Current Concepts of Epigenetics in Testicular Cancer

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Abstract Testicular germ cell tumors (GCTs) are characterized into seminomas (SGCTs) and non-seminomatous testicular germ cell tumors (NSGCTs). Serum tumor markers (STMs) play an important role in testicular cancer as they provide useful information for diagnosis, staging, and detection of recurrence. Nonetheless, additional tumor markers for early diagnosis and therapeutic options are required to enhance specificity of serological diagnosis of testes cancers. Epigenetics is defined as inherited changes in gene expression that are not encoded in the DNA structure. Epigenetic changes include DNA methylation, histone modifications, and microRNA (miRNA) regulation. It is through the study of epigenetics that diagnostic methods for early detection and novel therapeutic strategies may be established for testicular cancer. We performed a comprehensive review of the English medical literature in PubMed by combining search terms including DNA methylation, histone modifications, microRNA (miRNA) regulation, epigenetics, and testicular cancer. DNA methylation is the most extensively studied epigenetic modification. It consists of the addition of a methyl group to nucleotide bases. It has been reported that SGCT contain reduced levels of DNA methylation compared to NSGCT. MiRNAs are small non-coding RNAs that regulate posttranscriptional gene expression. It has been suggested that miRNAs may play

a role in the pathogenesis of GCT. Specific expression patterns have been displayed by various miRNAs in patients with GCT. Histones are proteins intertwined with coiled, double-stranded genomic DNA that form a structure known as a nucleosome. The most widely studied histone modifications include acetylation, methylation, and phosphorylation. Methylation of histone proteins has been found in all types of NSGCT. Epigenetics may offer an additional and effective tool in establishing a diagnosis of GCT of the testes, including prognostic information and perhaps enabling targeted treatment in patients with testicular GCT.

Keywords DNA methylation · MicroRNA regulation · Histone modification · Testicular cancer

Introduction

The majority of testicular cancers are germ cell in origin [1]. For this reason, the terms testicular cancer (TC) and testicular germ cell tumors (GCTs) are often used interchangeably. In many parts of the world, GCTs are considered the most common neoplasm among young men (aged 15–40); however, overall, it is an uncommon disease with an incidence of 10 in 100,000 men [2, 3]. GCTs are further characterized into seminomatous germ cell tumors (SGCTs) and non-seminomatous germ cell tumors (NSGCTs). NSGCT include embryonal carcinomas, yolk sac tumors, and choriocarcinomas [4]. Both histologic groups arise from a common precursor lesion known as intratubular germ cell neoplasia unclassified (ITGCN). SGCNs and NSGCTs have distinct biologic features and metastatic potential, with NSGCTs bearing a higher propensity for early spread and poorer prognosis in advanced stages. In TC, serum tumor markers (STMs) play an important role in patient management. STM levels provide useful information for diagnosis, staging, response to therapy, and

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detection of recurrence. In this context, three STMs have been identified as prognostically significant by the International Germ Cell Cancer Collaborative Group (IGCCCG). These include alpha-fetoprotein (AFP), human chorionic gonadotrophin (HCG), and lactic dehydrogenase (LDH) [5, 6]. These biochemical markers are readily available but lack specificity for GCT of the testes. Additional tumor markers with enhanced specificity may be of beneficial for early diagnosis, guiding treatment, monitoring the response to treatment and signaling recurrent disease.

Similar to other genitourinary (GU) malignancies including kidney cancer, bladder cancer, and prostate cancer, epigenetic alterations are common during carcinogenesis and disease progression in testicular cancer. In our review, we discuss the potential application of epigenetics in the early detection and management of testicular cancer.

Methods

We performed a comprehensive review of the English medical literature in PubMed by combining search terms including DNA methylation, histone modifications, microRNA (miRNA) regulation, epigenetics, and testicular cancer. The relevant articles were reviewed and we summarized the findings.

