**Cell Death &
Differentiation**

PERSPECTIVE

Sex disparity in cancer: roles of microRNAs and related functional players

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Abstract

A sexual dimorphism at the cellular level has been suggested to play a role in cancer onset and progression. In particular, very recent studies have unraveled striking differences between cells carrying XX or XY chromosomes in terms of response to stressful stimuli, indicating the presence of genetic and epigenetic differences determining sex-specific metabolic or phenotypic traits. Although this field of investigation is still in its infancy, available data suggest a key role of sexual chromosomes in determining cell life or death. In particular, cells carrying XX chromosomes exhibit a higher adaptive potential and survival behavior in response to microenvironmental variations with respect to XY cells. Cells from females also appear to be equipped with more efficient epigenetic machinery than the male counterpart. In particular, the X chromosome contains an unexpected high number of microRNAs (miRs), at present 118, in comparison with only two miRs localized on chromosome Y, and an average of 40–50 on the autosomes. The regulatory power of these small non-coding RNAs is well recognized, as 30–50% of all protein-coding genes are targeted by miRs and their role in cell fate has been well demonstrated. In addition, several further insights, including DNA methylation patterns that are different in males and females, claim for a significant gender disparity in cancer and in the immune system activity against tumors. In this brief paper, we analyze the state of the art of our knowledge on the implication of miRs encoded on sex chromosomes, and their related functional paths, in the regulation of cell homeostasis and depict possible perspectives for the epigenetic research in the field.

Introduction

Growing amounts of evidence are showing the influence of sex (i.e., biological determinants) and/or gender (that includes socio-cultural matter) on pathological conditions and clinical outcomes [1, 2]. Differences have been detected in a number of either transmissible or non-transmissible diseases. In particular, epidemiology clearly suggests that, apart from reproductive organs, several forms of cancers of great relevance, e.g., melanoma or leukemias, clearly display a gender disparity in terms of incidence, prevalence, or response to therapy [3]. Although a general female advantage has been observed by these epidemiological studies, few data have emerged so far explaining this trend [4]. Recent results also suggest that sex-specific differences could take place in cell death programs, representing critical features for the identification of sex-specific chemotherapeutic targets [5]. As a general rule, cells from males and females (here called male cells, XY, and female cells, XX) respond differently to injuries possibly because of their different capability to face cellular stress [6]. In particular, the same stressor can preferentially induce apoptosis in male cells, and survival by induction of autophagy in female cells. Of notice, these results come from studies carried out with non-transformed cells, essentially vessel cells (such as vascular smooth muscle cells or endothelial cells and fibroblasts) [7–9]. This difference is probably due to the greater capacity of the XX cells to prevent and repair the damage than XY ones [8]. In addition, XY and XX cells are differently susceptible to various cytotoxic agents. For

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instance, XY neurons were reported to be more sensitive to excitotoxicity than XX neurons, which appeared more prone to staurosporine-induced apoptosis [9]. These gender differences in cell susceptibility to an exogenous stress, that could be applied to non-cancer cells of different histotype, seem to be related to the inability of XY cells to maintain intracellular levels of reduced glutathione, paralleled by an increased activity of Superoxide Dismutase (SOD) and catalase activity in XX cells as observed either in vitro or in in vivo studies [2, 6–13]. Both genetic and hormonal differences have been hypothesized to be at the basis of all these disparities contributing to sex-specific phenotypes [14].

Discussion of literature data

The genetic issue

Many observations on the differences between XX and XY cells are derived from studies carried out with in vitro models. This suggests that at least part of the observed disparities are independent from the effects of sexual hormones and could be directly imputable to genetic differences, including X and Y sex chromosomes. However, the study of the roles of these chromosomes in the maintenance of cell homeostasis and death is still at the beginning. One key point deals with the presence of the two X chromosomes in cells from females and one X chromosome in cells from males. As a general rule, the phenomenon of X-inactivation should theoretically re-equilibrate female and male gene expression. However, this is not always the case, as up to 15% of X-linked genes escape X-chromosome inactivation (XCI) leading, in females, to the presence of a second, functional copy of the genes located in the XCI-escaped regions [15, 16]. A systematic analysis of these genes has not been performed, due to the extremely heterogeneous scenario of XCI (escaper genes may display different levels of expression between tissues and subjects [17]), thus making it difficult to understand which role, if any, these escaper genes may play in cell-sex disparity. However, Dunford and coauthors recently reported that, among escaper genes, there are tumor suppressors (ATRX, CNKSR2, DDX3X, KDM5C, KDM6A, and MAGEC3) which, when mutated, are significantly associated with cancer in males [18]. On this basis, it has been suggested that the presence of a second functional copy of a gene in an XCI-escaper region could protect females from the negative effects of the mutated copy [18]. In few words, the presence of two X chromosomes, i.e., different alleles of the same gene, could provide a significant advantage to cells from females since they could counteract gene mutations leading to cancer [19]. However, it is possible that this sort of "XCI-

