Mechanism of Sex Differences in Bladder Cancer: Evident and Elusive Sex-biasing Factors

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Abstract. Bladder cancer incidence is drastically higher in males than females across geographical, racial, and socioeconomic strata. Despite potential differences in tumor biology, however, male and female bladder cancer patients are still clinically managed in largely uniform ways. While sex hormones, and sex chromosomes have been shown to promote observed sex differences, a more complex story lies beneath these evident sex-biasing factors than previously appreciated. Advances in genomic technology have spurred numerous preclinical studies characterizing more elusive sex-biasing factors that include epigenetics, X chromosome inactivation escape genes, single nucleotide polymorphism, transcription regulation, metabolism, immunity, and many more. Sex-biasing effects can be leveraged for future efforts to provide precision medicine based on a patient’s biological sex. In this review, we will highlight key findings from the last half century that demystify the intricate ways in which sex-specific biology contribute to differences in pathogenesis as well as discuss future research directions.

Keywords: Sexual dimorphism, bladder cancer, sex hormones, sex chromosomes, epigenome

INTRODUCTION

Bladder cancer (BC) is one of the top non-reproductive cancers exhibiting stark male and female differences: men experience greater incidence by 3–5 times and females are more often diagnosed with advanced disease [1]. Although these differences have been well-documented over the last half century, underlying causes remain obscure.

Sexual dimorphism was once erroneously thought to occur only after the development of the gonads and subsequent secretion of sex hormones. This understanding naturally promoted a strong attribution to sex hormones as the cause for male and female differences in development and diseases. Now, we have a deeper appreciation that the baseline unequal chromosomal distribution between biological sexes—such as evidently illustrated by the X
Fig. 1. Sex-Biasing Factors of Bladder Cancer. Evident sex-biasing factors differentiating male and female bladder cancer (BC) incidence such as environmental risks, sex hormones, and sex chromosomes have historically been emphasized but do not accurately illustrate the full scope of factors driving sex differences. Many more hidden yet important factors, which we describe as elusive factors, that may contribute to higher male BC incidence include the following: epigenetics, gene regulation and expression, X chromosome inactivation escape genes, metabolism, the microbiome, long non-coding RNAs, imprinting, and immunity.

EVIDENT SEX-BIASING FACTORS

Behavioral and environmental factors

Tobacco smoke is a major risk factor for BC [11]. Unequal smoking patterns between males and females provides a reasonable explanation for observed sex disparities. However, prevailing differences in BC incidence among males and females with similar smoking levels validate the hypothesis that smoking itself is not the sole cause of gender disparities in BC [12–14].

Due to the historic workplace of men, higher chances of occupational exposures compared to women—especially for workers near aromatic amines, leather, paint, industrial machines, and aluminum—were once deemed as likely factors driving higher BC incidence in men. In 1990, however, the National Bladder Cancer Study by Hartge et al., showed that environmental exposure and cigarette smoking did not fully explain sex differences in BC [14].

For decades, attention has been skewed towards attributing environmental factors to explain male and female differences in BC incidence and prognosis. However, these aforementioned studies marked the beginning of a turn away from environmental factors to look into sex as a biological variable (SABC) in bladder tumor development and therapy.

Sex hormones

Androgens and androgen receptors (AR)

Sex steroid hormones and their respective receptors in BC have been studied for nearly half a century. In 1972, androgens were first identified as a BC risk factor when castrated male mice were better protected against bladder carcinogenesis induced by N-butyl-N-(4-hydroxybutyl)nitrosamine.
Table 1