Results and Discussion

Epigenetics

Epigenetics is defined as inherited changes in gene expression that are not encoded within the DNA structure [7]. Epigenetic alterations include DNA methylation, miRNA regulation, and histone modifications. DNA methylation has been the most extensively studied. It refers to the formation of a covalent bond by the addition of a methyl group (CH₃) to the carbon-5 (C-5) in cytosine residues of CpG dinucleotides. Regions of the genome containing a high density of CpG dinucleotides are commonly known as “CpG islands” [8]. This process is carried out by a group of enzymes known as DNA methyltransferases (DNMT) which use S-adenosyl-methionine (SAM) as the methyl donor [9]. Methylation of these regions may result in reduced expression or even silencing of the genes involved. Another important epigenetic mechanism is that of histone modification. This includes acetylation, methylation, phosphorylation, ubiquitination, sumoylation, and ADP ribosylation of histone proteins. Of these, acetylation and deacetylation of lysine residues play a major role [10]. There is evidence indicating that histone modifications play a role in carcinogenesis by altering expression patterns of regulated genes [11]. The third main epigenetic mechanism involved in carcinogenesis is miRNA regulation. These are small, non-coding RNAs that regulate the degradation and translation of messenger RNAs (mRNAs). These miRNAs

can carry out opposing functions within the same gene by behaving as oncogenes or tumor-suppressor genes (TSGs) depending on the tissue type and specific targets [12]. Therefore, identification of miRNA-regulated pathways may lead to a better understanding of the development and progression of urologic tumors including testicular cancer [13].

The following is a discussion on the association between the three main epigenetics, mechanisms, (DNA methylation, miRNA regulation, histone modification), and testicular cancer.

DNA Methylation

DNA methylation was first reported by Hotchkiss in 1948 when he discovered what he believed to be were artifacts designated as “epicytosine” and “epiguanine” from calf thymus DNA [14]. DNA methylation consists of the addition of a methyl group to nucleotide bases. Three types of base methylation have been described: adenine at the N-6 position, cytosine at the N-4 position, and cytosine at the C-5 position which is the most common among all organisms [15]. Methylation of these cytosine residues usually takes place in short CpG-rich regions with a CpG density of >60%, known as CpG islands. These are found in the promoter regions of 50% of all genes [16]. Hypermethylation of CpG islands located in promoter regions of TSG results in impaired gene expression of these TSGs. As such, there is a loss in their protective role in the development of tumors (Fig. 1.). Loss of gene expression in TSG affects several mechanisms of cell homeostasis including cell cycle, DNA repair, cellular adhesion and invasion, metabolism of carcinogenic agents, apoptosis phenomena, response to hormones, etc. [17]. The process of DNA methylation is carried out by a group of enzymes known as DNMTs. These three active DNMTs (DNMT1, DNMT3a, and DNMT3b) and one related protein lacking catalytic activity (DNMT3L) are present in mammals. DNMT1 shows a preference for hemimethylated DNA. It preserves the methylation patterns during cell division by maintaining methylation of hemimethylated CpG dinucleotides produced by DNA replication. DNMT3a and DNMT3b have been traditionally designated as *de novo* DNA methyltransferases. They are responsible for the establishment of DNA methylation patterns in germ cells and during mammalian development. As previously mentioned, DNMT3L is catalytically inactive and functions as a regulatory factor in germ cells [18].

Loss of DNA methylation was the first reported epigenetic change in human cancer [19]. DNA hypomethylation of oncogenes results in DNA overexpression, which in turn may result in carcinogenesis. It has been reported that SGCT contain reduced levels of DNA methylation compared to NSGCT [20]. Similarly, Netto et al. found that staining for methylation at the 5 position of deoxycytidine residues (5^mC) was markedly reduced and virtually undetectable in the majority of ITGCN and SGCT; however,

Fig. 1 DNA methylation of CpG Island carried out by DNA methyl-transferase. **a** Attachment of transcription factor to DNA strand activates RNA polymerase inducing transcription and gene expression. **b** Methyl group prevents transcription factor attachment to DNA strand producing transcription inactivation and gene silencing

significant staining was noted in NSGCT [21]. Furthermore, Smiraglia et al. found that SGCTs exhibit distinct epigenetic differences when compared to NSGCTs. By analyzing over 1400 fragments representing CpG islands through restriction landmark genomic scanning (RLGS), they demonstrated that NSGCTs exhibited variable degrees of CpG islands hypermethylation while SGCTs showed little or no hypermethylation. Moreover, they also demonstrated that SGCTs are more extensively hypomethylated in their chromatin than are NSGCTs [22]. Several promoter genes have been found to be hypermethylated in NSGCTs including RASSF1A, MGMT, BRCA1, HIC1, APC, CDH1, RARB, and FHIT [23]. Table 1 illustrates the methylation percentages of some of the genes analyzed for SGCT and NSGCT. Interestingly, it has been found that promoter hypermethylation of TSG involved in genitourinary malignancies including prostate and bladder cancer may be reversed with the use of flavonoids (quercetin, genistein, curcumin, etc) contained in fruits, vegetables, and other plant products.

