is still limited due to the small number of known genes regulated by imprinting. However, the statistics may increase because of the growing interest in epigenetics and the large number of genes predicted to be regulated by imprinting.

| Imprinted Gene | Oficial Name | Other Aliases | Chromosomal location | Cancer type | Reference |
|---|---|---|---|---|---|
| *DIRAS3* | DIRAS family, GTP-binding RAS-like 3 | *ARHI, NOEY2* | 1p31.1 | Ovarian and breast | [49] |
| | | | | Breast | [50] |
| | | | | Myeloma | [51] |
| | | | | Hepatocellular | [52,53] |
| | | | | Thyroid | [54] |
| | | | | Oligodendroglial | [55] |
| *PLAGL1* | pleiomorphic adenoma gene-like 1 | *RP3-468K18.1, LOT1, ZAC, ZAC1* | 6q24-q25 | Breast and ovarian | [56] |
| | | | | Gastric adenocarcinoma | [57] |
| | | | | Cervical | [58] |
| *PEG10* | paternally expressed 10 | *EDR, HB-1, MEF3L, Mar2, Mart2, RGAG3* | 7q21 | Hepatocellular | [59] |
| | | | | B-cell chronic lymphocytic | [60] |

| Imprinted Gene | Oficial Name | Other Aliases | Chromosomal location | Cancer type | Reference |
|---|---|---|---|---|---|
| *MEST* | mesoderm specific transcript homolog (mouse) | *PEG1* | 7q32 | Osteossarcoma | [61] |
| | | | | Lung | [62] |
| | | | | Breast | [63] |
| | | | | Uterine leiomyoma | [64] |
| | | | | Wilms tumors | [65] |
| *CDKN1C* | cyclin-dependent kinase inhibitor 1C (p57, Kip2) | *BWCR, BWS, IMAGE, KIP2, WBS, p57* | 11p15.5 | Gastric | [66,67] |
| | | | | Breast Lung | [68] |
| | | | | Gastric Hepatocellular Pancreatic Acute myeloid leukemia | [69] |
| | | | | Bladder | [70] |
| | | | | Hepatocellular | [71] |
| | | | | Rhabdoid | [72] |
| | | | | Osteosarcoma | [61] |
| | | | | Pancreatic ductal | [73] |
| | | | | Esophageal | [74] |
| | | | | Wilms | [75] |
| *DLK1* | delta-like 1 homolog (Drosophila) | *DLK, Delta1, FA1, PREF1, Pref-1, ZOG, pG2* | 14q32.2 | Hepatocellular | [76] |
| | | | | Multiple myeloma | [77] |
| | | | | Acute myeloid leukemia | [78] |
| *PEG3* | paternally expressed 3 | *hCG_1685807, PW1, ZNF904, ZSCAN24* | 19q13.4 | Glioma | [79, 80] |
| | | | | Ovarian | [81, 82] |
| *NNAT* | neuronatin | *Peg5* | 20q11.2-q12 | Pediatric acute leukemia | [83] |
| | | | | Wilms | [65] |
| *GNAS* | GNAS complex locus | *RP4-543J19.4, AHO, C20orf45, GNAS1, GPSA, GSA, GSP, NESP, PHP1A, PHP1B, PHP1C, POH* | 20q13.32 | Pituitary | [84] |
| | | | | Somatotroph adenomas | [85] |
| *IGF2R* | insulin-like growth factor 2 receptor | *CD222, CIMPR, M6P-R, MPR1, MPRI* | 6q26 | Wilms'tumor | [86] |