mediated protection" does not last for the whole life. Actually, it is reported that from middle age (around 55 years) onward, a phenomenon of age-related preferential inactivation of one X chromosome takes place [20–23]. This XCI skewing can lead to the expression of deleterious alleles and thus to an increased risk of morbidity. Actually, women that are offsprings of long-lived parents have a lower XCI skewing and lower prevalence of several diseases such as cardiovascular, skeletal, respiratory, neurologic diseases, and cancer with respect to the age-matched counterparts that were born from non-long-lived parents [24]. The reasons for this XCI skewing toward the chromosome containing the deleterious alleles are not known. However, this phenomenon has been observed since 1995 for many diseases including cancer [25–30].

On the other side, the presence in women of a number of non-inactivated X-linked alleles is associated not only to the protection against diseases but also to some immunological disorders, including autoimmune diseases [31]. This phenomenon is likely accounted by the fact that many XCI-escaper genes are involved in immune response. This issue will be further discussed later.

The epigenetic issue

Besides the genetic determinants mentioned above, a number of epigenetic factors, including microRNAs (miRs), have been hypothesized to play a role in cancer onset and progression. MiRs are in fact emerging as critical factors in post-transcriptional regulation of gene expression by influencing the main cellular activities, including cell proliferation and dissemination capabilities as well as cell death. These short non-coding RNAs (21–25 nucleotides) negatively modulate their target mRNAs by direct binding to the 3′untranslated regions (UTRs) with consequent translational repression or mRNA degradation, depending on sequence complementarity [32]. Notably, X chromosome contains an unexpected high number of miRs, at present 118, in comparison with only 2 miRs localized on chromosome Y, and an average of 40–50 on the autosomes [33]. The regulatory power of these small non-coding RNAs is well recognized, as 30–50% of all protein-coding genes are targeted by miRs according to complex interconnected circuitries as each gene might be regulated by many miRs and each single miR has up to hundreds of direct targets.

The role of miRs in cancer has been deeply analyzed demonstrating their tumorigenic as well as tumor-suppressor functions. As concerns sex chromosomes, it is important to highlight that the X-chromosome not only displays a high density of miRs, but also that several of them are involved in immunity regulation [34]. Moreover, the contemporary presence, on this chromosome, of genes coding for proteins playing immunological roles together

with a number of miRs impacting on immune system integrity and function may suggest the existence of sex-related loops functional to immunosurveillance (or failure of immunosurveillance) against tumor onset and progression [33]. Since the development of immunotherapeutic approaches recently gained the attention of physicians in the fight against cancer, the scenario depicted above appears to be of great interest. In particular, inhibitors of different immune checkpoints have been introduced for the treatment of different, otherwise non-responsive, tumors as well as, more recently, for first line treatment. This strategy stems from the ability of cancer cells to escape the surveillance normally exerted by the immune system via a series of key molecules, e.g., cytotoxic T-lymphocyte antigen-4 (CTLA-4) or programmed cell death protein 1 (PD-1), capable of inhibiting immune cells [35, 36]. In particular, PD-L1 and PD-L2 are transmembrane proteins that, by binding to their receptor PD-1, activate the PD-1/PD-L1 or PD-L2 pathway and induce T-cells to undergo apoptosis suppressing their activation. Hence, the immunosurveillance against tumors is hindered and tumor progression can take place [36].

Finally, it is important to mention that further possible epigenetic mechanisms could give rise to sex disparities. In particular, it has been observed that DNA methylation pattern of autosomes is different between males and females [37, 38]. In the first study [37], carried out in saliva samples, 580 autosomal sites showing strong differences between males and females have been detected. In the second study [38], performed on three independent cohorts of European subjects, 1184 CpG sites with different methylation levels between sexes were identified in blood cells. The same study also showed that two genes, CISH and RAB23, displayed a significant association between DNA methylation and expression in men and women. These two genes are involved in Hedgehog pathway [39] and inhibition of the JAK-STAT pathway [40]. Hedgehog pathway is crucial for response to injury, tissue stress, healing, and regeneration, whereas JAK-STAT pathway is fundamental for regulatory T-cell function [40]. Therefore, a sex-specific epigenetic control over these two pathways may in part account for the observed differences between men and women in immune and autoimmune responses [41].