Summary of sex-biasing factors and corresponding publications

<table>
<thead>
<tr>
<th>Sex-biasing factor</th>
<th>Male and Female Difference</th>
<th>Relevance in Bladder Cancer (Y/TBD)</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tobacco smoke</td>
<td>Behavioral risk of smoking</td>
<td>Y</td>
<td>[11–14]</td>
</tr>
<tr>
<td>Environmental toxins</td>
<td>Historic workplace of men yields greater exposure to environmental toxins</td>
<td>Y</td>
<td>[14]</td>
</tr>
<tr>
<td>Testes</td>
<td>Higher BC incidence in male mice with testes</td>
<td>Y</td>
<td>[15]</td>
</tr>
<tr>
<td>Androgens</td>
<td>Androgens promote BC development in rats and mice</td>
<td>Y</td>
<td>[15, 16]</td>
</tr>
<tr>
<td>AR</td>
<td>AR knockouts have decreased BC risk; AR gain of function have increased BC risk</td>
<td>Y</td>
<td>[23]</td>
</tr>
<tr>
<td>Goi/MAPK/MM9</td>
<td>Androgen independent pathway promoting BC</td>
<td>Y</td>
<td>[24]</td>
</tr>
<tr>
<td>p53</td>
<td>AR promoting BC in males by inhibiting p53 tumor suppressor activity; KDM6A inhibiting BC in females via p53 gene targets</td>
<td>Y</td>
<td>[3, 22, 25, 26]</td>
</tr>
<tr>
<td>CD24</td>
<td>Downstream AR target linked to BC cell proliferation in male mice</td>
<td>Y</td>
<td>[27]</td>
</tr>
<tr>
<td>CD44</td>
<td>AR directly represses CD44 suggesting AR’s stage-dependent tumor promoting capacity</td>
<td>Y</td>
<td>[28]</td>
</tr>
<tr>
<td>Wnt/β-Catenin</td>
<td>AR potentiating Wnt/β-Catenin pathway that induces BC proliferation</td>
<td>Y</td>
<td>[29]</td>
</tr>
<tr>
<td>CD8+T cell</td>
<td>Dysfunction of CD8+T cells mediated by androgens</td>
<td>Y</td>
<td>[35]</td>
</tr>
<tr>
<td>Estrogens</td>
<td>Estrogens suppressed BC development in rats</td>
<td>Y</td>
<td>[16]</td>
</tr>
<tr>
<td>ERα</td>
<td>Inhibitory effect on BC tumorigenesis in female mice</td>
<td>Y</td>
<td>[40]</td>
</tr>
<tr>
<td>ERβ</td>
<td>Promoting effect on BC tumorigenesis in female mice</td>
<td>Y</td>
<td>[41]</td>
</tr>
<tr>
<td>Progesterone</td>
<td>Multiparous mice with decreased tumor size; multiparous female patients with decreased BC risk</td>
<td>Y</td>
<td>[43–45]</td>
</tr>
<tr>
<td>XX vs XY</td>
<td>XX protective effects independent of estrogens</td>
<td>Y</td>
<td>[3]</td>
</tr>
<tr>
<td>LOY</td>
<td>Age-related LOY associated with shorter lifespan in men; loss of compensatory UTY on the Y chromosome as paralog of KDM6A may result in increased male BC risk</td>
<td>TBD</td>
<td>[54]</td>
</tr>
<tr>
<td>KDM6A</td>
<td>X chromosome inactivation escape gene conferring BC protection in females</td>
<td>Y</td>
<td>[3, 68]</td>
</tr>
<tr>
<td>PRC2/EZH2</td>
<td>Antagonistic relationship with KDM6A in bladder urothelium</td>
<td>TBD</td>
<td>[69, 70]</td>
</tr>
<tr>
<td>COMPASS</td>
<td>KDM6A is part of the COMPASS protein complex</td>
<td>TBD</td>
<td>[85]</td>
</tr>
<tr>
<td>Sex-specific gene regulation</td>
<td>Differential gene regulation in non-reproductive tissues</td>
<td>TBD</td>
<td>[57, 58]</td>
</tr>
<tr>
<td>Carbohydrates and amino acids</td>
<td>Higher serum levels in males</td>
<td>TBD</td>
<td>[88]</td>
</tr>
<tr>
<td>Lipids</td>
<td>Predilection for lipid biosynthesis in females</td>
<td>TBD</td>
<td>[89]</td>
</tr>
<tr>
<td>SULT1A1</td>
<td>Sulphotransferase gene decreased BC risk only in females</td>
<td>Y</td>
<td>[90]</td>
</tr>
<tr>
<td>UGT detoxification</td>
<td>AR represses UGT expression; UGT downregulation associated with tumor formation in mice and humans</td>
<td>TBD</td>
<td>[96, 99, 100]</td>
</tr>
<tr>
<td>Mitochondria/ROS</td>
<td>Higher ROS accumulation in males</td>
<td>TBD</td>
<td>[87]</td>
</tr>
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</table>

Note: TBD, to be determined; AR, androgen receptor; ER, estrogen receptor; LOY, loss of Y chromosome; KDM6A, lysine demethylase 6A; PRC2, methyltransferase of polycomb repressive complex 2; EZH2, enhancer of zeste homolog 2; COMPASS, complex of proteins associated with Set1; TOP2B, DNA topoisomerase 2 beta; UGT, UDP-glucuronosyltransferases; ROS, reactive oxygen species.