| Imprinted Gene | Oficial Name | Other Aliases | Chromosomal location | Cancer type | Reference |
|---|---|---|---|---|---|
| *TFPI2* | tissue factor pathway inhibitor 2 | *PP5, REF1, TFPI-2* | 7q22 | Prostate | [87] |
| *KCNQ1OT 1* | KCNQ1 opposite strand/antisense transcript 1 (non-protein coding) | *KCNQ1-AS2, KCNQ10T1, KvDMR1, KvLQT1-AS, LIT1, NCRNA00012* | 11p15 | Colorectal | [88] |
| *IGF2* | insulin-like growth factor 2 (somatomedin A) | *PP1446, C11orf43, IGF-II, PP9974* | 11p15.5 | Gastric<br>Hepatocellular<br>Insulinomas<br>Wilms' tumor<br>Bladder | [89]<br>[90]<br>[91]<br>[92]<br>[93] |
| *KCNQ1DN* | KCNQ1 downstream neighbor (non-protein coding) | *BWRT; HSA404617* | 11p15.5 | Wilms' tumors | [94] |
| *SLC22A18* | solute carrier family 22, member 18 | *BWR1A, BWSCR1A, HET, IMPT1, ITM, ORCTL2, SLC22A1L, TSSC5, p45-BWR1A* | 11p15.5 | Breast | [95] |
| *WT1* | Wilms tumor 1 | *AWT1, EWS-WT1, GUD, NPHS4, WAGR, WIT-2, WT33* | 11p13 | Wilms' tumors | [96] |
| *PEG3* | paternally expressed 3 | *hCG_1685807, PW1, ZNF904, ZSCAN24* | 19q13.4 | Glioma<br>Ovarian | [97, 98]<br>[99] |
| *H19* | H19, imprinted maternally expressed transcript (non-protein coding) | *ASM, ASM1, BWS, D11S813E, LINC00008, NCRNA00008, PRO2605, WT2* | 11p15.5 | Colorectal<br>Ovarian<br>Hepatoblastoma<br>Laryngeal squamous cell carcinoma<br>Testicular seminomas<br>Prostate<br>Head and neck<br>Ovarian<br>Osteosarcoma<br>Bladder | [100]<br>[101]<br>[102]<br>[103]<br>[104]<br>[105]<br>[106]<br>[101]<br>[107]<br>[108, 93] |

**Table 1.** Imprinted genes and cancers with LOI and DNA-methylation changes.

## 4. Imprinting and bladder cancer

Bladder cancer is the second-most common genitourinary disorder and the sixth-most common disease in the world. Genetic and epigenetic alterations (Figure 1) are mostly likely involved in the malignant transformation and progression of this tumor type [109].