Sex and immunity

A significant difference between the male and female immune system function has recently been described. As reviewed by Klein and Flanagan, the female immune system appears more efficient in a number of species, including humans [41]. Importantly, this different efficiency is present all along the entire human lifespan. From childhood to old age, the female immune system appears more powerful and able to better counteract infectious and non-infectious

diseases, including cancer [41]. This fact may be related to the different evolution of the two sexes in relation to their different and complementary biological functions. It means that, independent from the hormone-related functions, immunity is per se sex-biased, i.e., it could depend upon genetic or epigenetic matters. In addition, in females, this advantage can also become detrimental since the sex-biased nature of the immune system function can result in auto-immune diseases [31, 41, 42]. In this scenario, a role of sex has also been linked to PD-1 modulation [43, 44]. In patients with melanoma, the inhibition of PD-1/PD-L1 interaction resulted significantly associated with sex as the median objective response rate (ORR) was 54.6% among men and just 33.1% among women, and median progression-free survival (PFS) was 18 months vs. 5.5 months, respectively [43, 45]. The underlying reasons are not known, but they possibly involve the immune system sex disparity obviously based on sex hormones and other factors differently expressed in women and also on genetic, e.g., sex chromosomal-associated issues, and epigenetic signals, such as miRs.

Sex chromosomes and miRs

Based on the above findings, an miR-dependent regulation of molecules of relevance in modern immunotherapy such as PD-L1 should merit particular attention. Indeed, PD-L1 expression appears to be directly or indirectly controlled by several X-linked miRs (Fig. 1 and Table 1). According to TargetScan 7.1 (www. targetscan.org), miR-106b, miR-20b, and miR-513, all three localized on chromosome X, are putative repressors of PD-L1 by direct binding to their 3′UTRs. Specifically, miR-106b and miR-20b, which are part of the miR-106–363 cluster, including miR-106a, 18b, 20b, 19b2, 92a2, and miR-363, were reported to play an oncogenic role in different tumors [46]. Furthermore, miR106a was reported to downregulate the anti-inflammatory cytokine IL-10 [47] and the whole cluster was suggested to play a role in both innate and adaptive immunity [48]. Interestingly, miR-106b and miR-20a, together with miR-221, were proposed as biomarkers for early detection of gastric cancer [46]. Other miRs, such as miR-513 and miR-514, members of the X-linked primate-specific miR-506–514 cluster, have been associated with cancer, particularly with melanocyte transformation, melanoma promotion, and sensitivity to BRAF inhibitors [49, 50]. In addition, in biliary epithelial cells, miR-513 is downregulated by interferon- γ (IFN-γ) and regulates PD-L1 translation by direct targeting, thus suggesting an miR-mediated gene regulation of responses to IFN-γ [51].