(BBN) than uncastrated males, and female mice treated with testosterone had increased BC risk compared to control female mice [15]. Preclinical studies further suggested that androgens induce while estrogens suppress carcinogen-induced BC development in rats [16]; clinical observation of men having decreased BC mortality after treatment with 5α-reductase inhibitors or dihydrotestosterone (DHT) blockers further solidified the relevance of androgens [17]. Ultimately, these studies were hypotheses generating, as it remained unclear how exactly androgens initiated or potentiated BC. The next round of breakthrough studies came from molecular characterization of the androgen receptor (AR).

Using patient samples, Laor et al., observed greater levels of AR in tumor tissue compared to normal tissue, and females had relatively lower levels than males [18]. High grade tumors—although intuitively expected to exhibit increased AR—paradoxically had less AR than low grade tumors. It should be noted that other studies showing conflicting results could be due to a lack of standardized staining and scoring.
Usage of genetic engineered mice (GEMs) subsequently unlocked a series of insightful studies. Mice with AR gene knockout (ARKO), either germline [21] or urothelium-specific knockouts [22], were less susceptible, while mice expressing extra AR (hAR$^{GOF}$) [23] were more susceptible to BC induced by a bladder-specific carcinogen. The genetic evidence convincingly demonstrated the essential role(s) of AR in promoting bladder tumorigenesis. Findings from Miyamoto et al., further implicated how AR and androgens did not completely rely on one another to mediate their cancer driving effects [21]. For example, DHT treated and untreated male ARKO mice developed bladder carcinoma at rates of 25% and 0%, respectively, suggesting the function of androgens via a non-AR pathway. Consistent with these implications, Chang and colleagues recently reported a membrane androgen receptor (mAR-SLC39A9) that functions through a noncanonical AR pathway Ga\(\alpha\)/MAPK/MM9 to promote bladder cancer [24].

Mechanistic studies on AR have led to the identification of downstream target genes and molecular pathways. Initial characterizations of AR knockout mice suggested that tumor promoting activity of AR depends on the p53-PCNA DNA damage repair pathway [22]. Indeed, somatic loss-of-function of p53 tumor suppressor gene is frequently observed in BC [25, 26]. The notion that AR inhibits p53 tumor suppressor activity during bladder tumor initiation has been further supported by the observation that inactivation of p53 and Rb by SV40T antigen completely blunted the effect of AR [22]. The activity of AR in inducing BC cell proliferation in vitro [21, 27] also appears to be dependent on $CD24$, a glycosyl phosphatidylinositol-linked sialoglycoprotein—a direct downstream target of AR [27]. Interestingly, inactivation of $CD24$ is protective against BBN-induced BC in male but not in female mice, suggesting that the sex-biasing activity of AR is in part mediated by $CD24$ in vivo [27]. Moreover, an elegant study by Theodorescu and colleagues demonstrated that AR directly represses gene transcription of $CD44$ [28], the receptor for hyaluronic acid—a biomarker and potent driver of progressive disease in multiple tumor types, suggesting that AR may function in a stage dependent manner in promoting bladder tumor initiation but suppressing tumor progression.

Ectopic activation of the $Wnt/\beta$-Catenin signaling pathway in the bladder urothelium induces luminal bladder tumor in mice [29]. Like the BBN-induced bladder carcinogenesis model, this GEM model of BC also displays a striking sex difference: the incidence in males is 45% vs 3% in females when the $Wnt/\beta$-Catenin pathway is constitutively activated in the bladder urothelium. Mechanistically, constitutive $\beta$-Catenin activation induces nuclear translocation of AR while conversely, activation of the AR pathway potentiates the $Wnt/\beta$-Catenin signaling in bladder urothelial cells. Similar genetic interactions between androgen and $Wnt/\beta$-Catenin signaling pathways are also found during the masculinization of external genitalia [30]. Together, these observations suggest that synergism between the AR and $Wnt/\beta$-Catenin pathways may enhance sex differences in development and diseases.