However, a comprehensive analysis of the effects of these polyphenolic compounds in testicular cancer is yet to be seen [24].

MicroRNA Regulation

MicroRNAs (miRNAs) are small non-coding RNAs that are typically 19–35 nucleotides in length and regulate posttranscriptional gene expression [7, 25]. The overexpression of miRNAs in tumor cells has been found to be oncogenic whereas miRNAs that show reduced expression are seen as tumor suppressor in nature [26]. Alteration of gene expression by regulation of miRNAs is thought to be distinct from DNA methylation. Therefore, the mutual influence between these two epigenetic mechanisms is largely overlooked [10]. For example, Chen et al. were able to demonstrate that overexpression of miR-199-a-3p restored the expression of the TSG MGMT and APC in testicular cancer cells by affecting DNA methylation of their promoter regions [27]. Similarly, Ferreira

et al. reported that epigenetic silencing of CpG island hypermethylation of other classes of non-coding RNAs such as transcribed ultraconserved regions (T-UCR) or small nucleolar RNAs (snoRNAs) also occurs in human neoplasia [28]. The potential role that miRNAs may play in the pathogenesis of GCT has been suggested by a GCT-specific expression pattern displayed by miR-371-373, miR-302, and miR-146 [25]. Furthermore, a recent study reported that the miRNA miR-371a-3p allowed the identification of patients with GCT with a sensitivity and specificity of 84.7 and 99%, respectively, thus outperforming both HCG and AFP testing. Tumor-specific release was indicated by a decrease in serum miR-371a-3p levels postoperatively [29]. Dysregulation of various miRNAs has also been found to affect a process known as epithelial mesenchymal transition (EMT) seen in other genitourinary malignancies including bladder and kidney cancer [30, 31]. However, no known miRNA affecting EMT has been identified in association with testicular cancer. EMT is a transient process in which epithelial cells acquire a mesenchymal phenotype by losing intercellular adhesions. These are normally maintained by the group of proteins E-cadherins. This results in an increase in cell motility. As cell motility increases, cell adhesion decreases until the cell ultimately detaches from the surface epithelium gaining access into the hematogenous or lymphatic routes or alternately directly into adjacent structures. Table 2 illustrates a list on miRNAs implicated in the development of testicular cancer and other genitourinary malignancies. Characterization of miRNAs is performed by using two approaches: studying expression of known miRNAs by hybridization-based techniques or discovery of novel miRNA molecules by cloning and sequencing [7, 32].

Histone Modification

Histones are proteins intertwined with coiled, double-stranded genomic DNA that form a structure known as nucleosome, the basic unit of eukaryotic chromatin. Each nucleosome is composed of a core of four pairs of histone molecules (octamer) H2A, H2B, H3, and H4 and the histone linker H1 [33]. All of the histone molecules share a common structure which consists of a central “fold domain” and “tails” that contain NH₂ and COOH terminals. Histone modifications typically target the “tail” domain at different amino acid positions. These modifications include acetylation, methylation, phosphorylation, ubiquitination, sumoylation, and ADP ribosylation. The modification of DNA and histones can result in the inhibition or activation of genes. This occurs by a process that regulates chromatin remodeling between the transcriptionally active euchromatin and transcriptionally silent heterochromatin [7, 34]. Previous studies have illustrated the use of histone modifications as markers for diagnosis and prognosis of various genitourinary malignancies including kidney and bladder cancer

[35, 36]. Similarly, it has been reported that methylation of histone H3 at lysine 4 (H3-K4; gene activating) and lysine 9 (H3-K9; gene silencing) has been detected in all histological subtypes of NSGCT. This suggests a possible association between histone H3-K4 and H3-K9 methylation and abnormal gene expression in NSGCT [37]. Furthermore, Almstrup et al. observed that carcinoma in situ (CIS) cells from GCT display low levels of repressive histone modifications at H3K9me2 and H3K27me3 but high levels of H3K9 acetylation and H3K4 methylation [38]. Conversely, in a study with a series of 36 spermatocytic seminoma (SS) samples, no correlation between a specific epigenetic mark and the size of the SS cells was identified. This study was the first comprehensive analysis of epigenetic alterations including histone modifications at H3K9me2, H3K4me1, H3K4me2/3, H3K9ac, and H2A.Z [39].