PD-L1 transcription is induced by hypoxia-inducible factor-1α (HIFα) and signal transducer and activation of transcription-3 (STAT3) factors, directly acting on its
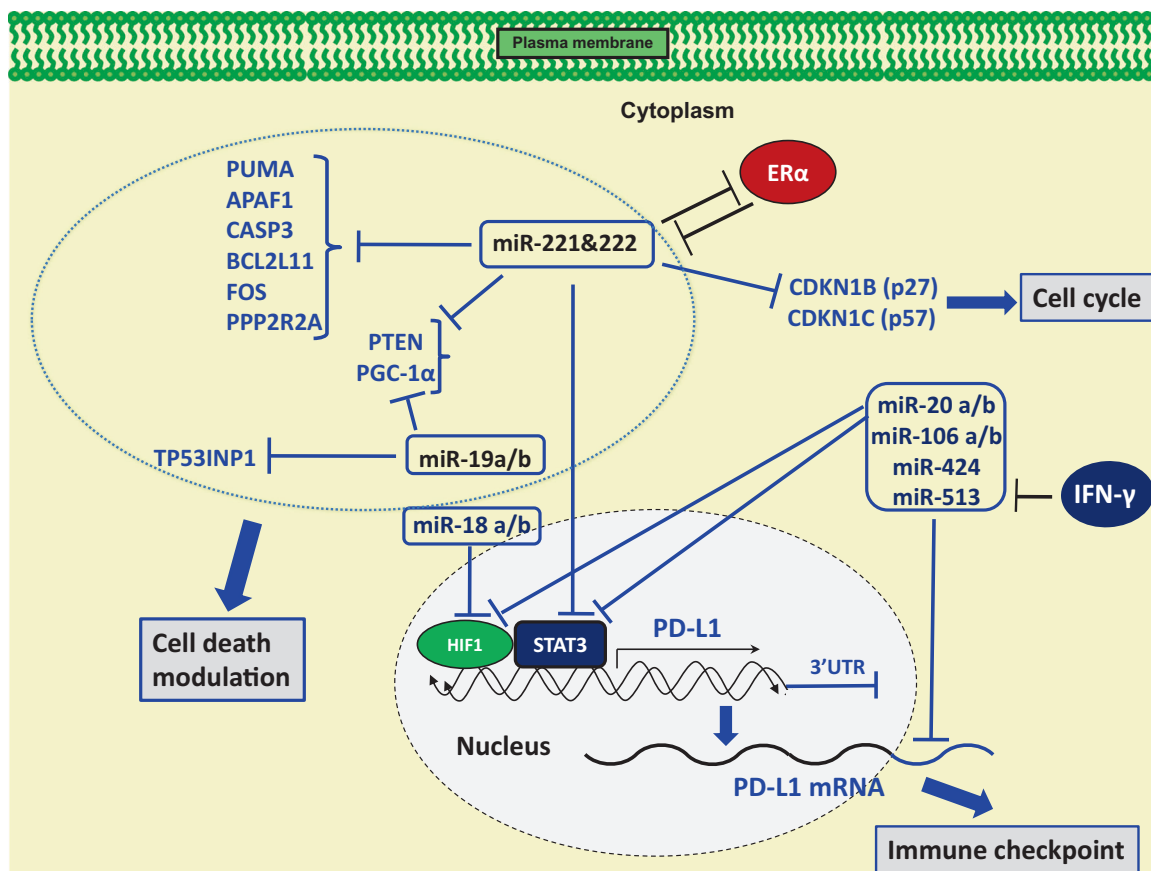
Fig. 1 X-linked microRNAs regulating cancer cell homeostasis. Schematic representation of miR-based, direct or indirect, regulation of PD-L1, as an example of immune checkpoint key molecule. Additional relevant genes targeted by the indicated miRs and involved in cell cycle and cell death modulation are also shown. See text for details. PD-L1 programmed cell death protein ligand, HIF1α hypoxia-inducible factor-1α, STAT3 signal transducer and activation of transcription-3, ER α estrogen receptor α, IFNγ interferon γ, PTEN phosphatase and tensin homolog, PUMA p53 upregulated modulator of apoptosis, APAF1 apoptotic protease activating factor-1, CASP3 caspase 3, BCL2L11 BCL2-like11, PPP2R2A protein phosphatase 2, regulatory subunit b, α, TP53INP1 tumor protein p53-inducible nuclear protein 1, PGC-1α peroxisome proliferator-activated receptor-gamma coactivator 1-alpha, CDKN1B -p27kip1 cyclin-dependent kinase inhibitor 1b, CDKN1C-p57kip2 cyclin-dependent kinase inhibitor 1c

promoter [52]. These transcription factors are regulated by miR-221&222 and by miR-18 and miR-19, the former couple specifically localized on chromosome X and the latter encoded on both chromosome 13 and chromosome X. As also miR-20a/b and miR-106a/b are produced from different miR gene clusters, apparently derived from genetic duplications, it is important to discriminate the contribution of X-chromosome-encoded miRs, looking for their possible role as functional modulators of female immunity [53, 54]. Both miR-18 and -19 are able to potentiate the nuclear factor-κB (NF-κB) activity, in turn favoring inflammation [53, 55]. Likewise, miR-20b appears to target both HIF-1a and STAT3 transcription factors [56], thus repressing PD-L1, either by direct pairing to its 3′UTR or indirectly through the downregulation of these two activating transcription factors. It is important to point out that, according to Targetscan, miR-20 and miR-106 are putative controllers

also of PD-L2, in agreement with its predominant post-transcriptional regulation. Finally, another X-linked miR, miR-424, directly targets either PD-L1 or CD80, thus possibly regulating both the PD-L1/PD-1 and CD80/CTLA-4 pathways further supporting the option of X-linked disparities associated with immune checkpoint responses [57].