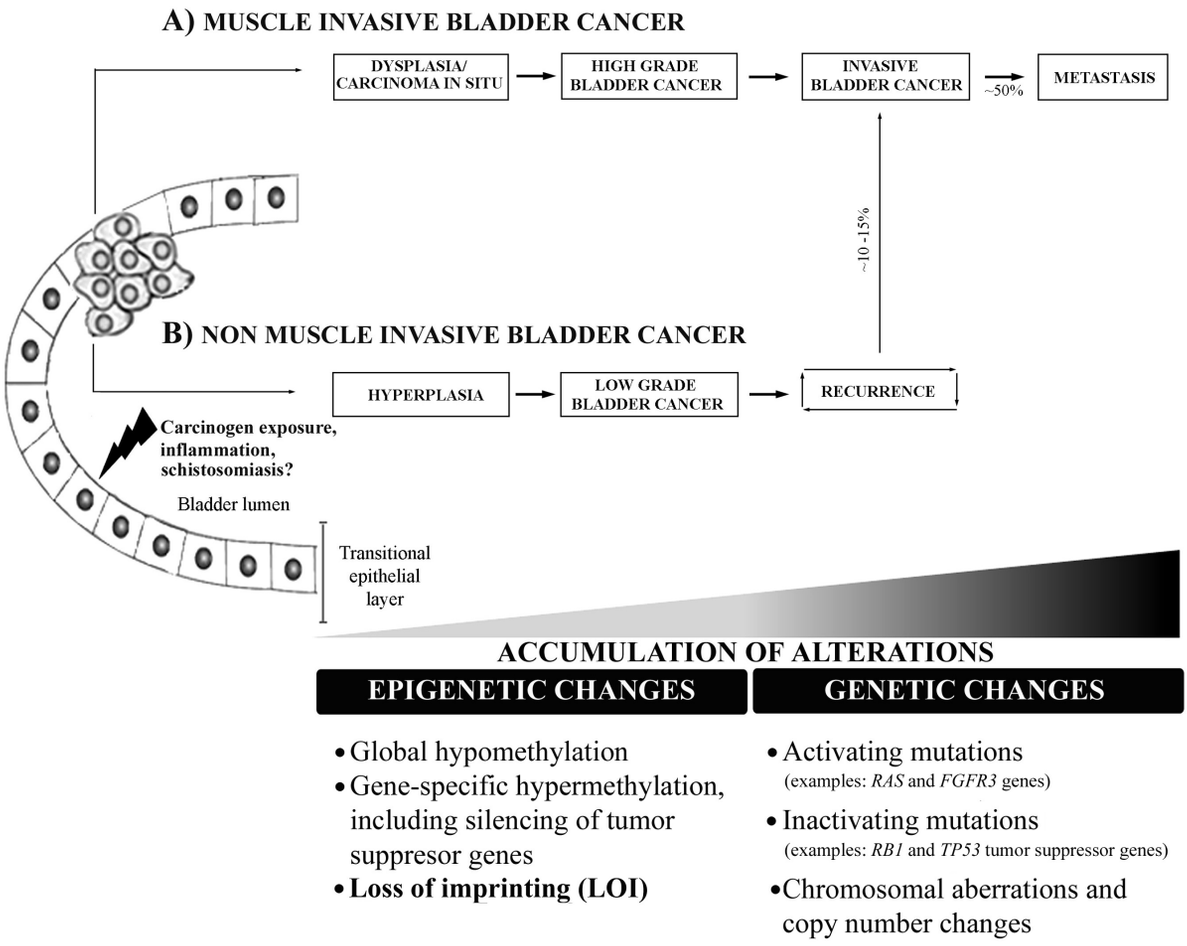


**Figure 1.** Urotherial carcinogenesis is a complex process resulting from the accumulation of genetic and epigenetic changes. Molecular and genetic analysis provide a framework for the characterization of molecular pathways (such as *RAS, FGFR3, RB1, TP53*-associated pathways) leading to tumor formation and clonal expansion. These pathways has been correlated with clinical and pathological parameters of both non-muscle and muscle invasive bladder cancer (A and B). Among other epigenetic changes, loss of imprinting (LOI) could lead to gene expression imbalances and contribute to the carcinogenesis process.

Currently, the diagnosis of bladder cancer is based on histological, pathological and morphological parameters and provides only a generalized outcome for patients [110]. In addition, the gold standard to detect and monitor bladder cancer is cystoscopy, which is an invasive and expensive method [111] even though this method shows poor performance in detecting low-grade tumors [112]. An understanding of cancer biomarkers will provide an opportunity to diagnose tumors earlier and with greater accuracy. Biomarkers can also help to identify those

patients with a risk of disease recurrence, progression and metastasis as well as predict which tumors will respond to different therapeutic approaches [113].

Although there are numerous studies reporting aberrant DNA methylation of several tumor suppressor genes in bladder cancer, studies regarding LOI in this tumor type are sparse.

### 4.1. Catenin (cadherin-associated protein), alpha 3 gene — *CTNNA3*

The *CTNNA3* gene encodes a novel alpha-catenin, alphaT-catenin, that has related functions to alphaE-catenin, a well-known invasion suppressor gene necessary for the formation of cell-cell adhesion complexes. In support of the hypothesis that *CTNNA3* is a new imprinted gene, Oudejans *et al*. [114] demonstrated that the 10q21.3 region containing the *CTNNA3* gene shows parent-specific imprinting patterns and that the transcription of this gene is down-regulated in placental tissues of androgenetic origin. It was later demonstrated that the *CTNNA3* gene is subjected to imprinting in early placental tissues with preferential expression of the maternal allele in the first trimester placental tissues [115]. However, it was observed that *CTNNA3* imprinting depends on the trophoblast cell type because the expression in the extravillous trophoblast is biallelic, whereas the expression in villous cytotrophoblast is maternal and monoallelic. The expression of alphaT-catenin is also lost in villous syncytiotrophoblast as well as in extravillous trophoblast following epithelial-mesenchymal transition, similar to the imprinting pattern of the cyclin-dependent kinase inhibitor 1C (*CDKN1C*) gene, also known as p57KIP. Taken together, these findings suggest that both genes share a conserved regulatory mechanism that correlates with an early step in placental development.

To the best of our knowledge, there is only one report in the literature describing the frequency of monoallelic versus biallelic expression of *CTNNA3* in urothelial carcinomas of the bladder [116]. Approximately 35% of informative bladder cancers showed monoallelic expression, which was specifically associated with the tumor tissue. Furthermore, the *CTNNA3* transcript levels were significantly lower in tumor samples compared with the controls, all of which displayed biallelic expression. These data suggest that epigenetic alterations of *CTNNA3*, such as monoallelic expression, may disrupt key molecules involved in the protein interactions in adherens junctions, such as beta catenin and E-cadherin, making *CTNNA3* a candidate marker for disease progression.