Discussion

It is reasonable to view epigenetic modifications as a potential therapeutic target given their feasible reversibility [40]. Several molecules to target these modifications have been developed, most notable being DNA methyltransferases inhibitors (DNMTi) and histone deacetylase inhibitors (HDACi).

DNMTi

Platinum-based combination chemotherapy serves as the most common form of medical therapy for GCT. A previous study revealed a close correlation between promoter hypermethylation of tumor-associated genes in GCT and chemotherapy resistance. Moreover, they demonstrated that cisplatin induces *in vivo* promoter hypermethylation [41]. This could explain the temporal response to cisplatin-based chemotherapy as well as disease relapse. Furthermore, a relationship was also found between 5-methyl cytosine content and chemotherapy sensitivity in GCT, when a single cisplatin-refractory seminoma was found to have a hypermethylated pattern not commonly observed in SGCT [42]. Overall, these data suggest an association between the global DNA methylation status and response to chemotherapy, indicating that the degree of methylation may dictate the chemotherapy sensitivity of GCT [43]. Previously silenced TSG through promoter hypermethylation may be reactivated by DNMTi, resulting in the arrest of tumor growth and propensity for invasion and possibly enhancing tumor sensitivity to chemotherapy. Interestingly, a recent study demonstrated the use of two compounds obtained from natural sources (epigallocatechin-3-gallate and laccic acid) as effective DNMT1 inhibitors. Gene demethylation and reactivation was achieved with both compounds in several cancers [44].

HDACi

HDACi are categorized into five groups (aliphatic acids, benzamides, cyclic tetrapeptides, hydroxamic acids, and short-chain fatty acids) according to their chemical composition and structure [45]. Since previous studies have highlighted the expression of HDAC in different histological subtypes of GCT, compounds targeting these epigenetic modifications may play an important role in testicular cancer therapy [46]. The HDACi depsipeptide was observed to induce apoptosis of TCam-2 cells (a cell line that resembles SGCT), highlighting its potential therapeutic utility in SGCT [47]. Moreover, a recent study suggested the use of a combination of suberoylanilide hydroxamic acid (SAHA) and valproic acid as a treatment option for Leydig testicular cell carcinoma in the radio-iodine-base setting [48]. Similarly, a phase I trial tested the tolerability of combining SAHA with epirubicin for the treatment of testicular and prostate cancer among other malignancies with promising results [49].

Conclusions

STM continues to serve an important role in testicular tumors. Epigenetics may offer an additional and effective tool in diagnosis, prognosis, and in establishing targeted treatment. The possibility of reversing epigenetic modifications is encouraging and continues to motivate research in the development of novel targeted therapies.

Appendix

Table 1 Hypermethylation percentages of promoter genes in seminoma and NSGCT [50]

Gene	SGCT	NSGCT
RASSF1A	0–40	21–83
MGMT	0–24	20–69
SCGB3A1	0	54
HOXA9	0	26
APC	0	24–29
FHIT	0	6–29
HOXB5	0	13
CDH13	0–6	9–12
CDH1	0	4–11
RARB	0	5–6

Table 2 MiRNAs involved in various genitourinary tumors [10, 27, 29, 51]

MiRNA	Tumor
miR-371a-3p	Testicular
miR-199a-3p	Testicular
miR-367-39	Testicular
miR-372-3p	Testicular
miR-373-3p	Testicular
miR-21	Prostate
miR-126	Prostate
miR205	Prostate
miR-200	Bladder
miR-127	Bladder
miR-1224	Bladder
miR-9	Renal
miR-34c	Renal
miR-223	Renal

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