How these X-linked miRs could provide a protection to female cells but not to male ones is still a matter of debate. A possibility that has been envisaged some years ago is that some of these X-linked miRs lie in the chromosomal regions that escape XCI and therefore are expressed at higher levels in female cells [33]. A precise map of XCI-escaper miRs is still missing and this should be the objective of specific research. However, it has been observed that in patients with active lupus 18, X-linked miRs are more expressed in CD4+ T-cells from women with respect to men, and five of them are regulated by demethylation [31].

Table 1 X-linked miRNAs putatively involved in direct or indirect regulation of the PD-1/PD-L1 axis

microRNA ID	X-chromosome position	Regulation	Role in cancer	3′UTR target	Gender disparities	References
hsa-miR-106a	chrX: 134170198-134170278 [-]	ER	Proliferation, invasion, migration and drug resistance	PD-L1; PD-L2; HIF-1; STAT3	F>M in systemic lupus erythematosus	[31, 46, 86]
hsa-miR-18b	chrX: 134170041-134170111 [-]	ER	Proliferation, metabolism	HIF-1	–	[53, 86]
hsa-miR-19b	chrX: 134169671-134169766 [-]	ER	Proliferation, invasion, drug resistance	HIF-1	–	[53, 86]
hsa-miR-20b	chrX: 134169809-134169877 [-]	ER	Proliferation, invasion, autophagy, drug resistance	PD-L1; PD-L2; HIF-1; STAT3	–	[46, 56, 86]
hsa-miR-221	chrX: 45746157-45746266 [-]	ER, AR	Proliferation, migration, invasion	STAT3	F>M in metabolic syndrome	[52, 63, 87, 88]
hsa-miR-222	chrX: 45747015-45747124 [-]	ER, AR	Proliferation, migration, invasion	STAT3	F<M in whole heart or isolated cardiomyocytes	[52, 63, 64, 87]
hsa-miR-424	chrX: 1345466714-1345467611 [-]	–	Proliferation, invasion, apoptosis	PD-L1	F>M in systemic lupus erythematosus	[57]
hsa-miR-513	chrX: 147213463-147213591 [-]	–	Proliferation, migration, apoptosis	PD-L1	–	[51]

This table summarizes microRNA target genes and/or sex-hormonal regulation and, when described, their sex-biased (F>M or F<M) expression in different pathophysiological conditions. F>M and F<M indicate higher or lower expression in females respect to males

ER estrogen receptor, *AR* androgen receptor, *PD-L1/2* programmed cell death protein ligand 1/2, *HIF-1* hypoxia-inducible factor-1, *STAT3* signal transducer and activation of transcription-3

This could suggest that these five miRs are XCI escapers, or, alternatively, that they could be demethylated by an XCI-escaper gene.

Among the X-linked miRs, miR-221&222 are the most extensively studied in tumors of different origins where they act as oncomirs controlling the development and progression of the tumor through the down-modulation of several key targets [58]. However, their possible impact on sex differences detected in tumor incidence and progression is still neglected. Literature on this argument appears to be focused on the study of the impact of hormones on cancer. For instance, it has been suggested that melanoma could be classified among the hormone-sensitive tumors according to complex, overlapping actions played by estrogens and androgens, particularly by the opposite effects of α and β estrogen receptors (ER) [59]. In particular, ERβ has been reported as capable of inducing autophagy-mediated cell death both in post-mitotic cells and proliferating cells, whereas ERα has been suggested to induce proliferation in transformed cells and autophagy in post-mitotic cells [60, 61]. Interestingly, among the number of X-linked miRs, six of them putatively bind and regulate ERα, including miR-221&222 [62]. Specifically, miR-221&222 inhibit ERα mRNA translation by direct binding to its 3′UTR, being in turn repressed by ERα according to a negative feedback loop [63].

Conversely, some insights are derived from studies carried out on miR-221&222 in cardiac cells. In fact, these two miRs display lower expression levels in females with respect to male murine cardiomyocytes contributing to sex-dimorphic cardiac phenotypes [64]. The mechanism underlying this disparity appears to involve endothelial nitric oxide synthase (eNOS) modulation via the unblocked expression of miR-221&222 direct target Ets-1 [65]. These data could be of relevance in view of the different cardiotoxicity of cancer chemotherapy in males and females [66].