### 4.2. Cyclin-dependent kinase inhibitor 1C gene — *CDKN1C*

In humans, the imprinted gene *CDKN1C* is located at 11p15.5. This gene is expressed from the maternally inherited allele and encodes the $p57^{KIP2}$ protein, an inhibitor of cyclin-dependent kinases. *CDKN1C* is considered a candidate tumor suppressor gene because of its location on a frequently deleted genomic region in human cancers, biochemical activities and imprinting regulation [117]. The imprinting of this locus is controlled by an ICR located ~ 700 kb from the *IGF2*/*H19* genes towards the centromere. The paternal allele of *CDKN1C* is silenced by the long non-coding *LIT1*/*KCNQ1OT1* RNA that originates from the differentially DNA-methylated KvDMR1 [11], where resides the promoter for this gene.

In bladder cancer, the down-regulation of *CDKN1C* can be explained by multiple mechanisms, including a switch of both alleles toward a paternal imprinting pattern as indicated by DMR hypomethylation and described by Hoffman *et al*. [70]. The other mechanisms proposed by the author include a loss of heterozygosity (loss of expression of the maternal allele) and the hypermethylation of the promoter region, although this mechanism cannot be the only one responsible for the down-regulation of *CDKN1C*. Other studies have indicated that *CDKN1C* is a putative tumor suppressor gene in bladder cancer due to the reduced mRNA and protein levels compared with normal tissue. By immunohistochemical analysis, it was observed that the presence of the $p57^{KIP2}$ protein was detected in only 25.8% of the samples but in 100% of normal urinary bladder mucosa [118] suggesting that a decrease in $p57^{KIP2}$ expression may be a biomarker for bladder cancer. Furthermore, the decreased expression of *CDKN1C* mRNA was frequently observed in a study using samples of urothelial carcinoma tissues and cell lines. Interestingly, loss of *CDKN1C* transcripts was correlated with the loss of *H19* mRNA expression [119].

### 4.3. *H19*-imprinted maternally expressed transcript (non-protein coding) / insulin-like growth factor gene (*IFG2*)

The *IGF2* and *H19* genes are located in the human chromosome at 11p15.5. The imprinted cluster in this region has been implicated in a variety of cancers. Initially, the *H19* gene was thought to be involved in human cancer because of its potential tumor suppressor activity. When tumor cell lines were transformed with an expression vector containing this gene, there were morphological changes and a delay of growth [120]. However, later studies suggested that the *H19* gene has oncofetal characteristics due to abundant expression in some human fetal tissues and tumors arising from these tissues [121].

Although the mechanism of *H19* activity is controversial, it has been shown that the expression patterns of several genes are altered in the presence of *H19* RNA expression. These genes have been linked to potentially malignant cellular processes such as invasion, migration and angiogenesis. Additionally, the expression of some genes with functions in cell adhesion was inversely correlated with *H19* expression, which may lead to the development of more invasive tumors [122].

The *H19* gene produces a 2.3-kb non-coding RNA transcript that is capped, spliced and polyadenylated. No protein product has been identified. Recently, Cai and Cullen [123] showed that the *H19* transcript can function as a primary miRNA in humans and mice. These authors suggested that although this miR-675 is a derivative of the *H19* gene, it does not have a defined role, although it is possible that it functions as a regulator of mRNAs.

The *IGF2* gene encodes the insulin-like growth factor II protein, which is structurally homologous to insulin, and promotes growth and plays a role in metabolic processes in various cell types [45]. *IGF2* is regulated in a precise manner to maintain the monoallelic expression, which highlights the importance of gene dosage. The LOI of *IGF2* was first observed in the Wilms' tumor [46, 47], and subsequent studies have found that aberrant imprinting or LOI of *IGF2* is linked to many types of tumors.

Investigation into the role of the *H19* in bladder cancer began in 1995. Ariel *et al*. [121] suggested that the *H19* gene was a potential cancer marker because it was prominently expressed in more malignant and invasive transitional cell carcinomas as well as in *in situ* carcinomas, demonstrating unpredictable behavior with high rates of recurrence, progression and metastasis. These data were later confirmed, demonstrating that *H19* expression was specifically associated with tumors, with no detection of expression in normal urinary bladder mucosa, suggesting that *H19* may have oncogenic properties in the bladder urothelium [124].