Studies have also suggested the roles of androgens in dampening the host immunity against cancer to therefore contribute to increased incidence and mortality of male patients to cancer [31]. For example, androgens have been shown to be immunosuppressive by inhibiting the function of macrophages, NK cells and T cells. Due to persistent antigen stimulation and hostile tumor microenvironment (TME) for tumor-infiltrating T cells, tumor-specific T cells are known to undergo programmatic dysfunction and exhaustion with diminished ability to mount effector function against cancer [32]. The mechanism of molecular programming of T cell exhaustion is not entirely clear, but the process involves key transcriptional and epigenetic changes centered around important transcription factors including Tcf7 (encoding Tcf1) and TOX [33, 34]. Using single cell RNA sequencing coupled by transcription studies, we have recently discovered that androgens can promote CD8$^+$ T cell exhaustion in the TME by directly transactivating Tcf7 [35].

Overall, we have taken significant strides to characterize androgens/AR pathway in male BC incidence and progression. However, key questions remain: Are androgens and AR solely responsible for sex differences in BC development? Will targeting these pathways have potent therapeutic value? While clinical trials (e.g., NCT02605863, NCT01234519, NCT02788201, NCT02300610)—such as ones evaluating the efficacy of existing hormone therapy for prostate cancer on recurrent NMIBC or advanced BC—had been initiated to test preclinical findings, trials consisted of small cohorts and in some cases were suspended due to insufficient enrollment. Meaningful clinical application would necessitate clinical trials with a larger patient cohort that includes both males and females.
Estrogens, Estrogen Receptors (ERα and ERβ), and Progesterone

Menopause provides a natural way to observe the effects of estrogen depletion in women. In a 2006 prospective study, post-menopausal women had increased BC risk and early age menopausal onset yielded even greater risk, suggesting the protective effects of estrogens [36]. These findings are preceded by Bertram and Craig’s initial observation of estrogen’s protective effects in female mice contrasted with testosterone’s effects to increase BC risk in male mice [15]. As straightforward as this seems, a closer look at the relationships between estrogens, estrogen receptors, and progesterone reveals a more complex story.

Estrogens can function through two canonical nuclear receptors—ERα and ERβ—each responsible for potentially distinct functions. In rat and mice BC, ERα is inconsistently detected, thus initially viewed to have no prognostic value [37, 38]. However, in 2014, Hsu et al., showed that ERα knockout mice developed tumors faster than females with ERα, suggesting ERα’s inhibitory role in tumorigenesis [39]. Contrastingly, ERβ is expressed in human tumor tissues and increases with higher stage and grade BC [40]. In female mice, depleting ERβ slowed down tumor growth [41]. Herein suggests a cause for sex disparities: while females have ERβ to drive BC likes males have AR, females also have a protective pathway via ERα that males may not have. It has also been shown that tamoxifen given before, during, or after BBN administration conferred protection from urothelial carcinogenesis for female mice, pointing to the prospects of Tamoxifen or other selective ER modulators as a chemoprotective agent against BC for female patients [42].

Progesterone may also play a role. Parous female mice—those who gave birth and thus experienced changes in progesterone—developed smaller bladder tumors than nulliparous mice [43]. Similarly, multiparous female patients had decreased BC risk [44, 45]. Thus far, there have not been follow-up studies to investigate the underlying mechanisms behind these observations; it would be interesting to see if progesterone mechanistically acts independently of ER receptors, alongside ERα, and/or antagonistically with ERβ to decrease BC risk in females.

Ultimately, sex hormones can only be partially responsible for BC sex disparities. Given that the median age of BC diagnosis is 73—an age when sex hormone levels have markedly decreased—how can sex hormones be the sole responsible player? Other critical sex-biasing factors must also be at play.

Sex chromosomes

In mammals, there is an unmistakable genetic inequality between sexes: males have one copy of the X and Y sex chromosomes, while females have two copies of the X chromosome. Activity of Sex-determining Region Y (Sry) encoded by the Y chromosome initiates differentiation of the gonads into the testes instead of ovaries and androgens secreted by the testes further induce male-specific sex differentiation. Because of the dominant roles of gonadal hormones, sex chromosomes have historically been overlooked for their potential function to induce male and female BC differences and has, only in more recent years, been studied for its unique sex biasing effects. A turning point began after the 2008 epidemiological study revealed how Turner syndrome patients—phenotypic females missing completely or partially one copy of the X chromosome (i.e., XO)—had higher BC risk than XX females [46]. In the same vein, Klinefelter’s patients—phenotypic males with one or more extra X chromosome(s) (e.g., XXY)—had lower risk of solid tumors than XY males [47]. While these studies suggested the potential roles of sex chromosomes, they were correlative and could not definitively conclude the independent effects of the X and Y chromosome because confounding factors such as chronic urinary tract infections, altered levels of sex hormones, or other pre-existing comorbidities were not accounted for.