Programmed cell death and miRs

Once again, cells from males and females seem to act differently when headed for death if subjected to the same exogenous stress, as female non-tumor cells are more prone to the autophagic protective effects, whereas male cells more frequently undergo apoptosis and/or necrosis [7, 8, 11, 12, 67, 68]. Looking for factors underlying this disparity, one option to consider is the involvement of the X-chromosome-linked inhibitor of apoptosis (XIAP) whose reduction can promote apoptosis and/or autophagy besides restoring sensitivity to chemotherapeutic drugs [69]. A sex-related loop would possibly involve 17β-estradiol (E2) that via ERα activates the miR-23 family and p53, in turn decreasing XIAP and inducing apoptosis [70]. Interestingly, miR-23a was reported to be differentially expressed by cells

from male and female murine brains and XIAP was indicated as a mediator of sex-related responses after stroke [71]. Notably, also miR-23c, belonging to the same miR family, is localized on chromosome X.

An additional Armadillo family member was recently identified as localized on chromosome X (Xq21.33-q22.2). This tumor-suppressor gene, called ALEX1 (Arm protein lost in epithelial cancer), was downregulated along with progression in several different solid tumors originating from epithelial tissues and its restored expression resulted able to inhibit proliferation and induce apoptosis [72]. For this gene, a differential tissue-specific sex hormone regulation and a critical role of its chromosomal localization has recently been hypothesized [73].

Finally, numerous miRs have been reported to regulate apoptosis, autophagy, and necrosis, also connecting the crosstalk between these types of cell death. Here again, some of these miRs are located on chromosome X. Examples are miR-374a, which is involved in the autophagic process through the inhibition of autophagy-related 5 (ATG5) and Ultraviolet Radiation resistance associated (UVRAG) proteins, and miR-504, which acts on the expression of the key tumor suppressor p53 [74]. Of interest are the X-linked oncomir-221&222, as among their direct targets include a relevant number of proapoptotic proteins such as phosphatase and tensin homolog (PTEN), p53 upregulated modulator of apoptosis (PUMA), apoptotic protease activating factor-1 (APAF1), and caspase 3 (CASP-3) [58, 75, 76], as well as the BCL2-like11 (Bcl2L11)-Bax/Bak axis [77]. Additional targets of miR-221&222 are TP53INP1, PPP2R2A, and PGC-1a, recently described for their participation in cell death, either through apoptosis or autophagy [78, 79]. Last but not the least, beclin-1, a key player in autophagy, was demonstrated as a new target of miR-221 [80] (see Fig. 1).

Conclusions

Human genome studies evidenced the presence on the X-chromosome of an unexpectedly high number of genes and miRs. This apparently non-casual localization might suggest the existence of X-linked functional circuitries, possibly contributing to sex-associated specificities, e.g., in immune responses. This fact, together with the presence of oncogenes apparently escaping XCI, could account at least in part for the sex disparity observed in several pathological settings. Furthermore, different possible mechanisms may account for the different sex-related expression levels of miRs, including X-linked transcription factors or cross-regulation by other miRs localized on X chromosome [81, 82].

A special case of miR-dependent, sex-specific regulation of immune responses and cancer immunosurveillance

discussed in this review is that of the PD-1/PD-L1 pathway, whose targeting with monoclonal antibodies (mAbs) has given really impressive therapeutic results [83, 84]. As mentioned, it has been observed that several miRs, either involved in the programmed cell-death processes or specifically targeting PD-L1, are localized on chromosome X. Therefore, an miR-based modulation of such pathway could be at the basis of many sex disparities observed between men and women in terms of stronger immunological responses and immunosurveillance [41, 44].

In this perspective, the need for further gender-specific research emerges in order to fill the gap between clinical data and our knowledge on the mechanisms underlying the detected gender disparity in the onset and response to therapy of different forms of cancer.

Finally, as very recently suggested by an Editorial appeared in Nature Medicine "the failure to assess the influence of sex chromosomes in studies of the genome doesn't necessarily boil down to a lack of tools: there is also a challenge of a lack of will. It takes a bit more effort to include sex chromosomes in certain genomic analyses, and so this step is sometimes skipped" [85]. We think the same stands for epigenetics, and time has come to deal with both issues in order to develop a real first-stage personalized approach to a number of life-threatening diseases.

Compliance with ethical standards

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