Disrupted *H19* imprinting was first demonstrated in bladder cancer in a small number of samples. Among the four informative samples (heterozygotes for a neutral genetic polymorphism), two tumors showed biallelic expression of the *H19* gene. The same study showed LOI of the *IGF2* gene in three cases [93]. LOI of *IGF2* and *H19* at low frequencies was also described by another study in which only 12.5% and 22.2% of informative samples for the *H19* and *IGF2* genes, respectively, demonstrated this alteration. A DNA methylation analysis of the DMR showed a consistent decrease in the percentage of methylation from normal to tumoral tissue in the methylated allele. In both the methylated and unmethylated alleles of the *IGF2* DMR, the average amount of methylation decreased from normal to tumoral bladder tissue, showing a relationship between the altered methylation in the DMR and a loss of imprinting pattern in bladder cancer [125].

Most tumors in the urinary bladder are superficial, with a low risk of metastasis. In less than one third of the cases, the tumor is invasive and compromises the muscle layer. Despite this low risk of metastasis, bladder cancer has a high risk of recurrence [126]. The *IGF2* gene was shown to have a role in invasion and metastasis in several types of cancer (reviewed in [127]. In bladder cancer, a recent study showed a connection between the increased levels of *IGF2* and cytoplasmic immunolocalization of E-cadherin in nonmuscle invasive tumors with 57% of analyzed tumors demonstrating LOI and cytoplasmic expression of E-cadherin. The study also demonstrated that E-cadherin may indicate tumor recurrence independently of tumor grade or stage [128]. The *CDH1* gene encodes a critical protein involved in epithelial adhesion. The process of epithelial-mesenchymal transition (EMT) has been identified as an important prognostic biomarker in bladder cancer [129] and plays a central role in the process of carcinoma cell dispersion [130]. Morali *et al*. [131] demonstrated that the IGF2 protein induced the spread and loss of cell-cell contacts in rat bladder carcinomas derived from NBT-II cells and decreased the mean tumor height from 6.8 μm to 4 μm after 3 hours of treatment with IGF2.

The ICR located upstream of the *H19* gene and its DMR contains seven CTCF binding sites. Takai *et al*. [108] analyzed these sites in normal human embryonic ureteral tissue and found that only the sixth site demonstrated allele-specific methylation, whereas the others sites were methylated. In the analysis of the sixth site in six samples of human bladder cancer, two cases showed hypomethylation of the paternal allele, and the CpG islands in the maternal alleles of the remaining cases were sporadically methylated. The methylation status of the sixth CTCF-binding site was also investigated in human bladder cancer and normal bladder tissues. The authors suggested that the hypomethylation of the paternal allele observed in bladder cancer was nearly absent in normal bladder tissue. This hypomethylation could be more prevalent

than methylation in the maternal allele at this locus and might play a role in the overexpression of *H19* in advanced-stage bladder cancer, as reported by Cooper *et al*. [124].

Carcinogen exposure is one of the mechanisms implicated in the development of human bladder carcinomas. In a mouse study that induced bladder cancer by N-butyl-N-(4-hydroxybutyl) nitrosamine exposure, the expression of *H19* was first noted in the lamina propria (the drug was administered for 5 weeks) and posteriorly in epithelial cells (the drug was administered for 20-28 weeks). The alterations in *H19* expression levels were consistent with preneoplastic changes in the transitional epithelium of the bladder [132].

Because the *H19* gene is not expressed (or is expressed at low levels) in normal adult tissues but is expressed in tumors derived from tissues previously expressing it during the embryogenic period, *H19* could be exploited for alternative therapeutic approaches. In fact, regulatory sequences of *H19* were used in a vector that expressed diphtheria toxin (DT-A) or herpes simplex virus thymidine kinase (HSV-tk) that were then transfected into tumoral cell lines, including a cell line derived from bladder cancer, and injected in an animal model of bladder cancer. It was found that the expression of DT-A was specific to T24P bladder cancer cells compared with human fibroblast IMR-90 cells. The in vivo experiment showed that the weights of the tumors from DTA-PBH19-treated animals (with 3 doses) were significantly less than the tumors from the control animals. Similar results were observed in animals treated with the *TK-H19* construct and ganciclovir (GCV) in a single dose, although the tumors started to resist the growth-inhibitory effects of the *TK-PBH19* and GCV treatment after the eighth day of treatment. These initial findings demonstrated that the *H19* regulatory sequence was capable of driving expression of therapeutic genes [133].