To directly examine the role of sex chromosomes (XX vs XY) in BC risk, we used age-matched “four-core genotype (FCG)” mice, which consist of four sex types: two testes-bearing types with either XX or XY and two ovaries-bearing types with either XX or XY [3, 35]. The FCG technology innovatively uncouples the sex chromosome effect (SCE) and gonadal hormone effect (GHE), thereby enabling quantitative evaluation of independent as well as interactive effects of these central sex-biasing variables [48]. We treated FCG mice with the bladder-specific carcinogen BBN and monitored BC development and overall survival of these mice [3]. Cox proportional analysis confirmed the independent sex-biasing effects of testis or androgen hormones with a hazard ratio 4.714 (vs ovary, 95% CI = 2.77–8.28). This study also unequivocally demonstrated that the sex chromosome complement is a sex-biased risk fac-
Independent and cooperative effects of sex chromosomes and
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Fig. 2. Independent and Interactive Effects of Sex-biasing Factors.
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ELUSIVE SEX-BIASING FACTORS

Genetic Imbalance

X Chromosome Inactivation (XCI) Escapes

One copy of the X chromosome in XX female cells
is normally inactivated in a random manner through
a process called X Chromosome Inactivation (XCI).
This dosage compensation mechanism ensures that
the X-linked genes are expressed at comparable lev-
els between XX and XY cells. However, XCI is an
imperfect process—a fraction of genes escapes XCI,
leading to higher levels of XCI escapes expressed
in XX females than in XY males. In humans, nearly
23% of the X-linked genes escapes XCI [55], which
supports the hypothesis that XCI escapes contribute
to sex differences in cancer. By analyzing paired
tumor–germline exome sequencing data from 4,126
patients across 21 tumor types from TCGA and Broad
Institute data sets, Lane and colleagues identified
that loss-of-function mutations of six X-chromosome
genes located at the non-pseudoautosomal region
(ATRX, CNKSR2, DDX3X, KDM5C, KDM6A, and
MAGEC3) are more frequently mutated in males than
females [5]. KDM6A is an important example of XCI
affecting sex differences and will be later discussed.
However, none of the other candidates are associ-
ated with BC outcomes, suggesting that the effects
of XCI escapes are cancer type dependent. By interro-
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somatic mutation frequency of CTNNB1 in liver hepat-
ocellular cancer is significantly higher in males than
females, implying that there are sex differences
in oncogenic mutational processes beyond the muta-
tional differences of X-chromosome-encoded genes
[7, 8].

Sexually dimorphic genetic architecture

Genetic architecture is a term to describe the
collective genetic variation linked to observed phe-
notypic differences. The advancement of genomic
techniques and curation of large genomic databases,
such as the Genotype-Tissue Expression (GTEx)
project [56], has made studying sex differences of
gene expression (GEX) and other sex biasing effects.

Table 2

Independent and cooperative effects of sex chromosomes and
gonadal hormones to drive sex differences in BC risk

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Loss of Y chromosome (LOY)—shown to be induced by smoking—frequently occurs in males
with BC [12, 49–52], but its lack of association to
tumor stage or patient survival has caused many to
initially question its clinical importance [12, 52, 53].
However, Forsberg et al., showed that age-related
LOY in men resulted in a median shorter lifespan
of 5.5 years compared to men without LOY, suggest-
ing that Y chromosomal effects on cancer risk cannot
be forgone [54]. Nevertheless, LOY is not associ-
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One potential role of the Y chromosome may be rele-
gated to epigenetic regulation which will be discussed
later.

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disease trait, or predisposition to a disease—may har- 466
bor SNPs to explain male and female differences in 467
BC incidence and mortality.

While sex differences in gene expression exists, 468
Lopes-Ramos et al., interestingly observed more sex 469
differences on the level of transcription regulation 470
than gene expression in a tissue specific manner: 471
87% of genes were differentially targeted, meaning 472
they experienced different regulatory pressures, but 473
70% of these differentially targeted genes, did not 474
show differential expression [57]. This means that 475
certain phenotypes may manifest in a latent man- 476
ner as time, age and stress differentially impact male 477
and female specific transcription factors. Meaning, 478
at any given time point, the potential for differential 479
expression may be higher than observed due to 480
differences in regulation hidden beneath more uni- 481
form expression. More recently, Oliva et al., used 482
version 8 of the GTEx database to include analy- 483
sis of cis sex-biased expression quantitative trait loci 484
(sb-eQTLs) and performed allelic expression analy- 485
sis between males and females [58]. Their findings 486
reiterated the concept that a seemingly similar phe- 487
notype between males and females is supported by a 488
diverse set of sex-specific checks and balances. Iden- 489
tified sex-biased genes were found to be involved with 490
pathways such as epigenetic methylation and drug 491
metabolism including CYP450 genes.

One limitation of these recent studies using the 492
GTEx dataset is the lower population data for blad- 493
der, endocervix, ectocervix, fallopian tube, renal 494
medulla than the rest of the tissue types ($n < 25$ vs. 495
$n = 73–670$), resulting in the exclusion of these five 496
tissues from the analyses. In general, this avenue of 497
research has not yet been extensively applied to study 498
sex differences in BC. Previous efforts of genome- 499
wide association studies (GWAS) have identified a 500
number of loci that are tightly associated with BC risk 501
[59–65]; but these efforts did not uncover sex-biasing 502
QTLs linked to BC. We believe that an alternative 503
strategy in analyzing gene regulation such as expres- 504
sion QTLs (eQTLs) could be insightful in explaining 505
male and female differences in BC incidence and 506
mortality by revealing the sex-specific variability in 507
response to the environmental signals and patholog- 508
ical states.

Sex specific selection constraints may be another 509
potential mechanism contributing to a sex-specific 510
genetic architecture. In two different studies, Ger- 511
shoni et al., found that both men and women had 512
reduced selection pressures on sex-specific genes 513
compared to non-sex specific genes [66, 67]. More- 514
over, the magnitude of decreased selection was 515
greater in men than women [67]. They postulate that 516
the reduced negative selection in male-biased genes 517
may contribute to a greater accumulation of delete- 518
rious mutations in men and in turn yield different 519
patterns of disease incidence. Whether this mecha- 520
nism is relevant in explaining BC sex differences is 521
not yet known and should be further explored.

Epigenetics: The sex epigenome

Epigenetic regulation lies at the heart of genetic 522
modification and phenotypic diversity. While barriers 523
studying this complex system may have dampened 524
rapid initial progress, its stable traction in recent 525
years along with the emerging concept of sex spe- 526
cific epigenetics—which we will collectively refer to 527
as the sex epigenome—brings a promising outlook to 528
translational application.

Modifications such as methylation and acetylation 529
of DNA and histones (as well as removal of the 530
modifications) define the simple yet ingenious way 531
for cells to selectively silence or promote expression 532
of specific genes to adapt to biological and envi- 533
ronmental changes. Abnormal or pathological states 534
often hijack or disturb this intricate epigenetic sys- 535	
In 2018, we identified a critical X-linked lysine 536
demethylase 6A (KDM6A or UTX) gene acting as a 537
tumor suppressor via the $p53$ pathway exclusively in 538
females [3]. In the BBN model of bladder carcino- 539
genesis, XY males are 12.39 times more likely than 540
XX females to develop and die from BC (Table 3). 541
In contrast, the BC risk ratio between sexes was 542
reduced by more than five folds to 2.349 in mutant 543
mice with the urothelium-specific $Kdm6a$ conditional 544
knockout. These observations strongly suggest that 545
$KDM6A$ is the primary sex-biasing tumor suppressor 546
responsible for the protective effects of the X chromo- 547
some in BC (Table 3) [3]. $KDM6A$—also known as 548
ubiquitously transcribed tetratricopeptide repeat on 549
chromosome X ($UTX$)—is commonly mutated in BC, 550
losing its normal histone demethylase function on 551
tri-methylated histone H3 at lysine 27 (H3K27me3), 552
making H3K27 available for acetylation. Transient 553
expression of $KDM6A$ in a human BC cell line (UM- 554
UC-13) elicited tumor suppressing results through its 555
demethylase activity, suggesting a cell’s ability to 556
retain epigenetic memory from even a temporary 557
expression of $KDM6A$ [3]. We verified these findings 558
using transcriptomic analysis of patient data from 559
TCGA project. Mechanistically, $KDM6A$ promoted expression of known canonical $Tp53$ gene targets
is a primary sex-biasing factor responsible for the protective effects of the X chromosome in BC.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>N(M, F)</th>
<th>HR</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (male vs. female)</td>
<td>29, 24</td>
<td>12.39</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Kdm6a^−/− (male vs. female)</td>
<td>19, 16</td>
<td>2.349</td>
<td>0.0300</td>
</tr>
</tbody>
</table>