Recently, a double promoter expressing DT-A was constructed with two regulatory sequences (*H19* and *IGF2*-P4) and tested in bladder cancer cell lines and animal models. The inclusion of two promoters was more efficient at lysing the cancer cell lines when compared to the single-promoter constructs, *H19*-DTA or *IGF2*-DTA. This increased efficacy was also observed in the growth inhibition of heterotopic bladder tumors, with a 70% reduction in tumor development compared to controls after three injections. The treatment of orthotopic tumors inhibited tumor growth, reducing the size of treated tumors to 86% of the size of tumors found in the control animals [134]. These findings suggest that this approach could be applied in cancer therapy.

### 4.4. Predict imprinted genes and bladder cancer

Although few studies have reported LOI in well characterized imprinted genes (such as *IGF2* and *H19*) in bladder cancer, there is a list of newly predicted imprinted genes already implicated in this type of tumor, some of them are candidates to diagnostic and/or prognosis markers.

A newly identified gene, *BLCAP* (bladder cancer associated protein), is a novel tumor suppressor gene candidate in human bladder cancer. This gene, also known as BC10 protein (bladder cancer-10 kDa protein), is located at 20q11.23 and encodes a small protein with unknown cellular functions. Although it has no homology to any known protein [135], it includes putative cytoplasmic domains at the N- and C-terminal ends, a SPXX motif and a

proline-rich area resembling the PXXP domain, which suggests that it may play a role in cell signaling [136]. Transcriptional down-regulation of this gene has been observed in different tumor types [137-138-139] including invasive bladder cancer [136]. In support of its role as a tumor suppressor, Fan *et al*. [140] showed that overexpression of *BLCAP* resulted in growth inhibition and induced apoptosis of human Ewing's sarcoma cells *in vitro*. In a recent study of 120 patients and validated with 2,108 samples, the authors confirmed that the loss of *BLCAP* expression is associated with tumor progression, high levels of nuclear protein expression and a poor prognosis, suggesting that *BLCAP* expression may be a prognostic biomarker[135].

*BLCAP* was initially considered a non-imprinted gene in human fetal tissues, with biallelic expression in the fetal brain, adrenal gland, heart, kidney, liver, lung and placental tissues, and showed an unmethylated promoter-associated CpG island in all tissues evaluated [141]. Recently, it was demonstrated that the *BLCAP* gene is imprinted in the human and mouse brains and this tissue-specific pattern may be regulated by the high levels of *NNAT* transcription in the brain [142]. The *NNAT* gene lies within the intron of the *BLCAP* gene [142] and is specifically expressed from the paternal allele in the central nervous system from mid-gestation through early postnatal development [141]. Since that *NNAT* gene may influences the imprinting of the *BLCAP* gene, it may be interesting to study the loss of imprinting of both genes in bladder cancer.

Another gene that may be regulated by genomic imprinting is the retinoblastoma tumor susceptibility gene (*RB1*). This important discovery was made in a genome-wide analysis of CpG methylation from the blood sample of a child with multiple imprinting defects. This study revealed a differential methylation pattern of a specific CpG island located within the intron 2 of the *RB1* gene. It was suggested that the presence of the CpG island resulted from a retrotransposition event in the KIAA0649 gene between exon 4 and an 18 bp segment of the 3'end of exon 3. The authors also showed that the CpG island 85 is unmethylated on the paternal chromosome and that this CpG island on the maternal chromosome is methylated, with a difference in gene expression favoring the maternal allele [143]. This finding was unexpected because the paternal transcripts were predicted to be more highly expressed than the maternal transcripts. According to the authors, this finding could be a result of transcriptional interference in which the lack of methylation of CpG number 85 and the expression of a transcript (2B-transcript) could interfere with the expression of the paternal allele. To explain this finding, Buiting *et al*. [144] proposed a model in which the binding of a transcriptional complex in the unmethylated 2B-promoter region (paternal) blocks the transcriptional complex that regulates the expression of an alternate transcript from the promoter located upstream to exon 1, resulting in a low abundance of the paternal allele. Recently, Nakabayashi *et al*. [145] confirmed the maternal methylation of the RB1 DMR in a study of rare reciprocal genome-wide uniparental disomy samples in patients with Beckwith–Wiedemann and Silver–Russell syndrome-like phenotypes.