N, sample size; M, male; F, female; HR, hazard ratio.

(CDKNA1 and PERP) and KDM6A conditional knockout mice had more than a 5-fold attenuation of sex differences—female knockouts had significantly worse survival, but male knockouts were not affected [3, 68]. Enhancer of zeste homolog 2 (EZH2), the methyltransferase of polycomb repressive complex 2 (PRC2), has an antagonistic relationship with KDM6A to control the methylation status of H3K27 in the bladder urothelium [69]. Thus, recovering UTX demethylase function indirectly through EZH2 inhibitors has proven to exhibit a therapeutic value in the preclinical settings [70]. Because KDM6A mutations are more common in women with non-muscle invasive BC [71], future studies should consider the viability of targeting KDM6A-related pathways in female BC patients with EZH2 inhibitors.

KDM6A conditional knockout male mice did not have worse survival compared to female counterparts. This may be due to the Y chromosome encoding for UTY, a paralog of KDM6A; UTY on the Y chromosome may compensate the loss or mutation of KDM6A on the X chromosome. It should be noted that UTY has been shown to have no or little detectable demethylase activity [72–77]. Overall, loss of Y could be a sex-biasing factor by resulting in the loss of a compensatory gene to KDM6A.

Independent from its demethylase action, KDM6A operates with the complex of proteins associated with Set1 (COMPASS) family, specifically two proteins called mixed lineage leukemia 3 and 4 (MLL3/KMT2C and MLL4/KMT2D), to mediate methyltransferase activity of H3K4me1 [78–84]. Drawing from current evidence, KDM6A serves to attenuate tumorigenesis by two different histone modifying mechanisms: (1) antagonizing the PRC2-dependent transcription repression via H3K27 tri-methylation and (2) promoting the COMPASS-dependent transcription activation via H3K4 mono-methylation [85]. While the potential sex-biasing roles of PRC2 and COMPASS complexes have not yet been proven in BC, their dynamic and diverse roles in development and cancer emphasizes the potential to leverage their actions to mitigate sex differences in BC.

DNA topoisomerase 2 beta (TOP2B) showed male-biased DNA methylation in BC patients, and Valrubicin—an anthracycline intravesical therapy used for BCG refractory patients—is designed to antagonize topoisomerase activity [6]. Interestingly, the side effects of Valrubicin have been shown to be mitigated by tamoxifen [86], suggesting sex hormones may alter drug efficacy [6]. According to the 2021 National Comprehensive Cancer Network (NCCN), Valrubicin recommendations are not specific to a patient’s biological sex. Thus, more research is warranted to elucidate how male and female sex epigenetics and hormones may differentiate BC patients’ response to Valrubicin.

Piecing together the epigenetic basis for BC sex disparities could reveal foundational mechanisms driving sex differences in BC patients. Thus, a systematic characterization of the sex epigenome in males and females could open many doors for precision medicine in BC. Ultimately, we believe that sex differences may be driven by a coordinated effect between three major players: the sex epigenome, sex hormones, and sex chromosomes and, moreover, interactions among these players may amplify the magnitude of sex differences (Fig. 2).

Metabolism

Metabolic differences between males and females are well-known, but its role in driving sex differences in cancer—especially in the metabolic reprogramming of cancer cells—has only been a more recent consideration [87]. At baseline, male serum has higher concentrations of carbohydrate and amino acid metabolites [88], while female cells have a predilection for lipid biosynthesis [89]. Rubin et al., posited that male cancers may specifically reprogram carbohydrate and amino acid metabolism and female dominated cancers may selectively reprogram the normal utilization of lipids [87]. It is not yet known whether this effect plays a part in increasing male bladder cancer risk.