The *RB1* gene was one of the first tumor suppressor genes discovered, and its loss of function has been reported in various tumor types. Rb1 protein interacts with a large and steadily growing list of cellular proteins and an even greater number of genes [146], reinforcing its central role in carcinogenesis. In bladder cancer, there are a large number of studies implicating

the *RB1* gene in tumoral development and progression. Aggressive tumoral behavior, such as in invasive high-stage muscle tumors, was associated with the down-regulation of *RB1* mRNA and protein in addition to altered mRNA expression of *TP16* and *CDK4* [147].

In some regions in the world, bladder cancer is associated with the urinary form of schistosomiasis. Abdulamir *et al*. [148] profiled the molecular markers in schistosomal and non-schistosomal bladder tumors and found lower expression levels of Rb protein in patient tumors not caused by parasitic infection and an association between down-regulation of the protein and late stages of the disease (III and IV) in the schistosomal and invasive non-schistosomal bladder tumors. These findings support the hypothesis that the Rb protein can be used as a prognostic marker and distinguish a tumor caused by infection from a tumor not caused by infection.

According to the model proposed by Buiting *et al*. [144], the loss of imprinting (demethylation of the maternal allele) could explain the lack/decrease in *RB1* gene transcripts mentioned above, highlighting the need to understand the mechanisms behind the down-regulation of the *RB1* gene. Furthermore, methylation of the RB1 gene promoter was evaluated in 45 patients with bladder cancer and in bladder cancer cell lines. However, the authors found unmethylated promoter-associated CpG island in all bladder cancer cell lines and primary tumors examined [149]. More recently, a study involving a large number of genes investigated the methylation status of 25 proven or suspected tumor suppressor genes in pT1G3 transitional cell carcinomas. The authors found that tumors displaying unmethylated *RB1* and *TP73*, among others genes, had higher progression rates in patients treated with non-maintenance bacillus Calmette-Guérion (BCG) [150].

These studies found an unexpected result compared with the studies of *RB1* gene expression, as the decreased expression of this gene could be linked to hypermethylation of the promoter. However, these studies did not examine the expression of the *RB1* gene; therefore, the association between the unmethylated promoter and cancer progression found in the study by Agundez *et al*. [150] could be due to the decreased expression of the gene associated with a loss of imprinting (demethylated maternal allele) at intronic CpG island 85.

*TP73* is a *TP53*-related gene that encodes a p73 protein that shares considerable homology with the tumor suppressor gene *TP53*, which was previously associated with the development of neuroblastoma and other tumors [151]. This gene is located at 1p36.3 and was shown to be a monoallelically expressed gene (reviewed in [152]) with maternal expression. Information about the imprinting of *TP73* gene in cancers is still limited and contradictory [153]. Kaghad *et al*. [151] demonstrated that p73 is a candidate for the putative, imprinted neuroblastoma suppressor gene; however, studies have shown a relationship between the loss of imprinting (biallelic expression and switching alleles) and some types of cancer, such as ovarian cancer [154], breast cancer [155] and gastric adenocarcinoma [156].

In bladder cancer, the loss of imprinting and an elevated expression of the *TP73* gene was suggested at first by Chi *et al*. [157], who found *TP73* biallelic expression in 52.2% of tumor samples analyzed but not in the normal tissue samples, with higher expression of the transcript in biallelic expressers (66.7%), whereas only 2 (18.2%) of 11 monoallelic expressers showed

high expression levels of this transcript. The authors also demonstrated that there is a positive correlation between high expression of *TP73* and tumor stage or grade. Based on these findings, it was suggested that the *TP73* gene is not a tumor suppressor in bladder carcinogenesis and that the loss of imprinting (activation of a silent allele) could contribute to the progression of bladder tumors. The overexpression of the *TP73* was also observed in 22 of 23 bladder cancer samples in a second study. However, when the allele-specific expression was evaluated, the biallelic expression of the gene was observed in all cancers and matched normal tissues [158].

## 5. Perspectives

It is well known that disruption of epigenetic processes can lead to altered gene expression associated with malignant cellular transformation. Still, it has been demonstrated that LOI occurs in a large variety of human cancers, however it remains to be determined if there is a commonality to the cell type which initially undergoes this alteration [159]. Moreover there is a need for greater knowledge of imprinted genes, since disrupted expression of them has been shown to have either oncogenic or tumour suppressing activity [11]. Future studies will provide new insights, particularly into interactions between products of imprinted genes in physiological pathways [9]. Among other epigenetic changes, the loss of imprinting in cancer may prove useful for advancing our knowledge and for development of new prognostic and therapeutic biomarkers.

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