Biotransformation of molecules such as hormones, neurotransmitters, drugs, and xenobiotics for proper usage, storage, and elimination is also an important metabolic process that has shown sex differences. In a case-control study, Zheng et al., observed that a His213 allele of a sulfotransferase gene (SULT1A1) conferred decreased BC risk only in females [90]. Further studies are needed to validate the clinical application of this finding as the female composition of the study was only around 24%.
The UDP-glucuronosyltransferases (UGT)-dependent detoxification pathway is responsible for eliminating xenobiotics and endobiotics [91]. The human UGT loci has been closely linked to BCa risk [59–65, 92–95] and in liver tissue, it has been shown that men have a higher expression of UGT enzyme UGT2B17, suggesting different enzymatic activity to metabolize carcinogens and chemotherapeutics between sexes [96]. Downregulation of UGTs is closely associated with tumor formation in mice [97, 98] and humans [99, 100]. AR represses expression of UGTs in the bladder [100] and the prostate [101], implying a sex-biasing role of the UGT detoxification pathway in BC. Despite the strong association, there is a lack of evidence demonstrating the causality. Currently, it is not well-established whether such sex differences persist in normal or cancerous bladder tissue. A notable preclinical barrier hindering current research is due in part to species differences between mouse and human UGT superfamily genes [102]. Humanized mouse models can overcome this barrier to elucidate the roles of UGTs in BC carcinogenesis and potential sex-biasing effects.

Mitochondria, another important player in metabolism, is maternally inherited and naturally exhibits strong sex specific activity in normal and pathological conditions [103]. One of the outputs of mitochondrial activity is reactive oxygen species (ROS) and interestingly, it has been suggested that female mitochondria may have a better ability to manage ROS levels—decreasing the accumulation of ROS build-up amidst a higher respiratory—than male mitochondria in the brain [87]. In some ways, this leads to a more susceptible tumor environment for males. It remains to be shown whether this phenomenon persists in male and female bladders. Searching for potential links between mitochondrial activity, ROS levels, and increased male BC risk may yield interesting findings.

**FUTURE DIRECTIONS AND CONCLUSION**

Sex disparities in BC morbidity and mortality are intuitively and clinically well accepted, but the full scope of mechanisms remains unknown. Preclinical publications have illustrated the roles of evident sex-biasing factors such as environmental toxins, androgens, estrogens, their respective receptors, and sex chromosomes (Table 1). But a deeper dive into the biology implies crosstalk between the aforementioned factors with more elusive effects (Fig. 1; Table 1). Thus, to adequately address the age-old question as to why males have greater BC incidence, we must adopt a framework of understanding that includes sex-biasing factors that have historically been elusive—these include XCI escapees, sex-biased transcriptional regulation, sexually dimorphic gene expression, the sex epigenome, metabolism, etc. With the advancement of genomic technology, numerous sex-biasing factors identified in other diseases remain to be characterized in BC. As mentioned before, androgen-mediated promotion of CD8 + T cell dysfunction contribute to sex differences in BC, highlighting the immunological basis of gender disparities in cancers [35]. The field has just barely scratched the surface when comes to the immunological basis of sex bias in cancer. In addition, age-related pathways, microbiome, genomic stability, imprinting, and noncoding RNA are all potential avenues that can be further explored.

In designing new experiments, we believe that a simple comparison between males and females is not adequate. Every study should always: 1) isolate the effects of each sex-biasing factor to eliminate potential confounding effects, 2) measure any potentiating or neutralizing effect one factor has on the other, and 3) adjust for sex specific age-related changes such as declining hormonal levels unique to men and women. Using proven strategies such as the FCG model is one way to address these points.

In this review, we highlighted preclinical results from the last decade with the goal to guide and direct future studies to formulate testable hypotheses that properly address how elusive and evident sex-biasing factors promote drastic male and female differences in BC. We hope that by tackling these specific areas of research, we can catalyze clinical improvement for males and females in the prevention, screening, and management of BC.

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AUTHOR CONTRIBUTIONS

CML and XL wrote and edited the manuscript. All authors contributed to the concept of the review.

CONFLICT OF INTERESTS

CML, ZL and XL declare no conflict of interests.

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